Published Online First: May, 2024
https://doi.org/10.21123/bsj.2024.9312
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

Identification of some biochemical indicators to indicate the contamination of some fish farms in Hilla city/ Iraq

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Received 09/09/2023, Revised 20/11/2023, Accepted 22/11/2023, Published Online First 20/05/2024

Abstract

In order to establish the possibility of determined biochemical indicators of possible pollution in Al-Forat fish farm (A) and Hussein Ajimi fish farm (B) as well as wild fish samples as a control from Shatt Al-Hilla River (C). Oxidative stress factors and antioxidant enzyme activity were determined in common carp *Cyprinus carpio* blood serum from two fish farms. Reactive Oxygen Species (ROS), Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Hydrogen peroxide (H_2O_2) performance were chosen as pollution bioindicators, as well as Total Protein (TP) as a general bio indicator for fish health. Results showed an increase in all antioxidant enzymes activities and oxidative stress factors in fishes from station B which recorded 34.52 µg/ml, 81.46 U/ml, 75.55 KU/L, 4.36 ± 0.4 U/l, 11.82 nmol and 41.13 mg/l for ROS, SOD, CAT, GPx, H_2O_2 and TP respectively in a significant difference ($P < 0.05$) from stations A and C, which indicate the heighten of pollution in the Hussein Ajimi fish farm B than from Al-Forat fish farm A and the control fish from Shatt Al-Hilla River C. The study confirms the possibility of using oxidative stress factors and antioxidant enzymes as biochemical indicators of fish farms pollution.

Keywords: antioxidants, biochemical indicator, common carp, contamination, Oxidative stress.

Introduction

Many studies have used fish as indicators for observing pollution in aquatic ecosystems[1]. By checking the activities and concentrations of many types of antioxidant enzymes and oxidative factors as well as several metabolites as bio-indicators to estimate the effect of contamination on the physiological homeostasis and enzymatic action inside fish [2,3] and also for observing obnoxious ranges of aquatic pollution [4]. Because of environmental pollutants effects, fishes are stressed and infectious diseases may arise, they are exposed to an oxidative impression of diverse contaminants that exist in the aquatic ecosystems [5].

In polluted environments, the water was converted to destructive ROS like anion superoxide (O_2^-). These free radicals can cause oxidative stress in the fish, a disturbance in the equilibrium between the production of ROS and antioxidant defense structure in the cell [6]. Oxidative stress is a condition associated with cell destruction caused by free radicals [7,8]. To avoid this tension the organism has

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developed antioxidant resistance tools[6,9]. The defense mechanisms of the cellular antioxidant detoxification system include antioxidant enzymes like the GPx/ glutathione reductase (GR) coordination, SOD, and CAT [10]. These antioxidants especially glutathione peroxidase take the most responsibility for ROS removal [11]. SOD is distributed widely in all organisms, it plays a crucial function in defense contrary to cell oxidative damage caused by high contents of ROS, via catalyzing the conversion of these superoxide anions to H_2O_2 and O_2 [11-14]. SOD was considered the first detoxification enzyme for free radicals created from the oxidative stress effect and operates as the first-line resistance structure versus ROS [15]. Increasing activity of SOD refers to excessive amounts of superoxide anion, and fishes were subjected to greater ranges of free radicals[6].

Catalase is a popular antioxidant enzyme that exists in the majority of aerobic reactions [15]. CAT or GPx destroyed H_2O_2 once SOD has dismutase the free radical into H_2O_2 by converting it to H_2O and O_2 [16]. Moreover, CAT is able to oxidize toxins such as phenols, alcohols, formic acid and formaldehyde at low concentrations of H_2O_2 [11,17]. SOD and CAT are essential antioxidant enzymes in the fish defense system against oxidative stress, which protect fish from tissue damage [11]. The increasing of CAT activity mentioned to excess presence of H_2O_2 inside fish, and they tolerate greater intensities of ROS, which lead up to oxidative stress [6].

GPx is an antioxidant that analyzes H_2O_2 to H_2O by using Glutathione GSH as an electron donor [15,18]. This enzyme is capable of catching superoxide anions to inhibit fat oxidation and preserve the internal equilibrium from oxidative stress [19]. Higher levels of fish GPx are signals of the excess presence of oxidative stress factors [15].

The study aimed to identify the possible pollution occurring in two fish farms, Al-Forat fish farm and Hussein Ajimi fish farm in Hilla City by measuring the actions of ROS, H_2O_2, SOD, CAT and GPx as biochemical indicators of oxidative stress as well as TP as a general bioindicator for common carp fish health.

Materials and Methods

Experimental fishes

Two fish farms were selected to estimate the pollution status, Al-Forat fish farm (A) and Hussein Ajimi fish farm (B) as well as control fishes was samples from Shatt Al-Hilla River (C) Fig. 1. The two farms are integrated productive farms which starts from egg production, fertilization, hatching, nursing of fries and fingerlings till the marketable size.

