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The possibility of using *Culex pipiens* (Diptera: Culicidae) larvae as a bioindicator of water pollution in Burullus Lake, Egypt

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Abstract

The urgent environmental issue of heavy metal pollution in aquatic environments has brought attention to Burullus Lake due to its environmental and economic significance. Therefore, this study focused on using the third-instar larvae of *Culex pipiens* as bioindicators of metal pollution in two Burullus Lake sites: Al-Bughaz (site 1, reference) and the front of Drain 7 (site 2, polluted). The water physicochemical analysis confirmed site 2's higher pollution levels. Metal concentrations in water and larval midgut tissues from site 2 were significantly higher than in site 1. All biochemical parameters estimated in site 2's larval midgut tissues showed greater impacts compared to site 1. Concentration levels of malondialdehyde and metallothionein along with expression level of heat shock protein 70 were notably higher in site 2's larvae. Antioxidant biological markers, like reduced glutathione, superoxide dismutase, catalase, and glutathione peroxidase, as well as cytochrome P450 activity decreased significantly in site 2's larvae compared to the reference larvae from site 1. Analysis of DNA damage parameters in the midgut cells of site 2's larvae revealed significant rises in tail DNA%, tail length, and tail moment. DNA damage was increased in site 2's larvae due to migration of DNA fragments. Ultrastructure observations of site 2's larvae revealed structural alterations along the epithelial cells of midgut tissue like destroyed microvilli, an irregular nuclear envelope, vacuolated cytoplasm, increasing lysosomal bodies, disintegrated mitochondria, and the appearance of spherites and dense vesicles. In conclusion, *C. pipiens* larvae are reliable bioindicators for metal pollution in aquatic environments due to their sensitivity to metal bioaccumulation.

Keywords: Burullus Lake, heavy metals, *Culex pipiens*, bioindicator, oxidative stress, DNA damage

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Introduction

Water contamination is a major issue on a global scale. It is considered a cause of diseases and death worldwide. Each year, approximately 1.4 million individuals die as a result of the effects of water contamination **(1)**. In Egypt, water resources are becoming more and more contaminated by dangerous chemical compounds due to the expansion of anthropogenic, industrial, and agricultural activities near water sources **(2, 3)**. The Burullus Lake, connecting with the Mediterranean Sea through the Al-Boughaz opening, has garnered considerable interest owing to its environmental and economic importance. There is growing public concern regarding the assessment of heavy metal

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distribution and contamination levels in the lake due to their potential detrimental impacts, long-term presence, and accumulation within aquatic ecosystems and their bioaccumulation in various biotic systems posing a threat to human health **(4-6)**. Therefore, the biomonitoring of heavy metal water pollution is needed to counter this problem.

Aquatic insects are effective bioindicators of metals in aquatic habitats reflecting amounts present in their environment **(7)**. Among aquatic insects, *Culex pipiens* (Diptera: Culicidae) stands out as a highly prevalent mosquito species found throughout Egypt. It serves as the primary carrier of *Wuchereria bancrofti*, responsible for filariasis (elephantiasis) in humans, as well as being a vector for Rift Valley fever viral infection and West Nile viral infection **(8)**.

Heavy metals influence biochemical and physiological processes in organisms **(9)**. In laboratory and field investigations, the usage of biomarkers is a strategy for evaluating ecosystem health and measuring biological effects **(10)**. In aquatic organisms, levels of lipid peroxidation and enzymatic and non-enzymatic cellular antioxidants indicate the oxidative stress status of the organism and can be utilized to assess environmental stress **(11, 12)**. Malondialdehyde (MDA), a byproduct resulting from lipid peroxidation, has been recognized as a marker for oxidative harm **(13)**. Additionally, antioxidant biomarker enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) have presented their efficacy as indicators of pollution across various organisms **(14).**

The cytochrome P450 (CYP450) enzyme plays a significant role in xenobiotic detoxification and drug metabolism in almost all living organisms and can be used as a biomarker for assessing their health status **(15).** Metallothioneins (MTs) are cysteine-rich proteins that regulate essential heavy metals (Cu, Zn, Fe, and Mn), neutralize poisonous heavy metals (Pb, Hg, As, and Cd), and sequester harmful metal ions by reducing the free cation forms that are considered toxic to organisms. Consequently, the measurement of MT aids in assessing the oxidative stress of aquatic organisms **(16)**. Heat shock proteins (HSPs) exhibit an essential function in reacting to non-living environmental stressors, like heat and chemicals, and consequently inhibit the induction of cell death in insects **(17)**. The heat shock protein 70 (HSP70) is a very effective biomarker for heavy metal contamination in insects **(18)**.

The comet assay is a method of microgel electrophoresis that can detect DNA damage at the single-cell level. It stands out as one of the prevalent, quick, and highly sensitive genotoxic indicators utilized extensively for assessing environmental hazards, such as heavy metals in insects **(19, 20).**

Ultrastructure modifications can serve as indicators of the impact of numerous anthropogenic contaminants on organisms **(21)**. Insects' midgut serves as the primary site for digestion and absorption, where the epithelium not only produces many digestive enzymes but also facilitates the uptake and transport of nutrients into the hemolymph **(22)**.

Hence, the current work was designed to explore the probability of using the third-instar larvae of *C. pipiens* as a sensitive bioindicator for heavy metal water pollution in Burullus Lake at two sites differing in heavy metal pollution degree using different biochemical and genotoxic markers, as well as ultrastructure changes.

Materials and Methods

Study area:

Burullus Lake stands out as one of Egypt's coastal shallow lakes, positioned as a coastal lagoon along the Mediterranean Sea, nestled between the two branches of the River Nile: the western Rosetta branch and the eastern Damietta branch (31° 22′ − 31° 26′ N and 30° 33′ − 31° 07′ E). Its depth typically ranges from 0.5 to 2.1 meters. This lake holds significant economic importance among the Egyptian northern lakes. It plays a pivotal role in the local ecosystem due to serving as a repository for

drainage water from surrounding agricultural areas and freshwater from the Brimbal Canal located in its western sector **(23)**. The present study was conducted on two different sites at Burullus Lake: Al-Bughaz (site 1) as a reference site and the front of Drain 7 (site 2) as a polluted site **(Fig. 1)**. The choice of these two locations was influenced by **Melegy et al.**'s **(24)** investigation into heavy metal contamination in Burullus Lake.

Fig. 1. Google map of Burullus Lake along the Mediterranean Sea, Egypt showing the two sampling sites: Al-Bughaz (site 1, reference) and the front of Drain 7 (site 2, polluted).

