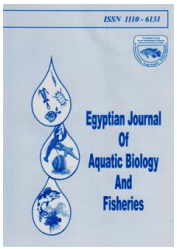
Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(2): 1631 – 1653 (2025)
www.ejabf.journals.ekb.eg
DOI: 10.21608/ejabf.2025.421007



Implications of Heavy Metal Accumulation on Biochemical and Genotoxic Aspects, and Relative Gene Expression in *Culex pipiens* Larvae

Salwa I. Sebak[1*], Mai I. Hussein[2], Heba M. Fangary[1]
[1]Zoology Department, Faculty of Science, South Valley University, Qena, Egypt
[2]Zoology Department, Faculty of Science, Suez University, Suez, Egypt
***Corresponding author: salwa_ibrahim@sci.svu.edu.eg**

ARTICLE INFO
Article History:
Received: Jan. 16, 2025
Accepted: April 2, 2025
Online: April 7, 2025

Keywords:
Culex pipiens,
Heavy metals,
Biochemical effects,
Genotoxicity,
Gene expression

ABSTRACT

Metal pollution is one of the most prevalent human pollutants to which mosquito larvae are subjected in agricultural and urban environments. *Culex pipiens* are rapidly adapted to polluted environments. The present research investigated the impact of exposure of *Cx. pipiens* larvae to copper chloride ($CuCl_2$) and cadmium chloride ($CdCl_2$) on the biochemical, genotoxic, and molecular responses. Various concentrations of $CuCl_2$ and $CdCl_2$ were evaluated for toxicity against late third-instar *Cx. pipiens* larvae. The larvae were treated for 24hr with the LC_{50} of $CuCl_2$ and $CdCl_2$. Copper chloride significantly accumulated in the *Cx. pipiens* larval tissues compared to $CdCl_2$. Biochemical studies revealed that these heavy metals increased total protein content, and catalase and peroxidase activities. Glutathione S-transferase, α-esterase, and β-esterase activities were reduced in $CuCl_2$ treatment while increased in case of $CdCl_2$ treatment. All parameters of the comet assay, viz., % of damage, tail length, DNA % in the tail, tail moment, and olive tail moment increased with varying levels relative to the control. Gene expression studies demonstrated significant down-regulation of pollution-biomarker genes, acetylcholiesteras (AChE) and cytochrome P450 (CYP450), and significant up-regulation of heat shock protein (HSP), superoxide dismutase (SOD) and metallothionein (MT) genes. In conclusion, *Cx. pipiens* larvae can be considered an effective bioindicator for heavy metal pollution, facilitating regular studies on metal pollution in aquatic ecosystems.

INTRODUCTION

In recent years, the accumulation of heavy metal ions in natural aquatic ecosystems has grown to be a major worldwide concern **(Aziz *et al.*, 2023)**. In such ecosystems, heavy metals, such as lead, cadmium, copper, arsenic, mercury, and iron normally exist at low concentrations (**Mireji *et al.*, 2008)**. However, they are considered carcinogenic and toxic when their concentrations increase beyond certain levels **(Liaqat *et al.*, 2023)**. Heavy metals can accumulate in the tissues of plants, insects, and mammals, resulting in many health consequences, including organ damage, cancer, and developmental disorders **(Singh & Kalamdhad, 2011)**. Natural activities, including wind erosion, forest fires, volcano eruptions, and biogenic processes release these pollutants

(Zaynab *et al.*, 2022). Moreover, human activities, such as mining, industry, and agricultural practices, including the application of inorganic fertilizers and pesticides share to introduce heavy metals into the environment **(Tariq *et al.*, 2016; Lekfeldt *et al.*, 2017)**. This ultimately affects invertebrates that reproduce in polluted aquatic environments **(Sowa & Skalski, 2019)**.

