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The Ability of Stressor Factors of Environmental Pollution to Induce ROS and 8-Ohdg Mediated Apoptosis in Fish Species of Suez Gulf, Red Sea

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Abstract

The Suez Gulf is one of the richest fishing grounds of Red Sea in the Egyptian sector, it receives pollution from different sources. The main objective of this study is to use some fish species as bioindicator to environmental pollution. Three fish species were sampled from 3 different sites; Site 1 (Clean Area), site 2 (Petroleum & fishing activities) and site 3 (Al-Attaka commercial landing site). The Collected fish samples were: A) *Dussumieria spp.*, B) *Scomberomorus Spp.* and C) *Sphyraena Spp.* The detection of elevated concentrations of total and fecal coliforms suggests the presence of microbial contamination. The results of oxidative stress displayed significant (P<0.01) increase in the level of ROS formation (259.0±10.4) in site-3 in comparison to site-1 (136.0±5.5) (control location). Results of DNA adducts revealed that site-3 showed significant (P<0.01) increase in the levels of 8-OHdG (18.37±1.47) compared with those in site-1 (5.75±0.77). Apoptosis results demonstrated significant (P<0.01) increase in the levels of apoptosis (32.79±2.92) in site-3 in comparison to site-1 (11.52±1.46) considered as control site. However, results of DNA barcoding did not reveal changes in the DNA sequence of the three fish strains. So, the present study suggested that oxidative stress resulted from environmental pollution could influence the stress biomarkers, however it, could not induce nucleotide replacements in fish strain under study.

Keywords: Pollution, Suez Gulf, Fish, Stress markers, DNA barcoding.

1. Introduction

Latterly, the environmental scientists have done much effort to save our environment from pollution risk. They concentrated on monitoring research studies of marine environment based on studding specific area, either by estimating the quality of coastal sediments [1, 2], seashells [3, 4], and/or water [5, 6]. Numerous studies on environmental monitoring have established the value of environmental indicators in assessing the origins and consequences of pollutants, coming to the conclusion that these indicators are the standard instrument for controlling environmental pollution [7-9]. The Red Sea, which connects to the Mediterranean Sea in the north and the Indian Ocean in the south via the Suez Canal, is located between Africa and Asia. Known for its abundant marine biodiversity and high endemism rates, the area is home to over 1,100 fish species and over 390 coral species. [10, 11].

The Red Sea coast extends to 1250 km from Suez to the Sudanese border. The marine environment provides human by different food resources and ecosystem services. Although the importance of marine environment to human, the anthropogenic activities cause deterioration to the marine environment. So, the periodic monitoring assessments for the marine environment have become urgently needed to predict and find solutions. Pollution sources in Red Sea differ from natural to manufactured sources. Industrial projects, petroleum industries, tourism, urban developments, shipping and fishing are considered the most common sources of pollution to the marine environment [12-14]

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Fish in Red Sea are the highly diverse group compared to other vertebrates. They are precious having economic and social values as the base for artisanal life styles of most coastal communities. Moreover, they assist important commercial fisheries [15]. Fish are considered as one of the important bioindicators in any aquatic system [16]. Fish are subjected to pollution and are used as indicators of the ecosystem health due to their nature living in aquatic environment and sensitivity to different pollutants [17].

In aerobic life, reactive oxygen species (ROS) are an unwanted byproduct. Their stable state level is determined by the balance between production and removal, which results in a particular level of ROS. "Oxidative stress" is the term used to describe when the dynamic balance is upset, leading to increased ROS levels and destruction of cellular components. It has been known that any stress usually leads to oxidative stress which may be due to the organism adaptation to different environmental stress factors [18].

The ability of dangerous substances to alter a cell's chromosomal and genetic code is known as "genotoxicity" [19]. Genotoxins are chemical pollutants that cause DNA/chromosomal spoil and were considered as main factors in carcinogenesis [20]. Apoptosis is an extremely controlled cellular process whereby the stimulation of specific death-signaling paths, cause death and deletion of cells from organism tissue. These paths are distinguished by intense variations in cellular structure causing self-destruction, and occur as natural development and aging [21]. Cell retraction, chromatin intensification, membrane leakage, and the formation of apoptotic bodies which macrophages phagocytose without inducing an inflammatory response are the distinctive morphological features of apoptosis [22]. Recently, DNA barcoding was applied to define unknown samples to enhance detection of new species [23]. The use of DNA barcoding led to significant advances in many complicated issues related to species taxonomy, biodiversity description in some regions and life history polymorphism [24].