A sampling of *Culex pipiens* larvae

Five samples of *Culex* larvae, each containing 25 larvae of the same size, were collected from each study site in May 2022. The larvae were caught using a 20 cm diameter metallic strainer equipped with an aluminum handle and then moved to glass containers filled with water from the lake. Within an hour, they were transported to the laboratory for sorting. At the Entomology Department of the Faculty of Agriculture, Alexandria University, the larvae were identified as third-instar larvae of *Culex pipiens*. After sorting, the larvae underwent a rinsing process with flowing water to remove any debris. Subsequently, the sorted larvae were kept at −20 °C until further analyses.

Analysis of water samples

The analysis of water from each site was conducted to evaluate its physicochemical properties and concentrations of heavy metals. To remove suspended particles, five random water samples were taken from each site, approximately 20 cm beneath the water's surface. These samples were filtered on-site using a polypropylene syringe equipped with a 0.45 µm millipore cellulose acetate filter, followed by acidification for conservation. Standard methods outlined by **APHA (25)** for analyzing natural and treated wastewater were employed to measure various physical and chemical properties of the water samples, including pH value, total alkalinity (mg/L), total dissolved solids (TDS, g/L), and dissolved oxygen (DO, mg/L). The concentrations of the foremost prevalent heavy metals, such as Cd, Fe, Pb, Cu, Mn, and Zn were determined in the water samples using graphite furnace atomic absorption spectroscopy (Berkin-Elmer model 2380) under specified conditions and detection limits in the manual for each metal **(26)**. The heavy metal concentration levels in the water samples were reported in mg/L.

Detection of heavy metal concentrations in the midgut tissues of *C. pipiens* larvae

The concentration levels of the same heavy metals estimated in water samples were analyzed in larval midgut tissues employing atomic absorption spectroscopy (Berkin-Elmer model 2380), following the method outlined by **Loring and Rantala (27)**. The average concentrations were represented as µg/g dry weight.

Biochemical analyses in the midgut tissues of *C. pipiens* larvae

Oxidative stress and antioxidant biomarkers

The midgut tissues of *C. pipiens* larvae collected from each site were washed with distilled water and dried on filter paper. Tissues were then weighed and homogenized. The resulting homogenate was centrifuged using an IEC-CRU5000 centrifuge at 6500 rpm and 4 °C for 30 min. The obtained supernatants were preserved at a temperature of −20 °C for further analysis. MDA concentration was determined using the procedure outlined by **Draper and Hadley (28)** and reported as nmol/mg of tissue. The level of reduced glutathione (GSH) was assessed using the technique outlined by **Beutler et al. (29)** and reported as nmol/mg of tissue. Furthermore, the antioxidant activities of SOD (mU/mg of protein), CAT (mU/mg of protein), and GPx (mU/mg of protein) were estimated utilizing the methods described by **Nishikimi et al. (30)**, **Aebi (31)**, and **Flohé and Günzler (32)**, respectively.

Cytochrome P450 (CYP450) monooxygenase

The CYP450 monooxygenase activity in the homogenates of *C. pipiens* larvae was determined using the **Brogdon** procedure outlined in the USA CDC's *Anopheles* research **(33)**. The preparation of NaOAc buffer (pH 5.0 using acetic acid) occurred by dissolving 0.25 M sodium acetate ($C_2H_2NaO_2$) in 800 ml of water. Next, 20 mg of 3,3',5,5'-tetramethylbenzidine (TMBZ) were dissolved in 25 ml of methanol, followed by the addition of 75 ml of 0.25 M NaOAc buffer at pH 5.0. The positive control stock solution was obtained by adding 10 mg of cytochrome C to 100 ml of 0.25 M NaOAc buffer at pH 5.0. About 100 µl of KPO_4 were dispensed into the wells of the plate containing larval homogenates. For the positive controls, 100 µl of the cytochrome C control solution was added to three wells. In the actual assay, 200 µl of TMBZ solution was dispensed into each test well. The reactions were initiated by adding 25 µl of 3% hydrogen peroxide (H_2O_2). The plate was then left to incubate for 5 min at ambient temperature. Subsequently, the absorbance was reported at 620 nm, and the activity of CYP450 was quantified in milliunits (mU) per milligram of protein.

Metallothionein (MT)

The concentration MT was assessed using an HPLC technique modified from **Alhama et al. (34)**. Initially, 150 µl of the sample homogenate underwent centrifugation at 3500 ×g for 10 min. Subsequently, the supernatant underwent further centrifugation at 22000 ×g and 4 °C for 30 min to obtain the S22000 fraction. The homogenization buffer was used to prepare a 10-fold dilution of the S22000 fraction. To modify the protein's structure, 125 µl of the diluted sample was combined with 108 µl of an incubation cocktail, containing 230 mM Tris (pH 9.5), 300 mM dithiothreitol, 100 mM ethylenediamine tetraacetic acid, and 10% sodium dodecyl sulfate, and then subjected to incubation in a water bath at 70 °C for 20 min. Following this, the incubation mixture was supplemented with 17 µl of 180 mM monobromobimane and allowed to incubate in darkness at ambient temperature for 15 min to label MT. MT I solution, prepared in 230 mM Tris (pH 9.5), was utilized as a standard solution, and the MT concentration was quantified as nmol/mg of protein.

Assessment of heat shock protein 70 (HSP70) expression in the midgut tissues of *C. pipiens* larvae by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique

Total RNA was isolated from midgut tissues of *C. pipiens* larvae using TRIzol reagent (Thermo Fisher

Scientific, USA). The concentration of larval RNA was estimated at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). To remove genomic DNA contamination, DNase I treatment (Fermentas, USA) was performed on 1.0 µg of total RNA per sample. Subsequently, first-strand cDNA synthesis was carried out in a 20 µl reaction volume using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The relative expression levels of the HSP70 in *C. pipiens* tissues were detected using the real-time qRT-PCR. The template for qRT-PCR comprised 1.0 µg of RNA. Specific primers targeting HSP70, and the housekeeping gene β-actin were utilized in this assay. Qiagen Rotor-Gene SYBR Green PCR Kit was employed for the qRT-PCR procedures in a 25 µl reaction mixture containing 1.0 µl of cDNA, 12.5 µl of SYBR Green, 2.5 µl of each primer, and 9 µl of H_2O. The qRT-PCR program included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of amplification at 95 °C for 15 sec and 60 °C for 10 sec. The assays were conducted on the Rotor-Gene Q platform using Rotor-Gene Q-Pure Detection version 2.1.0 (Qiagen, USA). The quantification of HSP70 mRNA transcript levels was performed using the 2-ΔΔCT method **(35)**. The primer sequences utilized for HSP70 were forward: 5′-(GAT GCA GTC ATC ACA GTT CCA GC)-3′ and reverse: 5′-(AAC AGA GAT CCC TCG TCG ATG GT)-3′. For β-actin, the forward primer was 5'-(ATG TTT GAG ACC TTC AAC TCG C)-3' and the reverse primer was 3'-(TAA CCT TCR TAG ATT GGG ACG)-5', as reported by **Provost-Javier et al. (36).**

Assessment of genotoxicity using comet assay

The comet assay, a single-cell gel electrophoresis technique, was utilized to evaluate DNA damage within the midgut cells of *C. pipiens* larvae, following the procedure outlined by **Singh et al. (37)**. Visualization of the slides was conducted using an epi-fluorescence microscope and a computer-based image analysis system (Comet Assay V software, Perspective Instruments). Comet scores were determined by analyzing 50 to 100 randomly selected cells per slide. Assessment of DNA damage involved calculating the percentage of tail DNA (%), tail length (µm), and tail moment (Arbitrary units).