The largest fish farm with Iraq is Al-Forat fish farm in a total area of 542.5 hectares (5425000 m^2) including water area of 418 hectares (4180000 m^2). It is located in Annana village toward the west of Abu-Garaq town at about 11 kms to the north-west of the center of Babylon province, on the latitude (32. 55065') and longitude (44. 38233´). The water source of this farm comes from Al-Hilla River[20,21]. The farm includes a hatchery with an annual production capacity of 60 million fingerlings, 46 nursery ponds with a total area of 7.5 hectares (75000 m^2), four ponds for fries and fingerling nursing with a total area of 35 hectares (350000 m^2), nine ponds for fish breeding with about 36-48.5 hectares an area for each, and six mothers ponds with an area of 0.39- 0.5 hectare for each[20]. Three types of carp are cultured there, common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*) and the silver carp (*Hypophthalmichthys molitrix*). The dominant cultured fish with more than 95% ratio is the common carp. Fish were fed a sink artificial diet manufactured locally in the farm site composed of raw materials (soybean meal, wheat bran, barley, corn) with total protein and fat percentages of 23%, 0.42% respectively. The feeding process was done by hand twice daily (field observations)[20].

The second station Hussein Ajimi fish farm is located in Albualwan village to the southwest of Al-Mahaweel district, on the latitude (32. 61292') and longitude (44. 37787´), in a total area of 60.5 hectares (605000 m^2) including a hatchery with an annual production capacity of 20 million fingerlings, 10 nursery ponds with a total area of 2.5

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hectares (25000 m^2), four ponds for fries and fingerling nursing and breeding with a total area of 45 hectares (450000 m^2), and two mothers ponds with an area of 0.2 hectares for each (2000 m^2)[21]. The water source of this farm comes from Shatt Al-Hilla River. The farm management used a monoculture system with common carp *Cyprinus carpio* fishes. Fishes were fed a sink artificial diet manufactured locally in the farm site consisting of the raw materials (fish meal, soybean meal, wheat bran, barley, corn, vitamins & minerals) with total protein and fat percentages of 32% and 12% respectively. The feeding process was done by hand three times or more daily (field observations).

Fishes from farm A were collected on the morning of 14/10/2022, and fishes from farm B were collected on the morning of 8/11/2022, while wild fishes from C station were collected on the morning of 15/11/2022. Some environmental factors of water in the three stations including temperature, salinity, dissolved oxygen and pH were recorded using YSI instrument model (556 MPS). A total of 120 fish were collected from the three stations using draft nets with mesh size 2×2cm, they were transferred to the laboratory using cooling boxes. Length (cm) and weight (g) were recorded after anesthetizing fish with clove oil for the purpose of collecting blood samples[22]. It was gathered from the heart using a 3 ml syringe, and then transferred to sterilized tubes, then left at room temperature for 2-3 hr. to be clot and transferred to the refrigerator for 24 hr. A refrigerated centrifuge model (5417 R) manufactured by (Eppendorf, Germany) was used to separate serum from the clotted blood at 6000 rpm for 5 minutes.

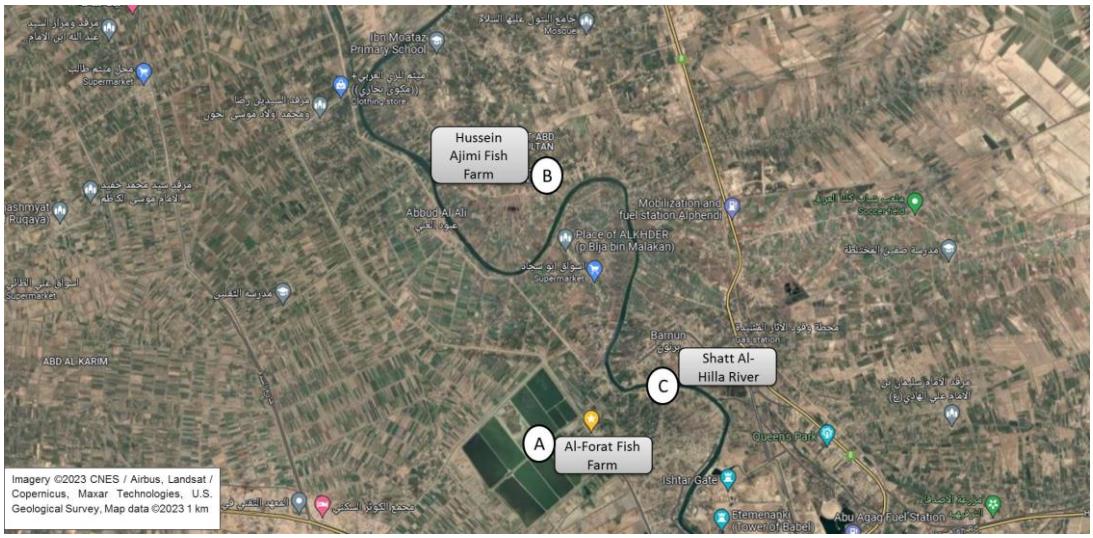

Figure 1. Map showed the location of the three study stations.