Based on the above-mentioned remarks, the present study was concerned about using some fish species as bioindicator for pollution in Suez Gulf at three sites with different levels of pollution. Our research aimed to analyze: Reactive Oxygen Species, 8-Hydroxy-2-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG) by HPLC and apoptosis in liver cells. Moreover, DNA barcoding was applied in this study as a tool to fully identify the tested fish species.

2. Materials and methods

2.1. Study location

The study area was chosen to be covered during consecutive survey trips in different seasons, starting with the first transect from Suez Gulf (Fig. 1). This transect was divided into three stations to represent different pollution levels: Site 1 (Clean Area), site 2 (Petroleum & fishing activities) and site 3 (Al-attaka commercial landing site).

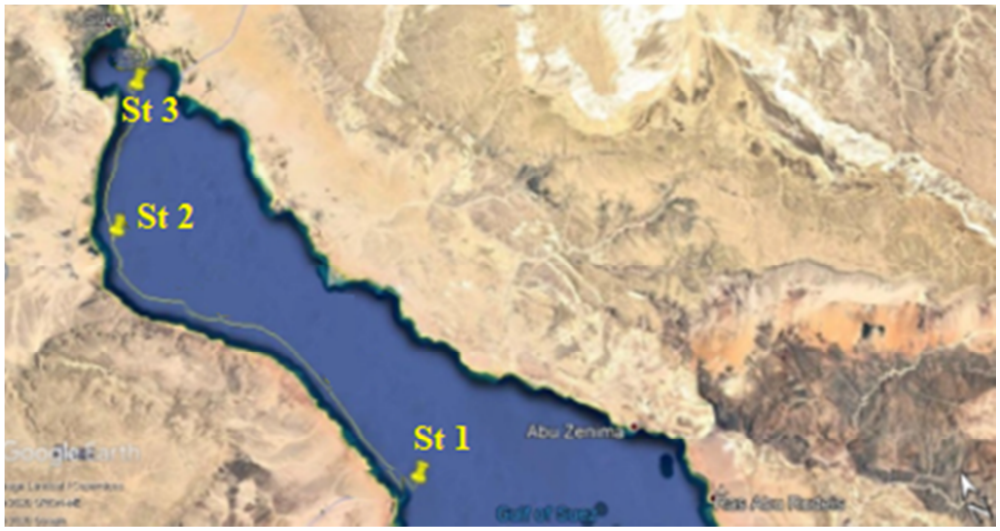

Figure 1: Sampling sites along from Suez Gulf

2.2. Fish Samples

The collected fish samples were identified referring to Morphological and morphometric characteristics. More details were characterized by molecular identification through DNA barcoding after transferring to Biotechnology and biodiversity conservation group, water pollution research Dept, National Research Centre.

2.3. Water Samples

Water samples for bacteriological experiments were obtained using sterilized glass bottles with a capacity of 1000 ml, which had been meticulously cleaned and thoroughly rinsed with distilled water prior to use [25].

2.4. Coliform Count

The enumeration of total coliform bacteria (TC) was performed through the multiple tube method, which identifies bacterial presence and estimates the most probable number (MPN) of total coliforms by inoculating measured water samples into tubes

with culture media. For fecal coliforms, the same volume of water samples was introduced into lactose broth, analogous to the procedure for total coliforms. After being kept at 37˚C for a period of time, the tubes showing positive results were then placed in an incubator at 44˚C for 48 hours. The probable number of fecal coliforms was determined using an MPN table in accordance with standard methods [25].

2.5. Evaluation of Reactive Oxygen Species generation

Liver tissues of collected fish were used to prepare single cell suspensions referring to Khalil e*t al*. [26]. The intracellular ROS production was measured by using a flow cytometer equipped and DCFH-DA fluorescent probe. At emission 525 nm and excitation 488 nm the cellular fluorescence was detected.