Ultrastructure examination of midgut epithelium of *C. pipiens* larvae

Midgut tissues from *C. pipiens* larvae sampled from the reference and polluted sites were separated and preserved by immersing them in a solution containing 4% formaldehyde and 1% glutaraldehyde (4F1G) in phosphate buffer solution (PBS) at pH 7.2, heated to 40 °C for 3 h. Following a 2-hour post-fixation step with 2% osmium tetroxide (OsO_4) in PBS, the samples underwent washing with the same buffer solution. Subsequently, the samples were exposed to dehydration through an ascending sequence of ethyl alcohol concentrations at 40 °C. They were then immersed in a mixture of Epon-Araldite within labeled capsules. Ultra-thin sections, approximately 0.06 - 0.1 µm thick, were prepared for examination under a transmission electron microscope (TEM) **(38)**.

Statistical analysis of data

The findings were presented as the means of five replicates ± standard errors (SE). The significance ($p \leq 0.05$) of differences between the mean values of each estimated parameter in samples of both sites, was assessed by the student's t-test. All statistical calculations were carried out using the IBM SPSS software package, version 25.

Results

Physicochemical characteristics of water samples

Table 1 illustrates the physicochemical properties of water at two sampling sites. The pH values were generally alkaline in both sampling sites. However, the value of pH in site 2 was higher than in site 1. Water samples obtained from site 2 revealed significantly ($p< 0.05$) higher concentration of total alkalinity as compared to that of site 1. In contrast, the water samples collected from site 2 reported significant decreases in the concentrations of TDS ($p< 0.01$) and DO ($p= 0.001$) as compared to those of site 1.

Table 1. The physicochemical parameters of water samples were obtained from two sampling sites: Al-Bughaz (site 1, reference) and the front of Drain 7 (site 2, polluted) at Burullus Lake.

Physicochemical parameters	Sampling sites		*t*-value	Sig. (2-tailed)
	Site 1	Site 2		
Hydrogen ion (pH)	8.06 ± 0.14	8.63 ± 0.26	-1.901-	0.130
Total alkalinity (mg/L)	291.66 ± 17.02	378.66 ± 21.36	-3.185-	0.033*
Total dissolved solids (TDS, g/L)	11.08 ±1.40	2.90 ± 0.18	5.781	0.004**
Dissolved oxygen (DO, mg/L)	9.70 ± 0.46	5.30 ± 0.20	8.685	0.001***

The presented values are means ± SE with significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$) calculated using Student's t-test.

Heavy metal concentrations in water samples

Figure 2 shows the mean concentrations of the estimated heavy metals in the water samples of the two investigated sites. The concentrations of all heavy metals in site 2 were significantly ($p < 0.01$) higher than those of site 1.

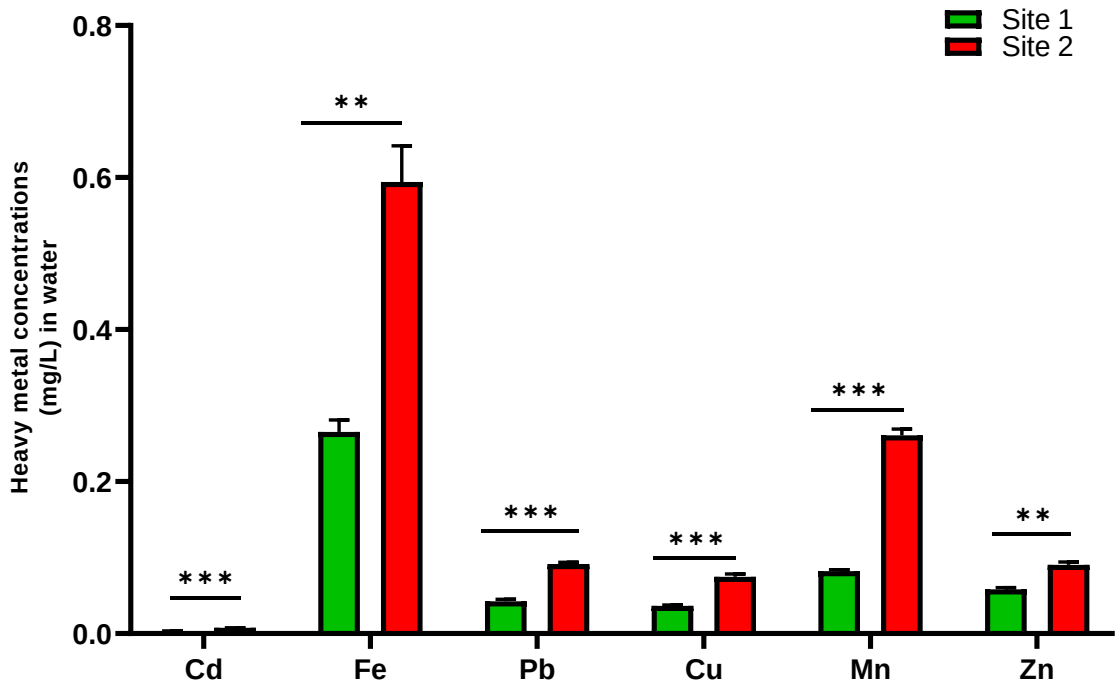

Fig. 2. Heavy metal concentrations (mg/L) in water samples obtained from two sampling sites: site 1 and site 2 at Burullus Lake. The represented values are means ± SE with significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$) calculated using Student's t-test.

Heavy metal concentrations in the midgut tissues of *C. pipiens* larvae

Figure 3 shows the mean concentrations of the estimated heavy metals in the midgut tissues of *C. pipiens* larvae obtained from the two selected sites. The results illustrated that the concentrations of all heavy metals were more significantly ($p< 0.01$) elevated in larvae obtained from site 2 as compared to larvae sampled from site 1.