Oxidative stress factors determination

Reactive Oxygen Species (ROS) was estimated according to [23] Ferric-xylenol orange (FOX1) assay method which is based on the oxidation of ferrous ion–*o*dianisidine complex to ferric ion by the oxidants contained in the plasma or serum samples. The assay works with two reagents, reagent 1 consists of (xylenol orange 150 µM, NaCl 140 mM and glycerol 1.35 M in H2SO4 25 mM); reagent 2 consists of (ferrous ammonium sulfate 5 mM and *o*-dianisidine dihydrochloride 10 mM in H2SO4 25 mM). The assay procedure included adding 140 µl of fish serum to reagent 1(900 µl) and reagent 2 (44 µl). The mixture was then shacken for 30 min at room temperature. The absorption was read in a wavelength of 560 nm utilizing a V-550 UV-vis spectrophotometer.

Hydrogen Peroxide (H_2O_2) was measured concerning the [24] procedure by adding 100 µl of fish serum to iced Trichloroacetic acid TCA 0.1% w/v, a refrigerated centrifuge was used to separate the supernatant at 15,000 rpm for 15 min at 4°C, the supernatant was added in a volume of 0.5 ml to a mixture of [10 mM phosphate buffer (pH 7.0) in a volume of 0.5 ml and 1 M KI in a volume of 1.0 ml], stirred gently. Absorption was measured within

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https://doi.org/10.21123/bsj.2024.9312
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

1 min at 390 nm using a V-550 UV-vis spectrophotometer. H_2O_2 is quantified as nmol in a calibration curve utilizing standard solutions with identified H_2O_2 concentrations.

Antioxidant enzymes activities determination

Superoxide Dismutase SOD was determined according to [25] rapid procedures depending on the capability of the enzyme to prevent pyrogallol autoxidation. The enzyme action is expressed as U/ml which is described as the quantity of SOD enzyme needed to inhibit 50% of pyrogallol autoxidation. The assay procedure involved adjusting the Spectrophotometer to zero reads using a buffer solution of Tris-EDTA. Absorptions of control and samples were stated at a wavelength 420 nm against Tris-EDTA buffer at zero time and after 1 minute of the addition of pyrogallol.

Catalase CAT KU/l estimated according to [26] assay by adding 0.2 ml of fish serum to 1 ml of substrate (H_2O_2 65 µmol per ml in sodium-potassium phosphate buffer 60 mmol/L, pH 7.4) and then incubated for 1 min at 37 °C. After that adding 1 ml of the reagent ammonium molybdate 32.4 mM, a yellow suspension which is a complex of molybdate with hydrogen peroxide created, the complex absorption was read at 405 nm using a Jasco V-550 UV-vis spectrophotometer.

Glutathione peroxidase GPx concentration was conducted corresponding [27] methods, and its principle was established depending on the reduction of glutathione GSH content after the fish serum interaction with H_2O_2 and NaN3. The assay Procedure included adding 0.1 ml of serum to [5mM of GSH/ 0.1 ml, 1. 25 mM of H_2O_2/ 0.1 ml, 25 mM of NaN_3/ 0.1ml and 0.05 mM of phosphate buffer (pH 7)], 2.5 ml is the total volume of mixture which incubated for 10 min at 37°C. Then 2 ml of 1.65 % $HPO3^{-2}$ was added to stop the reaction, centrifugation after at 1500 rpm for 10 min., taking 2 ml from the supernatant and mixed with [2 ml of 0.4 M Na_2HPO_4, 1ml of 1mM DTNB]. A yellow complex was formed, it incubated at 37°C for 10 min, then its absorption was read at 412 nm using a V-550 UV-vis spectrophotometer.

Total protein TP determination

TP mg/l in fish serum was estimated according to [28]. 100 µL of sample was added to 1.5 ml of 0.2 M phosphate buffer (pH 7.2), then centrifuged at 3000rpm for 10 minutes. An equal amount of 5% cold TCA was added to the supernatant, and left in the ice bath for 30 minutes. Precipitated protein was centrifuged, taking the sediment and dissolving in 0.2N NaOH/ 2.5 ml, then adding 0.1 ml of the mixture to a reagent of alkaline copper in a volume of 0.4 ml, putting the reactants in a shaker for 10 minutes, then adding 0.1 ml of Folin phenol reagent and left the mixture for 20 minutes at room temperature. Absorption was read at 650 nm using UV-VIS Spectrophotometer using a standard solution of Bovine Serum Albumin.

Collection of Fish, carefulness and handling were achieved according to Canadian Council on Animal Care guidelines for the utilization of fish in scientific research CCAC [29].

Data analysis

All values were shown as mean ± standard error. The significance of differences among the three stations was statistically investigated with one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) in a significant grade ($P < 0.05$) using IBM SPSS program (version 26) [30].

Results and Discussion

Study stations and experimental fishes

Table 1 shows some environmental factors of the three study stations' water. Results of temperature and dissolved oxygen showed considerable differences ($P < 0.05$) among the three study stations, the Hussein Ajimi fish farm B gave the highest value 28 ± 0.04 °c for temp. and the lowest value was 4.82 ± 0.05 mg/l for D.O., while statistical investigation for salinity showed no significant variances ($P > 0.05$) among the three sites. Data for pH presented no significant variances ($P > 0.05$) between farms A and B and significant differences ($P < 0.05$) among the two farms with site C.