Some metals, such as copper, iron, and zinc have important physiological and biochemical roles in organisms. Copper plays an essential role in sustaining the structures of organisms but its deficiency disrupts critical metabolic processes, while increased exposure leads to toxicity **(Schwartz *et al.*, 2003)**. Cadmium is a heavy metal that has no essential biological role even in small concentrations and is found as potentially toxic at low levels **(Kabata-Pendias, 2000)**. In Egypt, water resources are increasingly polluted by hazardous chemical compounds, especially heavy metals due to the proliferation of human activities near water sources **(Akhtar *et al.*, 2021)**. Aquatic insects accumulate heavy metals and serve as efficient bioindicators of metals in aquatic ecosystems, reflecting the levels present in their environment **(Mebane *et al.*, 2020)**. *Culex pipiens* (Diptera: Culicidae) is a notably prevalent mosquito species among water insects in Egypt. It serves as the principal vector of *Wuchereria bancrofti*, which is responsible for filariasis (elephantiasis) in humans, as well as transmitting Rift Valley fever virus and the West Nile virus infections **(Nebbak *et al.*, 2022)**.

The physiological and biochemical changes in the organism are linked to the negative impacts stemming from susceptibility to a contaminant or a pollutant **(Lai *et al.*, 2011)**. Biomarkers are employed in laboratory and field investigations as a method for assessing ecosystem health and quantifying biological impacts **(Dalzochio *et al.*, 2016)**. Antioxidant biomarker enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (Pox) have demonstrated their effectiveness as markers of pollution in many organisms **(El-Samad *et al.*, 2019)**. Glutathione transferases (GSTs) function as detoxification enzymes that are essential for the degradation of both internal and foreign toxic substances. They serve as important indicators for assessing the quality of freshwater environments, as their catalytic properties and gene regulation in aquatic organisms are closely associated with different ecological stresses **(Yang *et al.*, 2023)**. The level of acetylcholinesterase (AChE) is a critical neuro-behavioral metric utilized to validate the mechanical impacts of pollutant exposure in organisms **(Tilton *et al.*, 2011)**. The cytochrome P450 (CYP450) enzyme is crucial for xenobiotic detoxification and drug metabolism across nearly all organisms and serves as a biomarker for evaluating their health state **(Han & Lee, 2021)**. Heat shock proteins (HSPs) exhibit an essential function in reacting to abiotic environmental stressors, such as heat and chemicals, hence preventing cell death in insects **(King & MacRae, 2015)**. The heat shock protein 70 (HSP70) serves as a highly effective biomarker for heavy metal pollution in insects **(Hu *et al.*, 2019)**. Tissue protection against metal stress is thought to be provided by metallothionein (MT) induction in the insects gut which is considered the main organ

where the MT protein is present in its cells **(El-Gendy *et al.*, 2020)**. In addition to high cysteine content in MT, it can protect the cells from reactive oxygen species (ROS)-damaging impacts **(You *et al.*, 2002)**. The comet assay is a microgel electrophoresis method that identifies DNA damage at the single-cell level. It stands out as one of the prominent, quick, and highly sensitive genotoxic indicators widely employed for evaluating environmental risks, including heavy metals in insects **(Ceschi-Bertoli *et al.*, 2020)**.

The current work was designed to explore the probability of using larvae of *Cx. pipiens* as a sensitive bioindicator for copper and cadmium pollution, in terms of assaying certain biochemical, molecular, and genotoxic parameters.

MATERIALS AND METHODS

1. Rearing of *Culex pipiens*

Mosquitoe larvae were collected from the breeding sites in Qena City, Egypt (Latitude: 26° 09′ 60.00“ N; Longitude: 32° 42’ 59.99” E). They were raised in the insectary of the Zoology Department, Faculty of Science, South Valley University. Morphological identification of emerging adults was performed using taxonomic keys developed by **Harbach (1985)**. The adult mosquito colony was maintained under controlled laboratory conditions of 27±2°C and 65 ± 5 % relative humidity.