2.6. Analysis of 8-Hydroxy-2-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG) by HPLC

DNA was extracted from tested fish liver referring to Girgis et al. [27]. RNase (0.1mg/ml) was added to liver homogenates then incubated. Then we mix sodium acetate (3M) and ethanol (100%) with DNA at −20°C. Digestion of DNA was carried out using a CoulArray system (Model 5600). The UV detection was adjusted at 260nm. The HPLC was controlled and the data obtained and measured using CoulArray software.

2.7. Detection of apoptosis by acridine orange/ Ethidium Bromide

The morphological measurements of apoptotic variations in liver cells of fish species collected from various locations along Suez Gulf were conducted using a fluorescent microscope after being labeled with acridine orange/Ethidium Bromide (AO/EB) according to Czene *et al*., [28]. For each tested fish strain, about 100 cells were detected using fluorescent microscope (B2A filter) and necrotic cells /total cell count (×100). The apoptosis index was determined by substituting the percentage of necrotic cells with the percentage of observed apoptotic cells.

2.8. DNA extraction, PCR and sequencing

Three individuals from fish species were applied for DNA isolation following Malke *et al*., [29] for DNA barcoding by COI biomarker. The Cytochrome oxidase subunit one (COI) gene was amplified in a reaction mixture with total volume 25 µl involving 1x Taq polymerase buffer, 0.2 mM each dNTP, 0.4 uM of each primer, 2 mM MgCl2, 1 ng of sample DNA and 0.5 unit of Taq DNA polymerase / 2x Master Mix ampliTaq kit or both for PCR amplification of the gene. PCR amplification was performed with a thermocycler programmed PCR machine with different annealing temperatures to get the best condition for amplifying the genes. The primers used to amplify COI gene were: COI-F: 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3' and COI-R: 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' universal primers [30].

2.9. Statistical analysis

The analysis of all data was conducted utilizing the General Linear Models (GLM) procedure from the Statistical Analysis System [31], then by applying the Scheffé analysis to evaluate significant differences among fish samples. The analysis results are presented as mean ± SEM. All significance statements were determined considering a probability threshold of $P < 0.05$.

3. Results and Discussion

In this study, we are trying to set a reference point in order to measure the effect of different "environmental disturbances" on ROS generation, DNA adducts formation, apoptosis rate induction and DNA sequences alteration.

3.1. Fish Samples collection:

Three fish species were collected from the 3 selected sites, Suez Gulf and identified referring to Morphological and morphometric characteristics. Three fish species were identified and used in the following experiments in our study; *Sphyraena chrysotaenia* (*S. chrysotaenia*), *Scomberomorus commerson* (*S. commerson*) and *Decapterus russelli* (*D. russelli*).

Sphyraena chrysotaenia

An alien invasive species of barracuda, Family: Sphyraenidae. It's first record in Red Sea basin, by Egypt [32]. In addition to its economic value [33-35], *S. chrysotaenia* has exhibited the potential to affect the activities of invasive and native parasite-host relationships [36] .

Scomberomorus commerson

It is known as the narrow-barred Spanish Mackerel and it is a schooling migratory pelagic fish. The first record in Gulf of Suez: Egypt was by Bayoumi (1972). S. commerson is considered as a commercial species with high demand [37, 38].

Decapterus russelli

The Indian scad fish is a medium sized pelagic species. This fish is known to prey mostly on fish in the Levant, whereas crustaceans are major constituents in its diet [39].

3.2. Barcoding analyses and phylogenetic analysis

The DNA sequences were assembled and visually checked in CodonCode Aligner (CodonCode Corporation); Comparison with published studies and sequences. The Chromas Lite programme (available from Technelysium- Pty Ltd at the URL http://technelysium.com.au) was used to alter the raw sequences of COI genes that were the outcome. The GenBank database was compared using the BLAST algorithm with the partial COI gene coding sequences of the Egyptian samples of *D. russelli*,

Scomberomorus commerson and *Sphyraena chrysotaenia*. The MEGA11 software [40] package's Clustal W programme [41] was used to align COI sequences for *D. russelli*, *S. commerson* and *S. chrysotaenia* and other species of the genus Decapterus, Scomberomorus and *Sphyraena* that are present in the GenBank database.