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Table 1. Environmental factors recorded in water of the three study sites (mean ± S.E.).

Environmental factor	A	B	C
Temp. °c	27.8 ± 0.05[b*]	28 ± 0.04[a]	27.49 ± 0.06[c]
Salinity psu	1.55 ± 0.005[a]	1.4 ± 0.11[a]	1.44 ± 0.009[a]
D.O. mg/l	5.77 ± 0.09[b]	4.82 ± 0.05[c]	6.96 ± 0.07[a]
pH	7.37 ± 0.12[b]	7.37 ± 0.03[b]	8.22 ± 0.009[a]

A referred to the Al-Forat fish farm, B referred to the Hussein Ajimi fish farm and C referred to the Al-Hilla River. Temp. referred to Temperature, D.O. referred to Dissolved Oxygen and pH referred to potential Hydrogen.
*Different letters mean there were significant variances (P < 0.05) among stations.

Results for fish total length and total weight indicated no significant variances (P > 0.05) between farm A and station C and significant differences (P < 0.05) among farm B and other stations (Table 2). The mean total length and weight for common carp fingerlings in the three stations were 19.76 ± 0.88 cm, 130.97 ± 16.04 respectively.

Table 2. Total length and total weights for common carp fishes from the three study sites (mean ± S.E.).

Factor	A	B	C
Length cm	21.11 ± 0.99[a]	18.11 ± 0.35[b*]	20.07 ± 0.42[a]
Weight g	154.13 ± 16.27[a]	100.17 ± 7.08[b]	138.61 ± 5.64[a]

A referred to Al-Forat fish farm, B referred to the Hussein Ajimi fish farm and C referred to control fishes from the Al-Hilla River.
*Different letters mean there were significant variances (P < 0.05) among sites.

Oxidative stress factors and antioxidant enzymes activities

Results showed that fishes from Hussein Ajimi fish farm B gave the highest values for all oxidative stress factors and antioxidant enzymes 34.52 ± 1.53 µg/ml, 11.82 ± 1.59 nmol, 81.46 ± 4.13 U/ml, 75.55 ± 5.93 KU/L and 6 ± 0.27 for ROS, H_2O_2, SOD, CAT and GPx respectively, and this fishes presented significant variances (P < 0.05) with other fishes from Al-Forat fish farm A and control fishes from Shatt Al-Hilla river C in all physiological parameters Figs. 2-6 respectively). While fishes from farm A showed no significant variances (P > 0.05) with fishes in C station (control fishes) in ROS and H_2O_2 activities which were 7.69 ± 0.9 µg/ml and 3.74 ± 0.21 nmol respectively for farm A and 4.24 ± 0.22 µg/ml, 2.25 ± 0.28 nmol respectively for control fishes in station C (Figs. 1, 2 respectively). Nevertheless, the antioxidant enzymes activities in fishes from farm A had significant differences (P < 0.05) with fishes from station C which amounted to 57.55 ± 1.41 U/ml, 49.12 ± 1.95 KU/l and 4.36 ± 0.4 U/l for SOD, CAT and GPx respectively in fishes from Al-Forat fish farm A and 22.85 ± 0.27 U/ml, 21.45 ± 0.61 KU/l and 2.36 ± 0.13 U/l for SOD, CAT and GPx respectively in control fishes from Shatt Al-Hilla River C, (Figs. 4, 5, 6 respectively).

Total protein content

The highest value of serum TP was obtained in fishes from Hussein Ajimi fish farm (B) 41. 13 ± 2.23 mg/l, followed by fishes from Al-Forat fish farm (A) 20. 05 ± 1.09 mg/l, and after control fishes from Shatt Al-Hilla River (C) 19.24 ± 0.32 mg/l. Statistical tests exhibited significant variance (P < 0.05) among fishes from farm B and other stations A, C. While fishes from farm A presented no significant differences (P > 0.05) with control fishes in station C, Fig. 7.