2. Heavy metals tested

The heavy metal salts utilized in this study were copper chloride ($CuCl_2$) and cadmium chloride ($CdCl_2$). Each salt was solubilized in distilled water to prepare a 1000ppm stock solution. The stock solution was subsequently diluted to set up different concentrations of $CuCl_2$ (1, 2, 4, 5, 8, 10 and 12ppm) and $CdCl_2$ (5, 50, 100, 200, 300 and 400ppm).

3. Experimental bioassay

The toxicity of $CuCl_2$ and $CdCl_2$ to *Cx. pipiens* larvae was investigated using the above-mentioned concentrations. As recommended by the WHO protocol **(WHO, 2005)**, 20 late third-instar larvae (3-day-old) were released into a plastic cup (12cm in diameter and 7cm in height), containing 100ml of each heavy metal solution. A parallel control with 20 late third-instar larvae released into a cup containing 100ml of distilled water was also conducted. Each concentration as well as the respective control were replicated four times. Mortality was made 24h post-treatment, and then corrected versus that of the control using Abbott’s formula **(Abbott, 1925)**. Corrected mortality was then subjected to probit analysis **(Finney, 1971)** to estimate the LC_{50}, 95% confidence limits, heterogeneity, and slope.

4. Detection of heavy metal concentrations in *Culex pipiens* larval tissues

The concentration levels of copper chloride and cadmium chloride were detected in *Cx. pipiens* third-instar larval tissues 24h post-treatment with the LC_{50} of these heavy metals employing atomic absorption spectroscopy (Berkin-Elmer model 2380), following the method outlined by **Loring and Rantala (1992)**.

5. Biochemical studies

5.1. Sample preparation

The surviving larvae treated with the LC_{50} of copper chloride and cadmium chloride for 24h were collected individually. A glass Teflon tissue homogenizer (ST–2 Mechanic-Preczyina, Poland) was utilized on ice-cold conditions to homogenize the freshly collected larvae (250mg) of 100 treated larvae in one ml of distilled water. Each homogenate was centrifuged at 5000 × g for 15min at 4°C utilizing a cooling centrifuge. After removing the pellets, each supernatant was divided into 0.5ml multiple aliquots. These aliquots were subsequently stored at −20°C for further biochemical analyses, including total protein content and enzymatic activity assessments. The control homogenate was also prepared using larvae that were not exposed to heavy metals. All treatment and control groups were triplicated.

5.2. Determination of total protein content

Bradford (1976) method was employed to estimate the total protein amount. The bovine serum albumin (Sigma-Aldrich, Germany) and Coomassie Brilliant blue (G-250, Sigma-Aldrich, Germany) were used as the standard and reagent, respectivly. The total protein content was measured spectrophotomerically at 595nm with a Jenway-7305 UV/Vis spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK).

5.3. Determination of activities of antioxidant enzymes

The activity of catalase (CAT) was determined using Biodiagnostic kit # CA 2517 (Angstrom Biotech Pvt. Ltd., India) based on the protocol of **Aebi (1984)**. The CAT level was determined at absorbance of 240nm, and measured in units (U)/mg protein.

Hammerschmidt *et al.* (1982) procedure was used to measure the peroxidase enzyme (POX) activity. The enzyme activity was quantified at 420nm, and expressed as Δ optical density (O.D)/min/mg protein.

5.4. Determination of detoxifying enzyme activities

Glutathione S-transferase (GST) activity was measured following the methods described by **Habig *et al.* (I974)** using 1-chloro, 2, 4-dinitrobenzene as a reagent. The activity was measured at 340nm, and expressed as nanomole substrate conjugated/min/mg protein.

Van Asperen (1962) method was used to measure α- and β-esterase activity using α- and β-naphthyl acetate substrates, respectively. The activity was measured spectrophotomerically at 600nm.