3.3. Barcoding analyses

The universal primers amplified the target region in all tested species, generating 634 bp (Fig. 2). For more specific identification, DNA barcoding was followed by sending the purified PCR products for sequencing to Macrogen (Seoul, Republic of Korea) using an automatic ABI 370×1 DNA Sequencer (Applied Biosystem). DNA barcoding is a universal initiative that provides an integrated and functional genetic marker to catalogue and stocking marine biodiversity with significant conservation actions. The DNA barcoding technique is centered on a single part of the mitochondrial genome, chosen since it reflects conserved taxa suitable for primer design and covering polymorphism among different species [42].

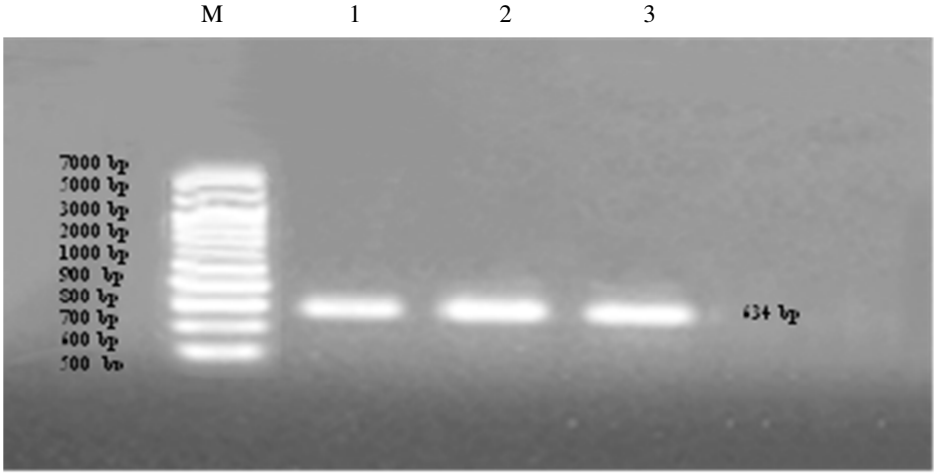

Figure 2: PCR products of COI gene of *D. russelli* (Lane1), *S. commerson* (Lane2) and *S. chrysotaenia* (Lane3)

3.4. Comparison with previously published studies and sequences

The COI sequences of samples fish species: *D. russelli* (acc. no. OP217100), *S. commerson* (acc. no. OP269543) and *S. chrysotaenia* (acc. no. OP269556) were deposited in GenBank/NCBI databases. This was conducted through: morphologically, BLAST comparisons for the detected sequences with their equivalents in the GenBank database, and the results phylogenetic tree. The present sequences of *D. russelli* showed 99% similarity with *D. russelli* (acc. no. KU499592.1), from Saudi Arabia. While Egyptian *Scomberomorus commerson* showed 100% similarity with *S. commerson* (acc. no. HM007790.1, KT251199.1, KF434774.1 and MT076823.1), 99% with *S. commerson* isolate (acc. no. MF737199.1) and lesser similarities with other *Scomberomorus* species, like 90 % with *S. niphonius* (acc. no. MF123123.1) and 89 % (acc. no. MW829380.1). On the other hand, Egyptian *Sphyraena chrysotaenia* showed higher similarities with *S. chrysotaenia* (acc. no. KY176643.1), but lesser similarities to other fish species of family Sphyraenidae, including, for example, 86% with *Sphyraena pinguis* (acc. no. JF952862.1).

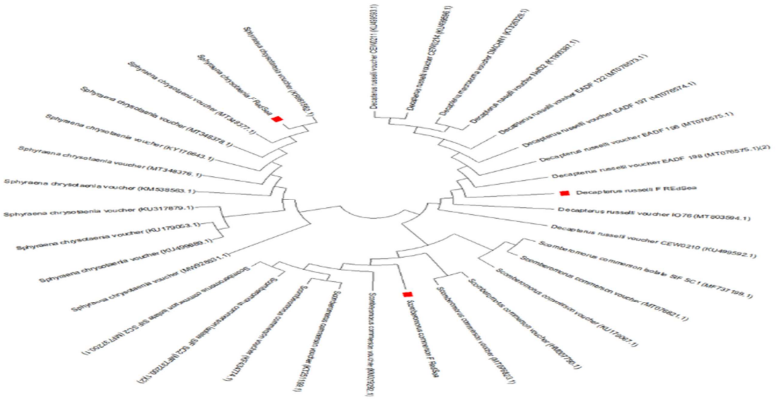

Figure 3: Maximum likelihood Phylogenetic tree for COI gene sequences of *D. russelli*, *S. commerson* and *S. chrysotaenia* collected from 3 different sites, the Red Sea and others retrieved from the GenBank database for the same species.