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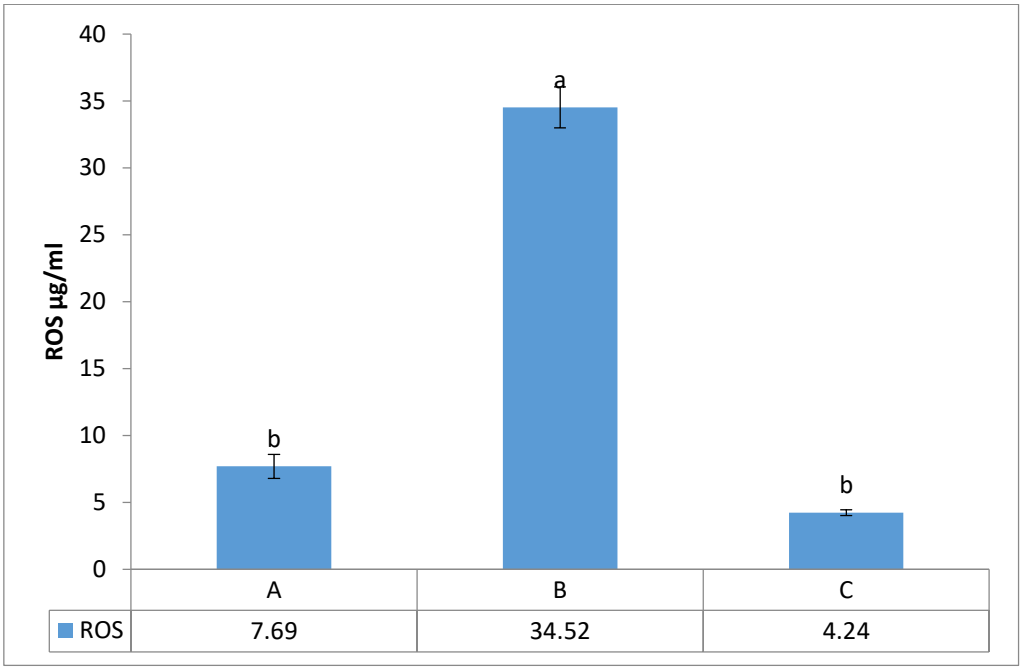

Figure 2. Reactive Oxygen Species ROS µg/ml activities in serum of common carp from the three study stations (mean ± S.E.).

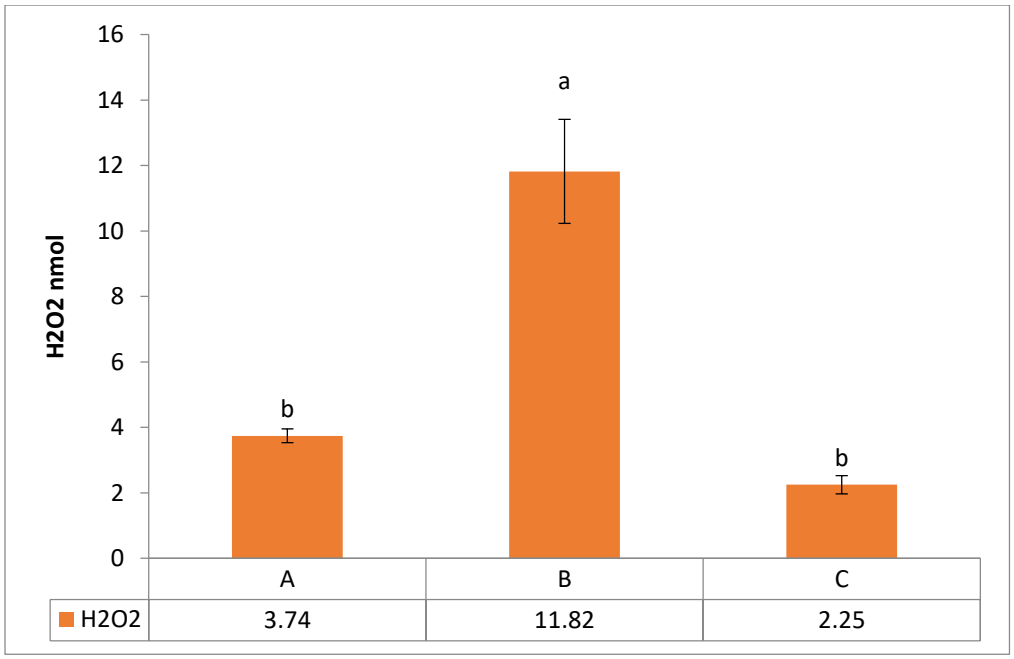

Figure 3. Hydrogen Peroxide H_2O_2 nmol activity in serum of common carp from the three study stations (mean ± S.E.).

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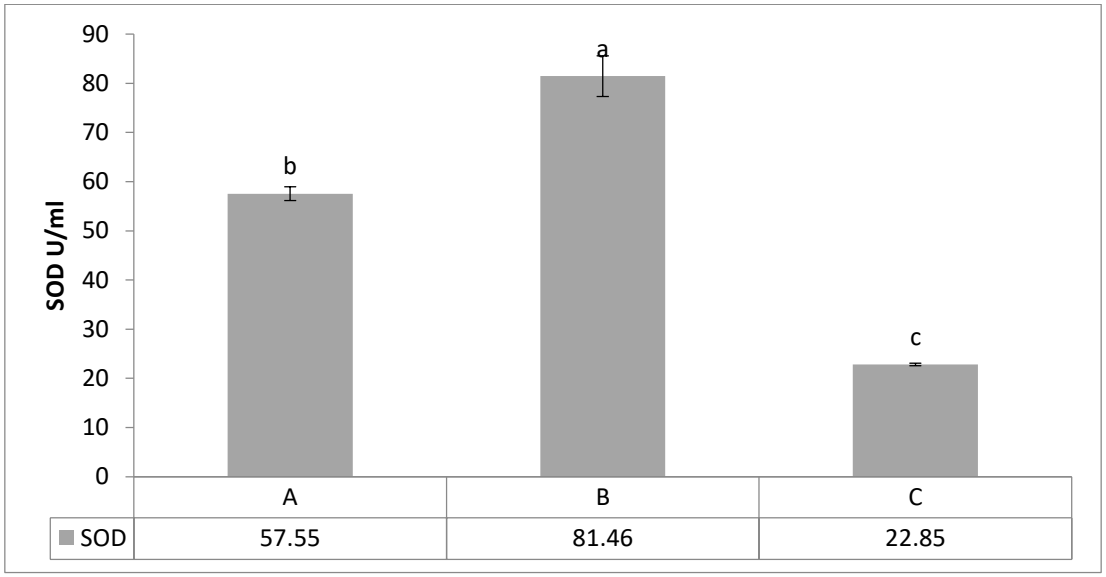