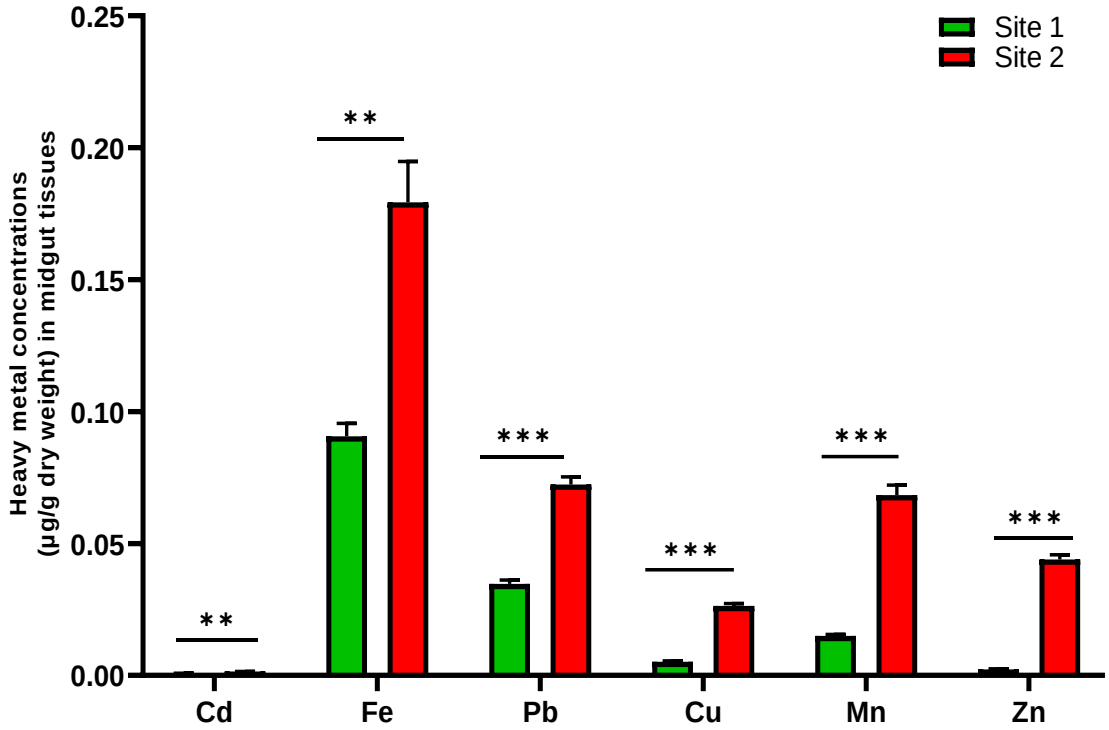

Fig. 3. Heavy metal concentrations (µg/g dry weight) in the midgut tissues of *Culex pipiens* larvae collected from two sampling sites: site 1 and site 2 at Burullus Lake. The represented values are means ± SE with significance levels ($^{*}p\leq 0.05$, $^{**}p\leq 0.01$, and $^{***}p\leq 0.001$) calculated using Student's t-test.

Biochemical analyses in the midgut tissues of *C. pipiens* larvae

The results illustrated variations in the biochemical markers in the midgut tissues of the studied larvae in response to heavy metal pollution, as illustrated in **Figures 4 & 5**. The MDA concentration in the larval midgut homogenate of the polluted group from site 2 was significantly ($p< 0.01$) amplified in comparison with the MDA concentration of reference larvae from site 1 **(Fig. 4a)**. The GSH level was significantly ($p= 0.00$) decreased in midgut tissues of larvae obtained from site 2 compared to those collected from site 1 **(Fig. 4b)**. Moreover, the activities of the antioxidant enzymes, including SOD, CAT, and GPx were markedly lower ($p= 0.00$) in the midgut tissues of the polluted group from site 2 than that detected in the reference larvae of site 1 **(Fig. 4c-e)**.

The CYP450 activity recorded a significant ($p< 0.01$) decrease in the midgut of polluted *C. pipiens* larvae of site 2 as compared to the reference larvae of site 1 **(Fig. 5a)**. At the same time, the MT concentration recorded a highly significant ($p= 0.00$) increase in the midgut tissues of larvae from site 2 as compared to the reference larvae of site 1 **(Fig. 5b)**.