Phylogenetic examination for COI genes revealed a close proximity between all *Decapterus* species, mainly for Egyptian *D. russelli* presented in this research and from Saudi Arabia as well as Norway, being the two species found in the same subclade in the phylogenetic trees of analyzed genes (Fig. 3). Interestingly, COI sequences of *S. commerson* collected from Egypt were located in a single subclade with another (acc. no. HM007790.1 South Africa, KM079292.1, India and MT076823.1, United Arab Emirates). In addition, a sample of *S. chrysotaenia* collected from Egypt in the current study was grouped together in a single subclade with (acc. no. KR861562.1) from United States.

3.5. Counts of bacterial indicators

The counts of bacterial indicators, particularly presumptive coliform counts, are regarded as critical measures of microbial quality [43]. The most probable numbers (MPN) of indicator bacteria in three sampling locations from the Suez Gulf during one year are illustrated in the accompanying (Fig. 4). Total coliform counts exhibited the lowest values at both site 1 (the reference point) and site 2, remaining within the permissible limits set by WHO [44] (T.C < 500 CFU/100 ml). Conversely, the highest values were recorded at site 3 (Fig. 4 A). Fecal coliform counts were similarly minimal at sites 1 and 2. However, with the exception of site 3, all water samples collected from the study area contained bacterial populations exceeding the acceptable thresholds established by WHO [44] (*E. coli* < 100 CFU/100 ml). This indicates a significant environmental pollution effect on bacterial counts, particularly with increased proliferation noted at site 3.

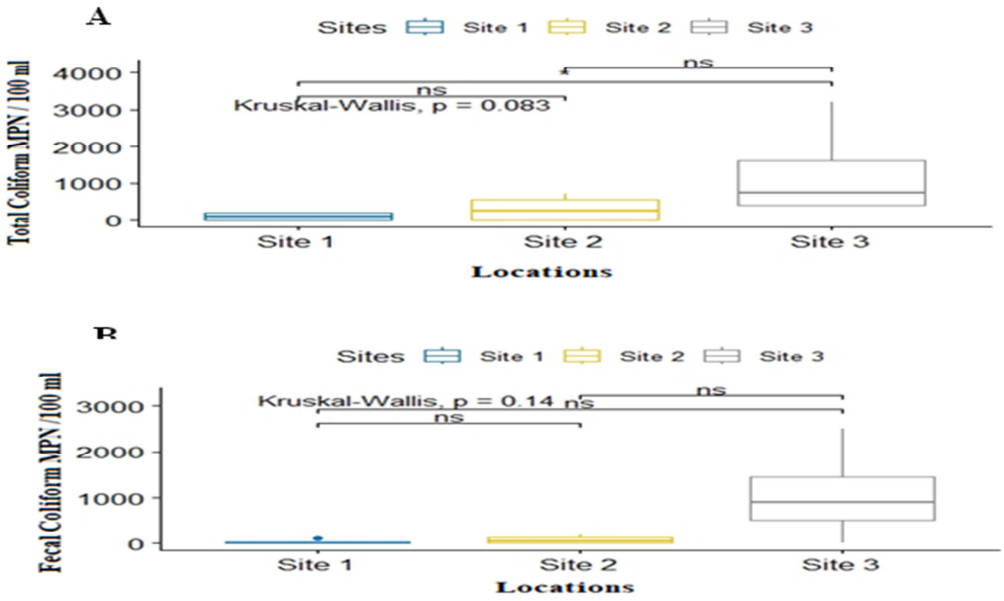

Figure 4: Box plots and results of the Krusskal-Wallis test of bacterial count of total coliform (A) and fecal coliform (B) in water samples recorded at the three sampling locations in Suez Gulf (*p, 0.05).