Figure 4. Superoxide Dismutase SOD U/ml activity in serum of common carp from the three study stations (mean ± S.E.).

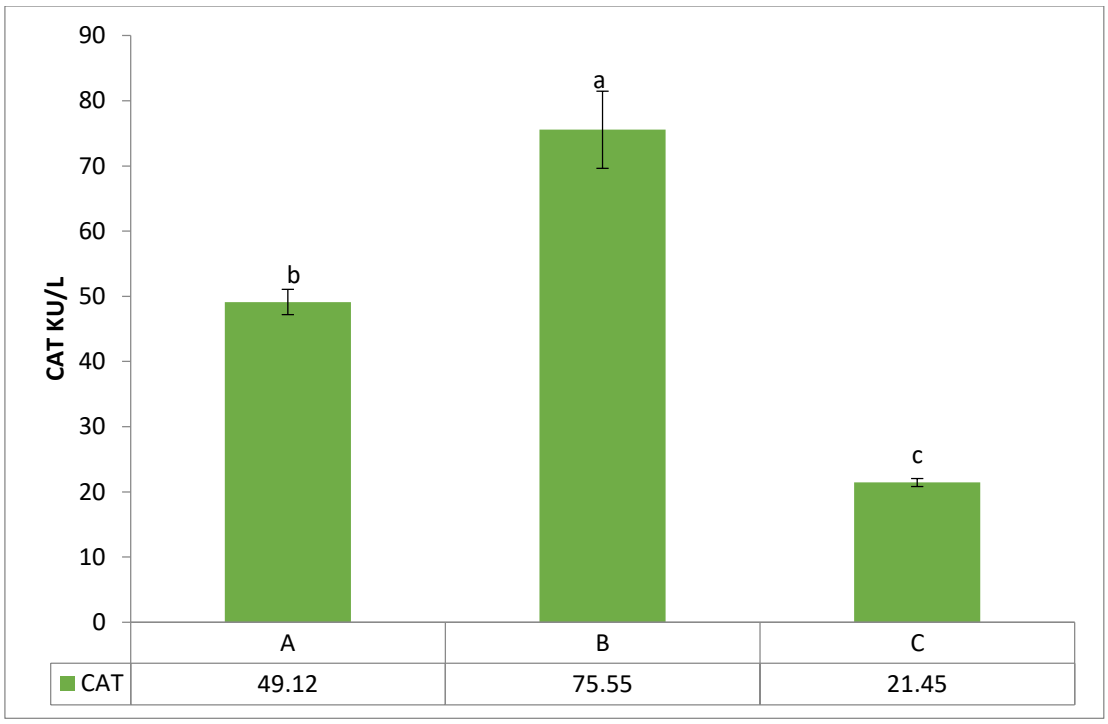

Figure 5. Catalase CAT KU/l activity in serum of common carp from the three study stations (mean ± S.E.).

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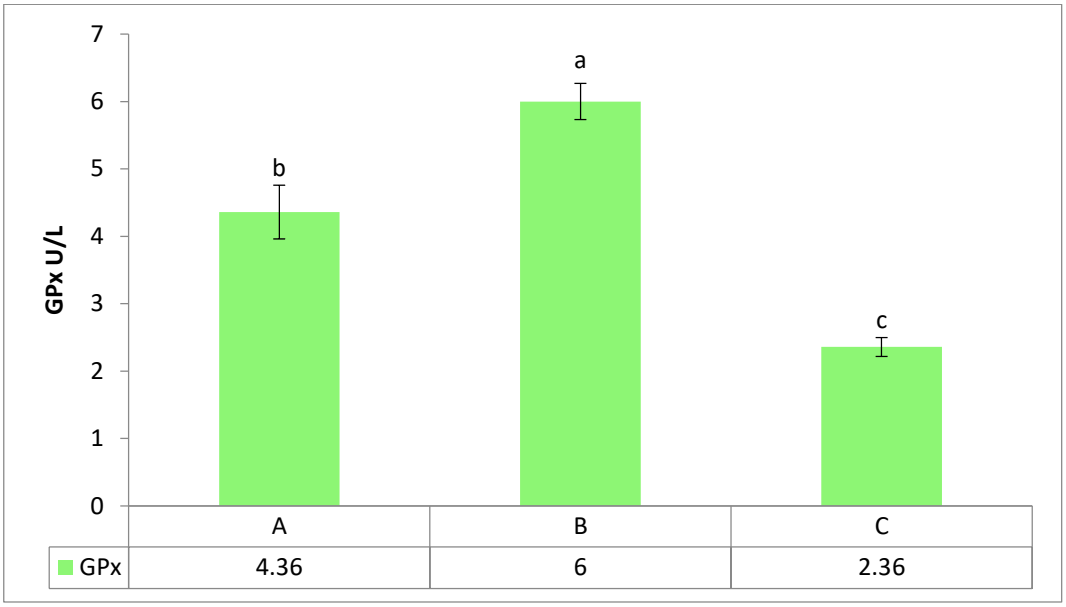