6. Genotoxic studies

A comet test (single-cell gel electrophoresis) was utilized to determine the damage on DNA according to **Singh *et al.* (1988)**. The samples were homogenized and centrifuged at 100 × g for 10min. The formed pellet was gently embedded in 1ml of homogenizing buffer to extract nuclei. To prepare and visualize the comet slides, the methods outlined by **Dua *et al.* (2013)** were followed. The ethidium bromide-stained

slides were examined and assessed using a digital color camera and an Axio fluorescence microscope (CD75V1A Zeiss, Germany) set to 400× magnification. Comet Score software version 1.5 (TriTek Corporation, Sumerduck, VA) was used to measure the parameters of the comet test (damage percentage, tail length, tail moment, tail DNA percentage, and olive tail moment) for 100 cells per treatment.

7. RNA extraction, cDNA synthesis, and quantitative PCR analysis (qPCR)

Total RNA was isolated from *Cx. pipiens* third-instar larvae by BIOZOL (Bioflux, Cat No. 10760055-1) 24 hours post-treatment with the LC_{50} of copper chloride and cadmium chloride, following the manufacturer's guidelines. One hundred ng of RNA was reverse-transcribed to create cDNA, using the GScript ULTRA First-Strand Synthesis Kit (GeneDireX, Inc.). The relative expression of cytochrome P450 (CYP450), superoxide dismutase (SOD) and heat shock protein (HSP) were assessed according to **Delhaye *et al.* (2016)**. The sequences of the used primers in the qPCR analysis are listed in Table (1). The reference gene was β-actin, and the $2^{-\Delta\Delta Ct}$ technique was applied following **Livak and Schmittgen (2001)**.

Table 1. List of primer names and their nucleotide sequence

Gene	Sequence (5'-3')	Reference
Acetylcholinesterase (AChE)	Fwd: ATCTGCGAGTTTCCGTTCGT Rev: CTTCTCGTCGTCCTGGTAGC	Acc#AY762905
Cytochrome P450 (CYP450)	Fwd: TCCAAGATCACGTGGCGAAA Rev: GGTGTCTGTTCCGGGGATTT	Acc # AY662654
Heat shock protein (HSP)	Fwd: CAAGCGAGCAAAGCACTAGC Rev: CGGTGCAAATCGCTTACGTT	Acc#HQ881846
Superoxide dismutase (SOD)	Fwd: GCATTGCGAAAACTTCCTTC Rev: TGCCCAGATCATCAATTTCA	**Delhaye *et al.*, 2016**
Metallothionein (MT)	Fwd: GCAACTGTGCCAGCAAGAAG Rev: GACAAGGGAGATGCGTCCTAT	Acc# AY433383
β-actin	Fwd: TGCGTGACATCAAGGAGAAGC Rev: CCATACCCAAGAAGGAAGGCT	**Tian *et al.*, 2016**

8. Statistical analysis

The Shapiro-Wilk test was initially used to check for normality in all datasets from the molecular, genotoxic, and biochemical investigations. A mean ± standard error (SE) was then used to express the data. One-way analysis of variance (ANOVA) and post hoc Tukey's tests for multiple comparisons were used to compare group means **(Midway *et al.*, 2020)**. A significant threshold of $P<0.05$ was established. IBM-SPSS Statistics v.25 (IBM, Armonk, New York, USA) was utilized for all statistical computations.

RESULTS

1. Toxicological effects

The results of the toxicity of copper chloride ($CuCl_2$) and cadmium chloride ($CdCl_2$) versus the third-larval instar of *Cx. pipiens* 24h post-exposure are shown in Table (2). Data illustrate that the two tested metals were highly toxic. Copper chloride was the most toxic heavy metal, with LC_{50} of 6.69ppm (Fig. 1A), while cadmium chloride showed less toxicity, with LC_{50} value of 265.913ppm (Fig. 1B). The mortality percentages of the larvae were positively correlated with $CuCl_2$ and $CdCl_2$ concentrations. Larval mortality percentage in copper chloride treatment increased gradually from 1.25% at 1ppm to 76.25% at 12ppm concentration. Whereas, it increased from 1.266% at 5ppm to 65.823% at 400ppm for cadmium chloride application. In the control group, the larval mortality was 0% in both treatments.