Factors that specifically influence bacterial life forms were measured to survey water quality within the Suez Canal. As temperatures rise, the dissolvability of oxygen and other gasses in water diminishes. When the water is exceptionally warm, it'll not contain sufficient oxygen for the life forms to outlive [45]. This study presents a considerable number of the monitored stations in the Gulf of Suez exhibited poor bacteriological water quality. There was a significant decline in bacteriological water quality across the entire region in site 3. This decline is likely attributed to a marked increase in tourist activities. Elevated concentrations of pollution indicator bacteria suggest a potential health risk to the public [46].

3.6. Variation of ROS levels in different sites

The present study illustrated the intracellular ROS generation in liver cells of fish species collected from the selected locations subjected to various pollution levels (Fig. 5). Free radical reactions and ROS are indicators to various physiological reactions of living organisms and in case of exposure to toxicity through different environmental pollutants enhanced an increase of oxidative stress resulted from ROS. Current information indicated that oxidative damage exists in aquatic organisms led to expand environmental and ecotoxicological research to aquatic animals to act as guards of environmental pollution [47]. The results of this study displayed significant (P<0.01) increase in the level of ROS formation (259.0±10.4) in

site-3 in comparison to site-1 (136.0±5.5) as considered as control location. Moreover, the level of ROS formation (220.0±9.5) in site-2 was increased significantly (P<0.05) as compared with those in to site-1 (136.0±5.5) (Fig. 5).

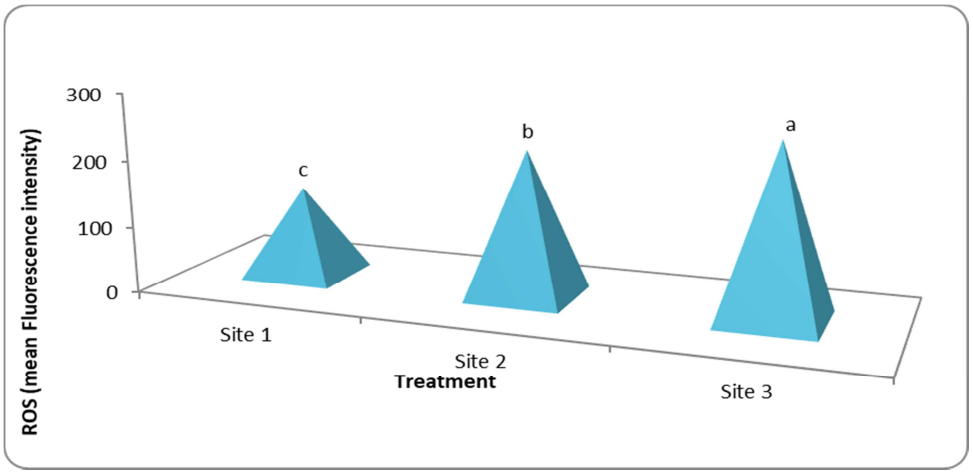

Figure 5: Intracellular ROS levels alterations in liver cells of fish species collected from different locations. a,b,c: Superscripts letters exhibiting significance differences between groups: Groups with various letters are significantly different at (p< 0.05).

It seems from our results that site 3 (Al-attaka) which is known as a polluted site showed the highest levels of ROS formation and this could be related to pollution in this site as a commercial landing site. Different pollutants can elevate the level of ROS formation, and cause oxidative stress and injury of tissues and cells [48]. Chemical toxicity induces high levels of ROS; for example, treatment with 4-tert-butylphenol-treatment induced formation of ROS and caused oxidative stress in grass carp hepatocytes (L8824) [49].

3.7. Generation of DNA adducts

A level of DNA adducts (8-OHdG) in liver cells of fish species collected from different locations. The following results revealed that site-3 showed significant (P<0.01) increase in the levels of 8-OHdG (18.37±1.47) compared with those in site-1 (5.75±0.77). Additionally, the levels of 8-OHdG (15.83±.85) in site-2 was increased significantly (P<0.05) when compared with those detected in site-1 (5.75±0.77) (Fig. 6). However, no significant differences were found in the levels of 8-OHdG between sit-2 and site-3 (Fig. 6).