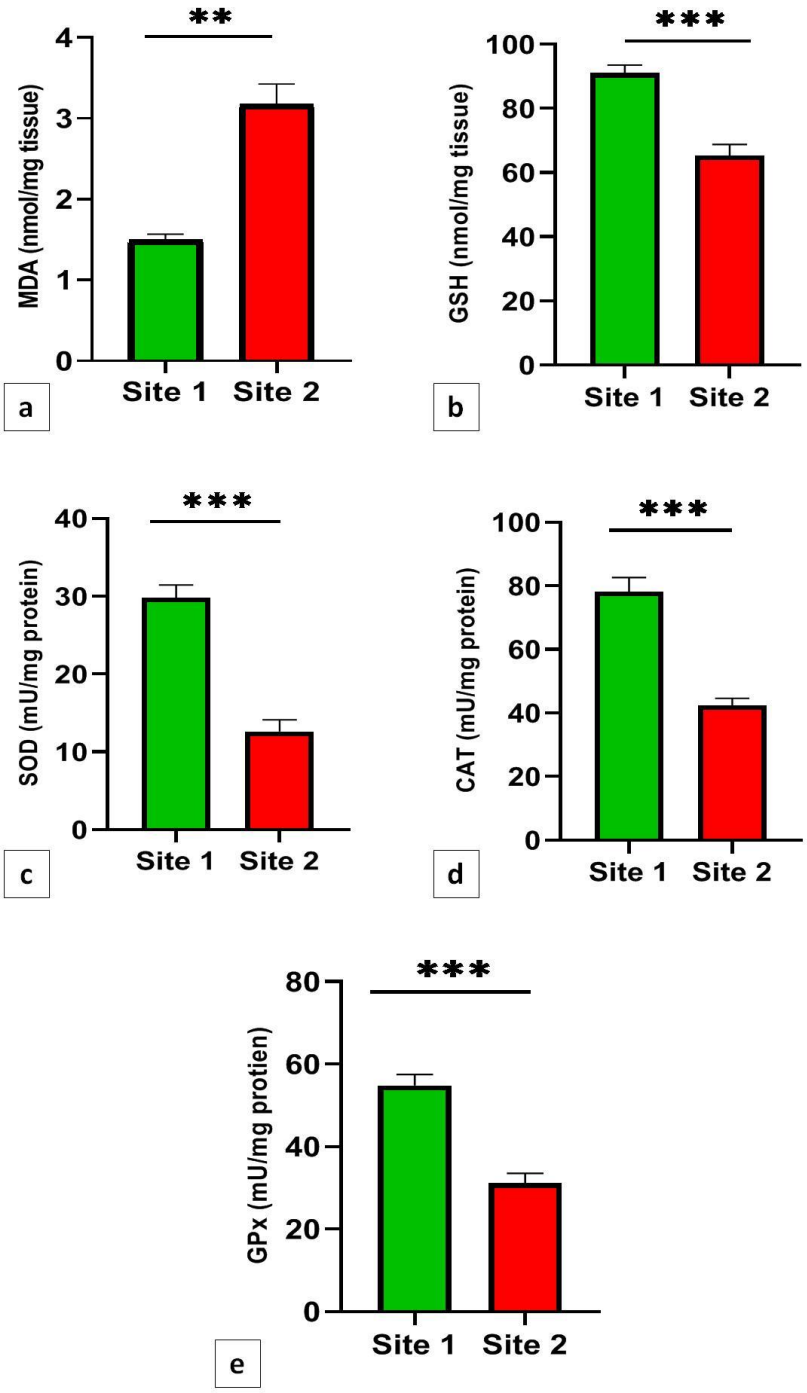

Fig. 4. Oxidative stress and antioxidant parameters in the midgut tissues of *Culex pipiens* larvae obtained from two sampling sites: site 1 and site 2 at Burullus Lake. **a:** Malondialdehyde (MDA), **b:** Reduced glutathione (GSH), **c:** Superoxide dismutase (SOD), **d:** Catalase (CAT), and **e:** Glutathione peroxidase (GPx). The represented values are means ± SE with significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$) calculated using Student's t-test.

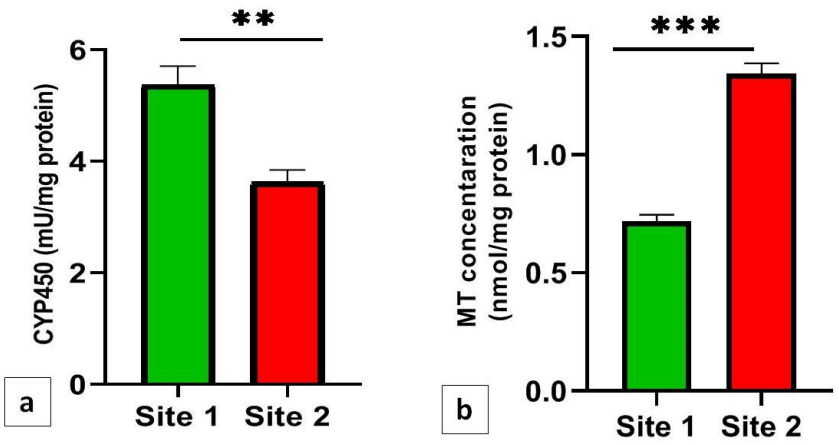

Fig. 5. Biochemical parameters in the midgut tissues of *Culex pipiens* larvae obtained from two sampling sites: site 1 and site 2 at Burullus Lake. **a:** Cytochrome P450 (CYP450) monooxygenase and **b:** Metallothionein (MT). The represented values are means ± SE with significance levels ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$) calculated using Student's t-test.

Expression of heat shock protein 70 (HSP70) in the midgut tissues of *C. pipiens* larvae

As illustrated in **Figure 6**, the expression level of HSP70 showed a highly significant ($p = 0.00$) increase in the midgut tissues of larvae from site 2 as compared to the reference larvae of site 1.

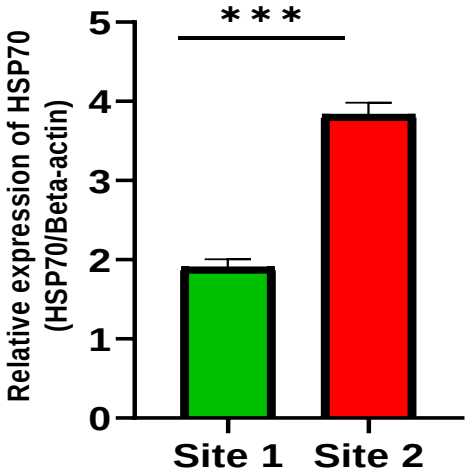

Fig. 6. Relative gene expression of heat shock protein 70 (HSP70) in the midgut tissues of *Culex pipiens* larvae collected from two sampling sites: site 1 and site 2 at Burullus Lake. The represented values are means ± SE with significance levels ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$) calculated using Student's t-test.

Genotoxicity in the midgut of *C. pipiens* larvae using comet assay.

Examination of comet microphotographs revealed that the DNA molecules extracted from the midgut cells of *C. pipiens* reference larvae did not exhibit detrimental alterations. Instead, they exhibited a symmetrical bright nucleus surrounded by a thin halo **(Fig. 7a)**. Conversely, the DNA of the polluted larvae exhibited a noticeable comet formation after electrophoresis due to the breakdown and migration of genomic DNA fragments **(Fig. 7b&c)**.

Analysis of DNA damage in larvae's midgut cells revealed high variations between reference larvae collected from site 1 and polluted larvae collected from site 2. Midgut cells of larvae from the contaminated site exhibited significant ($p \leq 0.001$) increases in all measured DNA damage parameters, evidenced by the migration of DNA toward the tail region. This migration was measured through the rising of fluorescence in the tail region, indicating a higher percentage of tail DNA, tail length, and a product of both measurements, known as the tail moment. In the reference larvae, the percentage of DNA in the comet tail was recorded at 5.94 ± 0.75%, the tail length was 2.61 ± 0.18 µm, and the tail moment was 1.77 ± 0.09 Arbitrary units. In larvae from the contaminated site, the percentage of fragmented DNA in the comet tail (27.42 ± 1.97%), tail length (18.50 ± 1.40 µm), and tail moment (43.58 ± 1.47 Arbitrary units) indicated high DNA damage **(Fig. 8a-c)**.

Ultrastructure of midgut epithelium of *C. pipiens* larvae

As shown in **Figure 9 a&b**, the epithelial cells of the midgut of the reference group of *C. pipiens* larvae from site 1 are provided with a striated brush border called microvilli. They have oval nuclei with regular nuclear envelopes. The cytoplasm of these cells is enriched in mitochondria, cisterns of the smooth endoplasmic reticulum, cisterns of the rough endoplasmic reticulum, and free ribosomes.

Alterations in the midgut epithelium were observed in the polluted group of *C. pipiens* larvae from site 2. The destroyed microvilli, irregular nuclear envelope, vacuolated cytoplasm, and increasing number of lysosomal bodies were observed in **Figure 9c.** Also, as shown in **Figure 9d**, spherites and dense vesicles, as well as disintegrated mitochondria, appeared in the midgut cells of polluted larvae from site 2.