Figure 6. Glutathione Peroxidase GPx U/l activity in serum of common carp from the three study stations (mean ± S.E.).

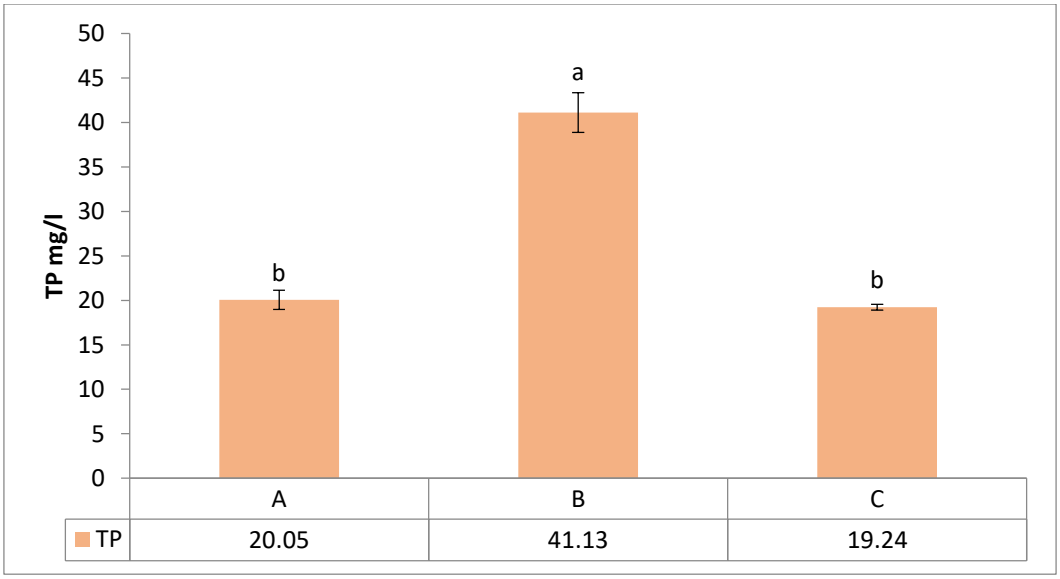

Figure 7. Total Protein TP mg/l activity in serum of common carp from the three study stations (mean ± S.E.).

Discussion

Oxidative stress biochemical markers present distinctive information about the aquatic ecosystem's health through their close association with the pollution that affects the health status of the living organisms present there [31]. Results of the study presented those fishes from the Hussein Ajimi fish farm (B) gave the highest values for all oxidative stress factors and antioxidant enzymes as well as total protein in significant variances ($P < 0.05$) from those in the Al-Forat fish farm (A) and the control fish from Shatt Al-Hilla River (C). This reflects the high pollution status of this farm and this current pollution may be due to the farm management method used. The activities practiced was obtained from the two farms authority, although the Hussein Ajimi fish farm used artificial

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diets with better nutritional values (32% total protein, and 12% total fat) than what was used in the Al-Forat fish farm, the wrong management and excessive provision of diets to the fish led afterheat to the accumulation of surplus feed at the bottom of the pond, which leads to rotting of the pond water and an increase in the percentage of pollution in it. We observed the Al-Forat fish farm, which is the largest fish farm in Iraq [32], despite the use of fish diets that are actually poor in the total protein ratio (23%), since the diet lacks fish meal, it is also, unlike the diet used in Hussein Ajimi fish farm B, with low-fat content (0.42%), and this type of low-fat diet reduced the percentage of pollution in the fish pond when utilized. In addition, the feeding rate of 5% of the body weight used in managing the fish ponds in the Al-Forat fish farm is a good percentage and does not lead to the accumulation of residual diets in the pond.. Also, the wide areas of fish ponds in the Al-Forat fish farm, the appropriate stocking densities, and the continuous pond water exchange operations also reduced the incidence of pollution (field observation), and the results obtained confirm this matter, since there are no significant differences ($P > 0.05$) between the Al-Forat fish farm, and the control fish from the Shatt Al-Hilla River in the oxidative stress factors ROS and H_2O_2, as well as TP.