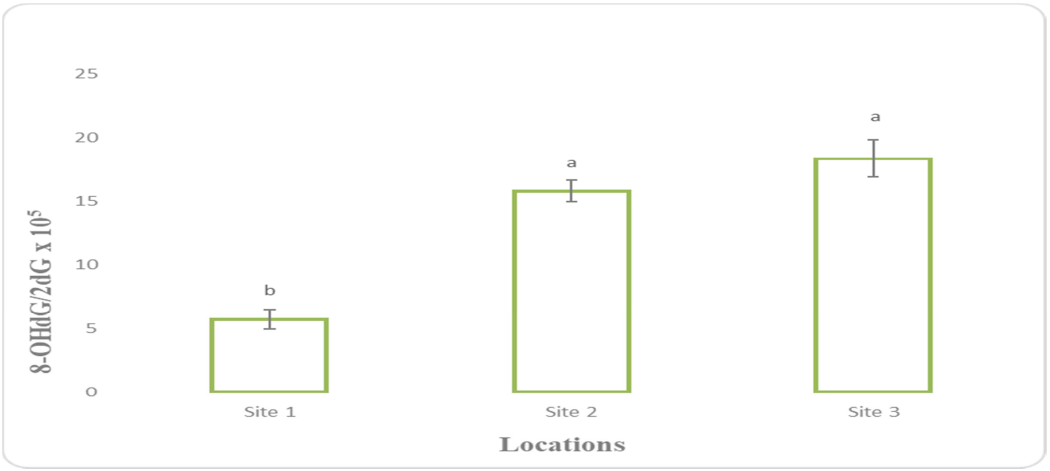

Figure 6: Generation of 8-OHdG in liver cells of fish species collected from different locations. a,b: Superscripts letters exhibiting significance differences between groups: Groups with various letters are significantly different at (p< 0.05); however, those with identical letters are non- significantly different.

DNA damage was studied in collected fish species from different sites. The levels of 8-OHdG (18.37±1.47) in site 3 (which considered as a polluted area) are higher than the other 2 sites. Similar results were obtained with Meier et al., [50] who found high records of DNA adducts in haddock fish liver exposed to various profiles of petrogenic or pyrogenic PAHs. DNA adducts are believed to be a critical biomarker of exposure in different organisms, particularly during first emergence after exposure to genotoxic, which may act a significant role in creating a mode of action for cancer [51]. DNA adducts in different fish species are stable and can be observed several months after removal fish to a clean environment [52, 53].

3.8. Apoptosis formation in fish species

The levels of apoptosis in liver cells of tested fish species collected from different sites are presented in Fig. (7&8). The results demonstrated significant (P<0.01) increase in the levels of apoptosis (32.79±2.92) in site-3 in comparison to site-1 (11.52±1.46) as considered as control site. Furthermore, the level of apoptosis (13.44±1.14) in site-2 was increased significantly (P<0.05) when compared to those detected in site-1 (11.52±1.46) (Fig. 7). Additionally, apoptotic cells were almost observed in fish species collected from site-3 and site-2 compared to necrotic and normal cells (Fig. 8). However, normal cells were almost found in fish species sampled from site-1 compared to apoptotic and necrotic cells (Fig. 8).

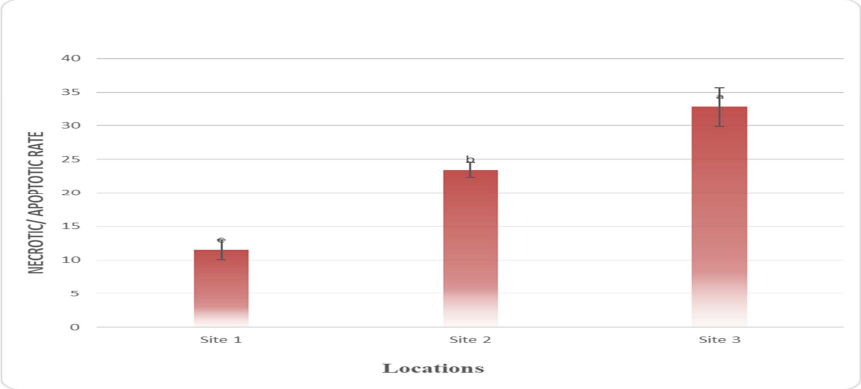

Figure 7: Alterations of apoptosis rate in liver cells of fish species collected from different locations. a,b,c: Superscripts letters exhibiting significance differences between groups: Groups with various letters are significantly different at (p< 0.05).