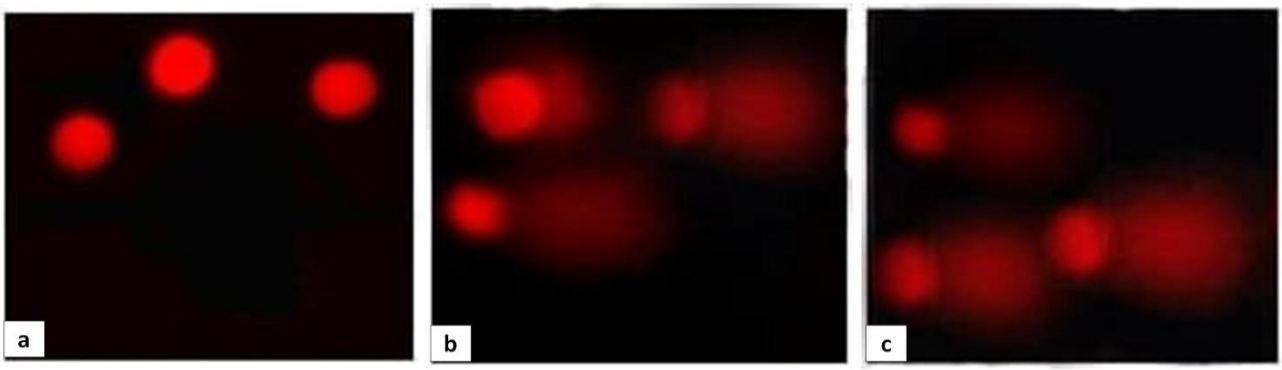

Fig. 7. Comet photomicrographs of DNA isolated from midgut cells of *Culex pipiens* larvae collected from two sampling sites at Burullus Lake. **a:** site 1 and **b&c:** site 2.

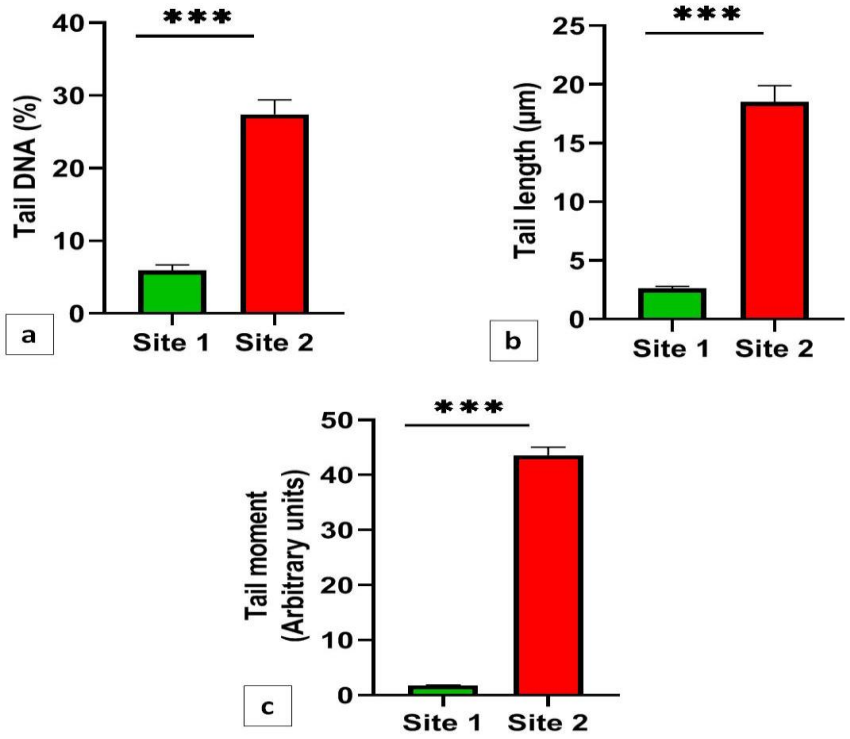

Fig. 8. DNA damage by comet assay measured in the midgut cells of *Culex pipiens* larvae collected from two sampling sites: site 1 and site 2 at Burullus Lake. **a:** Percentage of tail DNA (%), **b:** Tail length (µm), and **c:** Tail moment (Arbitrary units). The represented values are means ± SE with significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$) calculated using Student's t-test.

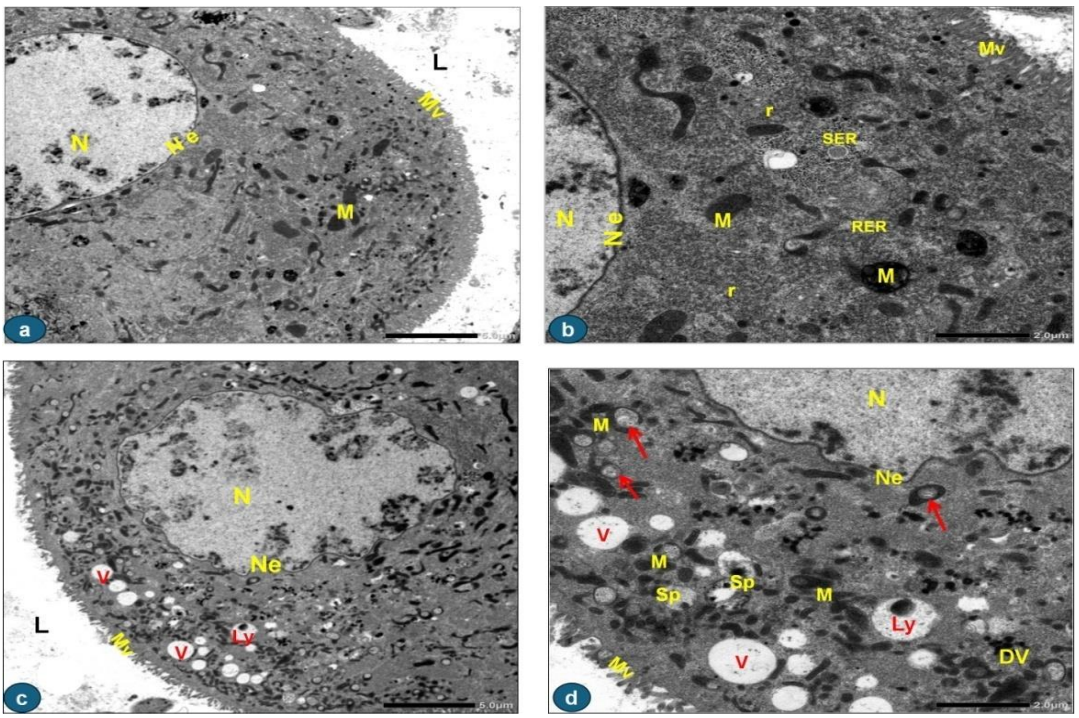

Fig. 9. Transmission electron micrographs (TEM) of the epithelial cells of the midgut of *Culex pipiens* larvae collected from two sampling sites. **a-b:** site 1 and **c-d:** site 2 at Burullus Lake. Lumen **(L)**, Microvilli **(Mv)**, Nucleus **(N)**, Nuclear envelope **(Ne)**, Mitochondria **(M)**, Smooth endoplasmic reticulum **(SER)**, Rough endoplasmic reticulum **(RER)**, Ribosomes **(r)**, Lysosomal body **(Ly)**, Vacuole **(V)**, Spherite **(Sp)**, Dense vesicle **(DV)**, and Disintegration in mitochondria **(arrows)**.