This study corresponded with many studies on evaluating the state of contamination in aquatic environments using antioxidant enzymes and oxidative stress factors in fish [33] used CAT, SOD, GPx, GR and GST in gills, muscles and hepatopancreas, of crayfish *Orconectes limosus* as biomarkers of contamination in the Danube River or seasonally changed antioxidant enzymes. Investigation by [31] suggested that the use of oxidative stress biomarkers (CAT, GSH, SOD) was important to estimate the effects of untreated wastewater on fish in the Ataturk Dam Lake [5], were chosen SOD and CAT in the blood of *Barbus m. petenyifish* as bioindicators to evaluate Lake Ohrid's healthy status affected by anthropogenic pollution [11]. Studies pointed out that CAT and SOD occupy essential roles in preserving Yangtze Sturgeon *Acipenser dabryanus* against starvation-induced oxidative stress. A Study by [1] showed that contamination with methyl parathion induced oxidative stress in *Catle catle* fish and levels of antioxidant enzymes had arisen to face the effects of high levels of ROS [34], found changes in the anti-oxidant enzymes and the oxidant factors of seminal plasma in common carp and rainbow trout with seasons change, and they attributed that to the increases in metabolic rates during the late phase of spawning [4]. Results confirm that the activities and expression of CAT, SOD, GST and MDA in *Oreochromis niloticus* liver can be used as bioindicators to estimate the effect of crude oil pollution [35]. Estimated the activities of SOD, CAT and GST in *Cyprinus carpio* plasma to notice the pollution status in the Sitnica River in Kosovo, the three antioxidant enzyme's activities were superior in fish from contaminated regions, they concluded that waste discharges from cities and industries are the main pollutants in this river.

Measuring the total protein content is considered a general bio indicator of the fish's healthiness. A rise in total protein content level may be an indication of the state of stress to which the fish are exposed [36]. Many studies have found a rise in total protein content associated with an increase in antioxidant enzymes and oxidative stress factors levels [37-40].

Conclusion

The study confirmed that the estimation of antioxidant enzyme activities (CAT, SOD and GPx) and oxidative stress factors (ROS and H_2O_2) in the serum of cultured *Cyprinus carpio* was beneficial to evaluate the environmental pollution status at the two fish farms.

The final conclusion of evaluating the healthy status of the two fish farms presence heightened of pollution in the Hussein Ajimi fish farm (B) than from the Al-Forat fish farm (A) and the control fish from Shatt Al-Hilla River (C).

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Acknowledgment

The authors would like to extend their thanks and gratitude to all the employees of the environmental research and studies center, where the study was carried out in the Laboratory of Biodiversity.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Babylon.

Authors' Contribution Statement

L. M. A.A. A. Research idea presentation, selection of study areas, collection of samples from the field, laboratory work, Methodology, data analysis, writing the original draft, and review and editing. Z. H. O. Collection of samples from the field, Methodology. J. M. S. Supervision, discuss results and contribute the final manuscript.

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تحديد بعض المؤشرات الكيميائية الحيوية للدلالة على تلوث بعض المزارع السمكية في مدينة الحلة / العراق

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الخلاصة

من اجل اثبات إمكانية استخدام بعض المؤشرات الكيموحيوية لاحتمال التلوث في مزرعة أسماك الفرات (أ) ومزرعة حسين عجيمي (ب) وكذلك عينات الأسماك النهرية كعينات سيطرة من نهر شط الحلة (ج). تم تحديد عوامل الإجهاد التأكسدي ونشاط الإنزيمات المضادة للأكسدة في مصل دم سمك الكارب الشائع *Cyprinus carpio* من مزرعتين سمكيتين. تم اختيار أنواع الأوكسجين التفاعلية (ROS) ، والأوكسيد الفائق (SOD) ، والكتلاز (CAT) ، وأداء الجلوتاثيون بيروكسيديز (GPx) ، وأداء بيروكسيد الهيدروجين (H2O2) كمؤشرات بيولوجية للتلوث ، وكذلك البروتين الكلي (TP) كمؤشر بيولوجي عام لصحة الأسماك. . أظهرت النتائج ارتفاع جميع أنشطة إنزيمات مضادات الأكسدة وعوامل الإجهاد التأكسدي في الأسماك من المحطة B حيث سجلت 34.52 ميكروغرام / مل ، 81.46 وحدة / مل ، 75.55 كيلو وحدة / لتر ، 4.36 ± 0.4 وحدة / لتر ، 11.82 نانومول و 41.13 مجم / لتر لكل من ROS و SOD و CAT و GPx و H2O2 و TP على التوالي بفارق معنوي ($P < 0.05$) من المحطات A و C ، تشير النتائج إلى زيادة التلوث في مزرعة حسين عجيمي السمكية B مقارنة بمزرعة أسماك الفرات A والأسماك النهرية C . تؤكد الدراسة إمكانية استخدام عوامل الإجهاد التأكسدي والإنزيمات المضادة للأكسدة كمؤشرات بيوكيميائية لتلوث المزارع السمكية.

الكلمات المفتاحية: اجهاد التأكسد، مضادات الاكسدة، مؤشر كيموحيوي، الكارب الشائع، تلوث.