Table 2. Toxicity of copper chloride and cadmium chloride against the 3rd larval instar of *Cx. pipiens*

	Concentration (ppm)	**Mortality %**	**LC_{50} (ppm)**	**95% Confidence limits (ppm) LCL – UCL**	**Slope ± SE**	χ^2 (df)	P
Copper chloride	1	1.25	6.69	5.26-8.998	3.36±0.25	33.47 (5)	<0.05
	2	1.25					
	4	22.5					
	5	56.25					
	8	61.25					
	10	67.5					
	12	76.25					
	Control	0.0					
Cadmium chloride	5	1.266	265.913	217.79-341.54	1.55±0.19	3.07 (5)	<1.61
	50	11.392					
	100	24.051					
	200	41.772					
	300	50.633					
	400	65.823					
	Control	0.0					

LCL: Lower confidence limit, UCL: Upper confidence limit.

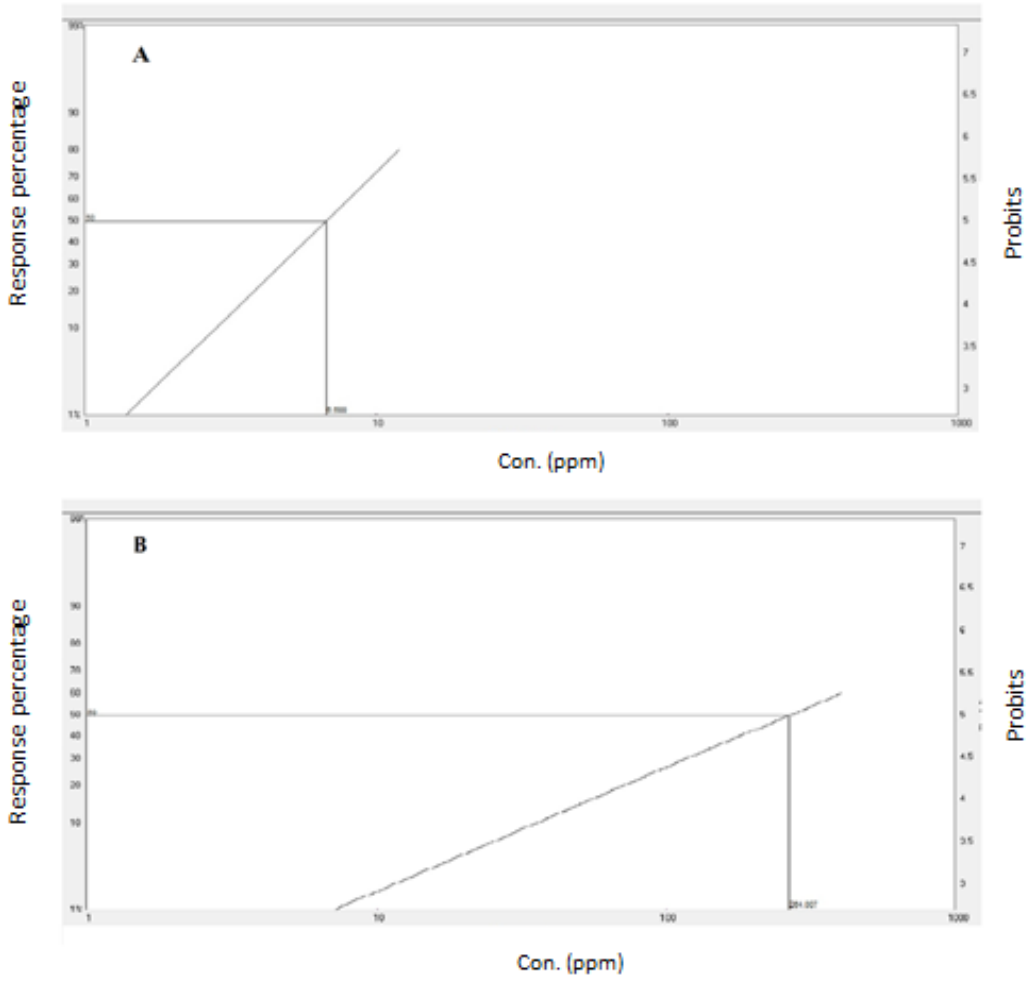


Fig. 1. Concentration-mortality regression line for the 3rd larval instar of *Cx. pipiens* treated with variable concentrations of **A)** Copper chloride; **B)** Cadmium chloride

2. Heavy metal concentrations in *Culex pipiens* larval tissues

The concentrations of copper chloride and cadmium chloride in the tissues of *Cx. pipiens* larvae 24h post-exposure with LC_{50} are shown in Fig. (2). The results display that the