Discussion

Water pollution is a significant global issue requiring regular evaluation of water resources, as it represents a major cause of death and illnesses on a global scale **(39)**. Heavy metals in water are a global concern. As, they enter the water bodies due to industrialization, urbanization, and chemical compound usage, posing significant threats to the aquatic environment and living organisms **(40)**.
Biological indicators offer numerous benefits over chemical methods in monitoring the health status of aquatic ecosystems. Biomonitoring using aquatic insects can offer information about the long-term habitat conditions, whereas physicochemical data only reveals the ecosystem's status when sampling **(41)**.

The present results of the physicochemical parameters of water samples revealed that the water obtained from the two sites was alkaline with a lesser pH value in the reference site. This finding aligns with that obtained by **Shaker et al. (42)**, who indicated that the highest values of pH may be due to the increasing phytoplankton activities which consume CO_2 during photosynthetic processes. The increase in the polluted site's total alkalinity may be due to the rise in bicarbonate concentration produced from organic matter decomposition by bacteria and precipitation of calcium carbonate. This outcome is consistent with **Shahat et al.**'s findings **(43)**. The level of TDS at the polluted site was found to be significantly lower than that at the reference location. The previous study on TDS in Brullus Lake water showed a negative relationship with water exchange between the Mediterranean Sea and the lake, where salinity increased from sites close to Al-Boughaz only (reference site). Water salinity decreased as we moved away from the Al-Boughaz, and it is also evident that the water of the Mediterranean Sea cannot reach sites in the central, western, eastern, and southern sectors of the lake **(44)**. Our finding indicated that the DO level in the contaminated site has significantly decreased, which may be related to the oxidation of chemical effluents and oxygen consumption in the breakdown of organic debris. This view agrees with the results reported by **Shahat et al. (43)**.

In recent decades, ecotoxicological investigations have attracted attention from all over the world. These studies evaluate anthropogenic environmental toxins by identifying organisms with deposited heavy metals. Insects serve as bioindicators to estimate the heavy metal accumulation in their environment, especially when there is direct contact with the environment **(45)**.

The midgut of larvae serves as an efficient chemical and physical barrier against potentially harmful substances that are consumed during feeding **(46)**. Numerous processes occur in the larval midgut, including antioxidant effects, detoxification, and expression of various isoforms of enzymes **(47)**. Therefore, the midgut tissues of *C. pipiens* larvae in our work presented a reflection of the elevation in heavy metal concentrations in the contaminated water sample. The current findings demonstrated that water and the midgut tissues of *C. pipiens* larvae from site 2 accumulated larger quantities of metals, including Cd, Fe, Pb, Cu, Mn, and Zn.

Exposure to environmental pollutants is linked to increased oxidative stress produced from increased cellular generation of reactive oxygen species (ROS), which have numerous detrimental effects on organisms **(48)**. The first line of defense consists of antioxidant enzymes like SOD, CAT, and GPx that can neutralize molecules that could become free radicals or that cause more radicals to be triggered **(49)**. Heavy metals affect oxidative equilibrium by boosting ROS generation and changing antioxidant enzyme activities that are regarded as significant indicators for harmful metal impacts **(50)**.

The present results revealed a significant elevation in MDA levels in polluted larvae, possibly due to heavy metal accumulations resulting in increased H_2O_2 and ROS levels that consequently cause lipid oxidation and decrease SOD activity and GSH content **(51)**. This result is corroborated by the finding of **Ihechiluru et al. (52)**, who demonstrated

a great positive relationship between MDA concentration and Pb concentrations in the dragonfly *Austroaeschna inermis* (Odonata, Telephlebiidae). Also, **Yuan et al. (53)** found that the MDA contents in the testes of male *Bombyx mori* larvae (Lepidoptera, Bombycidae) increased considerably with the concentration of Cd. Moreover, Cu and Zn exposure caused an increase in MDA levels in *Galleria mellonella* larvae, as reported in **Coskun et al.**'s study **(54)**.

In the group that was contaminated in our study, the GSH level was considerably lower. This finding is in the same line with the finding of **Sun et al. (55)**, who found that exposure to Ni reduced GSH levels in *Spodoptera litura* (Lepidoptera, Noctuidae). According to **Olakkaran et al. (56)**, *Drosophila melanogaster* (Diptera, Drosophilidae) showed reduced GSH levels in response to lead exposure. GSH binds with heavy metals to protect DNA, RNA, and protein molecules, and consequently, the activities of GSH-dependent enzymes, like GPx, are reduced in insects subjected to metals **(57)**. On the other hand, **Abdelfattah et al. (58)** illustrated that adults of *Aiolopus thalassinus* (Orthoptera, Acrididae) from the polluted site had an increase in GSH levels in their gut.

The midgut tissues of *C. pipiens* larvae taken from the polluted site showed a significant decrease in SOD activity. This decrease may rely on the consumption of this enzyme during the conversion process of 2 $O_2^{\bullet-}$ and $2H^+$ to H_2O_2 and O_2 as a result of the heavy metals accumulation **(59)**. The recorded result in our study is aligned with the findings of **Azam et al. (60)**, who demonstrated that extended exposure to metals causes a drop in SOD activity along with a decline in antioxidant capacity and an elevated degree of oxidative stress. Furthermore, **Wu et al. (61)** showed that the high concentration levels of Pb in *Oxya chinensis* (Orthoptera, Acrididae) decreased SOD activity. The present finding contradicts that of **Islam et al. (62)**, who observed that muga silkworms, or *Antheraea assamensis* (Lepidoptera: Saturniidae), exposed to heavy metals had higher SOD activity (Crudididae, Orthoptera).

As a result of the reduction in SOD activities in the present study, the CAT activity was also significantly decreased in the heavy metal-polluted *C. pipiens* mosquito larvae. CAT contributes to reducing the excess amount of H_2O_2 and converts it by the Fenton reaction into H_2O and O_2 **(63)**. When exposed to zinc, the tasar silkworm *Antheraea mylitta*'s total body tissue showed an inhibition in CAT activity **(64)**. CAT activity in gypsy moth larvae was considerably inhibited by Cd exposure, both at low and high levels **(65)**. Additionally, **Zhang et al. (66)** reported that female *Aleuroglyphus ovatus* (Sarcoptiformes, Acaridae) exposed to cadmium had a decrease in CAT activity. Conversely, **Saleem and Afsheen (67)** showed that water contamination with heavy metals increased CAT activity in three species of water striders (*Metrocoris communis*, *Limnogonus fossarum fossarum*, and *Aquarius adelaidis*).