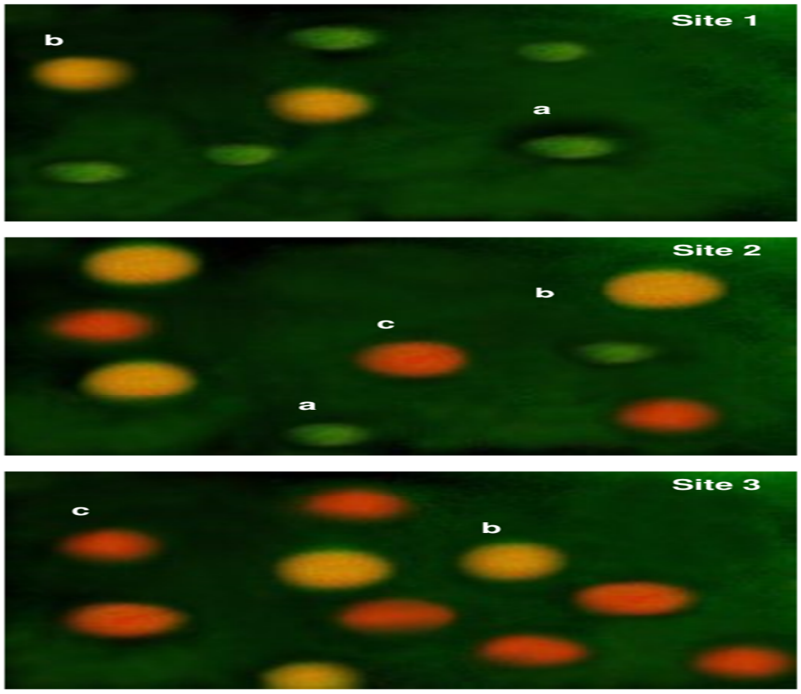

Figure 8: Fluorescent microscope examinations in liver cells of fish species indicating normal (a: living cells), apoptotic cells (b: apoptotic cells; c: necrotic cells) as checked by acridine orange/ Ethidium Bromide staining.

Apoptosis is known as an important toxicological indicator regulated by many parameters [54, 55]. In our results, high levels of apoptosis in liver cells of tested fish species were observed especially in site 3 which is considered the polluted site when compared to the other sites. High oxidative stress can accelerate procedure of oxidative phosphorylation in the mitochondria of organisms and lead to apoptosis besides, high levels of ROS and ATP liberation [56]. Matching with our results, pollutants in the aquatic environment enhanced the oxidative stress and apoptosis in common carp [57]. In some cases, oxidative stress can stimulate p53 and cause apoptosis [58].

4. Conclusion:

Fish are among the most important aquatic animals due to their economic and environmental impacts. The detection of total and fecal coliform bacteria and other chemical pollutants in the Red Sea possess a major threat to public health, marine ecosystems and economic efforts. Addressing these risks requires a comprehensive understanding of the underlying factors, effective monitoring and assessment, and implementation of appropriate mitigation measures. This study suggested that oxidative stress was able to induce apoptosis values due to increase the rates of DNA adducts and ROS generation at the polluted site3 as compared with other sites. However, DNA barcoding results did not reveal changes in the DNA sequences of the three fish strains. Therefore, the results suggested that oxidative stress induced by environmental pollution could affect stress markers but cannot induce nucleotide substitution in the fish strain under study. This research may contribute to a better understanding of the environmental health of the Suez Gulf and the effects of pollution on the local marine ecosystem.

5. Statements and Declarations

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

6. Competing interests

The authors declare that they have no competing interests.

7. Funding

There was no external support for this study.

8. Acknowledgements

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9. Author Contribution

All authors contributed in the manuscript. All the authors have given their approval to the final version of the manuscript.

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