Furthermore, the considerable decrease in GPx activity in the contaminated larval samples in our study could be attributed to the consumption of GPx during the inhibition and breakdown of free radicals and H_2O_2 **(52)**. The present finding agrees with **El-Saad et al. (68)**, who observed a declining level of GPx activity in *Apis mellifera* (Hymenoptera, Apidae) inhabiting a contaminated environment. Also, **Mese et al. (69)** observed a decrease in *G. mellonella*'s GPx activity in all treated groups that were exposed to Cu and Zn.

CYP450s are well known for their functions in the detoxification process, but they are also in charge of the oxidative metabolism of a wide range of structurally varied endogenous and exogenous chemicals, including fatty acids and steroid hormones, in insects **(70)**. In our study, the CYP450 activity in the contaminated larval group was at a lower level than the reference larvae. This result is compatible with the outcome of **Zhan et al. (71)**, who found that CYP450 activity decreased in the sixth larval instar of the cotton bollworm due to

chronic Cd exposure. Furthermore, lead and cadmium exposure significantly downregulated the expression of cytochrome CYP450 genes in *Anopheles gambiae* (Diptera, Culicidae) in the previous study of **Musasia et al. (72)**. In contrast, **Bernabò et al. (73)** discovered that *Chironomus riparius* (Diptera, Chironomidae) larvae exposed to copper had an elevated CYP450 enzymatic activity.

In insects, tissue protection against metal stress is thought to be provided by MT induction in the gut which is considered the main organ where the MT protein is present in its cells **(74, 75)**. In addition to high cysteine content in MT, it can protect the cells from ROS-damaging impacts **(76, 77)**. Others highlighted its function in maintaining the balance of zinc and copper **(78)**. According to **Egli et al. (79)**, *Drosophila* MT is essential for maintaining copper homeostasis and cadmium detoxification.

The current results demonstrated that the midgut tissues of larvae from site 2 had significantly higher MT concentration in response to heavy metal pollution than those from the reference site. Our finding was consistent with **Liu et al.**'s study **(80)**, showing that *O. chinensis*'s MT expression rose as the amount of Cd increased. The higher MT production is the mechanism for metal tolerance, as reported in the springtail *Orchesella cincta* **(81)** and *Chironomus javanus* fourth-instar larvae **(82)**.

HSP70 has a variety of roles in insects, varying across species and even within the same species based on physiological and environmental factors **(17)**. It is expressed at low levels in normal circumstances: however, its expression is enhanced for cellular protection during stressors brought on by temperature, pathogenic infections, and metals **(83)**. In the present work, the larvae with the largest level of heavy metal accumulation showed a considerable increase in HSP70 expression compared with that observed in the reference group. According to **El-Samad et al. (84)**, an elevation in HSP70 expression level occurred in *Pimelia latreillei* (Coleoptera, Tenebrionidae) in response to heavy metal contamination, and this result supports our findings. Overall, the high levels of stress proteins, MT and HSP70, in our study indicate an increase in ROS generation as a consequence of metals.

Heavy metals also impact cellular organelles, including cell membranes, mitochondria, lysosomes, endoplasmic reticulum, and nuclei, and interact with DNA, causing damage to it **(85)**. The *C. pipiens* larvae, obtained from the polluted site in our study, exhibited a notable elevation in DNA damage, evidenced by an increasing tail DNA%, tail length, and tail moment, in their midgut epithelial cells than larvae from the reference site. **Yousef et al.**'s study **(86)** noted that there is a direct link between increased DNA damage and metal exposure and ROS generation. Elevations in ROS may lead to DNA damage and degradation **(87)**. **Abdelfattah *et al.* (88)** demonstrated that *A. thalassinus* cells from contaminated areas have greater levels of DNA damage. **El-Gendy et al. (74)** noticed DNA damage in the midgut cells of *Trachyderma hispida* (Coleoptera: Tenebrionidae) in a heavy metal-polluted industrial area. Similar findings were revealed by **Yang et al. (89)**, who observed that *Drosophila* exposed to high Cd concentrations showed noticeably high DNA damage according to the comet assay study.

DNA damage can be attributed to many molecular pathways, such as nuclease activation, direct hydroxyl radical (OH-) reactivity with the DNA, or breakage originating from free radical reaction with deoxyribose residues. Furthermore, the creation of a basic site ultimately results in strand breakage, changes and degradations of nitrogenous bases, harm to the sugar moiety, the creation of cross-linkages between DNA and proteins, and harm to the DNA repairing machinery **(90)**.

According to the current findings, the exposure of the *C. pipiens* larvae to heavy metals in site 2 caused a variety of ultrastructure alterations along the epithelial cells of midgut. These symptoms manifested as epithelium destruction, microvilli degeneration, an irregular nuclear envelope, vacuolated cytoplasm, increasing number of

lysosomal bodies, disintegrated mitochondria, and the appearance of spherites and dense vesicles in the midgut epithelium. These outcomes are in the same line with **Miranda et al. (91)**, who observed disruption and injury in the midgut epithelium of *Aedes aegypti* (Diptera, Culicidae) as a result of exposure to $CuSO_4$. When the metal storage capacity of midgut cells is reached to their extent, the cells are decomposed releasing the accumulated metals into the lumen where they are expelled along with excrement. This process disrupts the midgut's functions and damages the epithelial cells **(92)**. The presence of vacuoles in the midgut cells of polluted larvae is a sign of cytotoxicity and could be a result of the leakage of lysosomal hydrolases into the cytoplasm **(51)**. The appearance of spherites and dense vesicles in the polluted larvae's midgut epithelial cells is attributed to the assimilation of excess metals *via* absorption through binding with metal-binding proteins. These metals are subsequently sequestered within intracellular granules and spherites, a mechanism protecting the surrounding midgut tissues from potential disruption resulting from chemicals **(93)**. Finally, the present study revealed that heavy metals can affect the larvae of *C. pipiens* in different ways, and this effect varies according to the pollution degree.

Conclusion

This study concluded that the third-instar larvae of *C. pipiens* can bioaccumulate heavy metals present in their aquatic habitats. As, their midgut tissues were very sensitive to heavy metal pollution resulting in biochemical, molecular, and ultrastructure modifications. Consequently, *C. pipiens* larvae can be considered an effective bioindicator for heavy metal pollution to make regular evaluations of metal pollution in the aquatic environment.

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Declaration of interest

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