concentrations of these two heavy metals were increased significantly in the treated larvae compared to those of the untreated larvae ($P< 0.05$). Moreover, copper chloride significantly accumulated in the *Cx. pipiens* larval tissues compared to cadmium chloride.

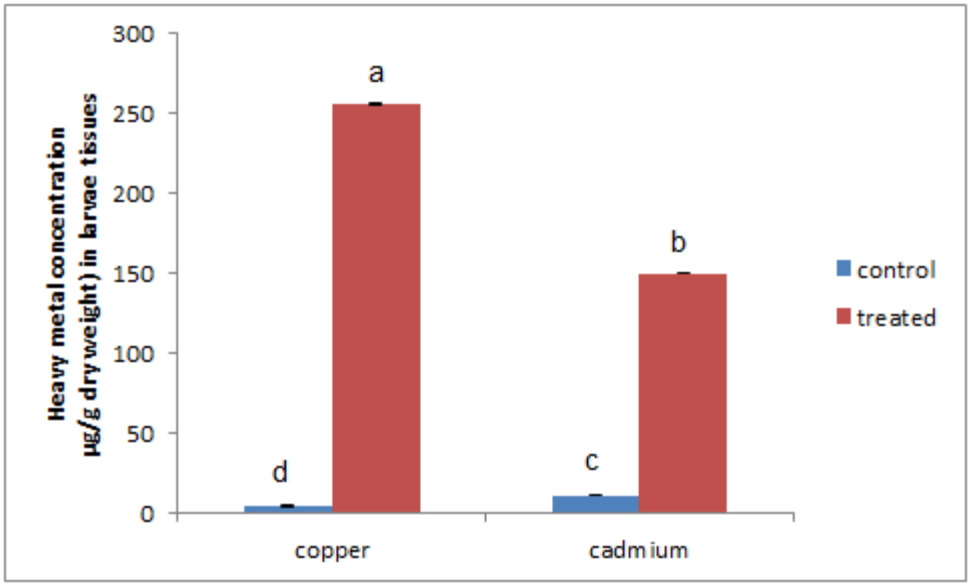


Fig. 2. Heavy metal concentrations (µg/g dry weight) in the 3rd larval tissues of *Cx.pipiens* 24h post-treatment with LC_{50} of copper chloride and cadmium chloride. Means with different letters are significantly different (Tukey's test: $P<0.05$)

3. Heavy metal biochemical effects on *Culex pipiens* 3rd - larval instar

3.1. Heavy metal effects on the total protein content

Treatment with copper chloride and cadmium chloride significantly increased the content of the total protein in the third-larval instar of *Cx. pipiens* relative to control ($P<0.05$) by 43.66, and 13.23%, respectively (Fig. 3A).

3.2. Heavy metal effects on the activity of antioxidant enzymes

Fig. (3B) showed that CAT activity was significantly increased relative to control post-exposure to copper and cadmium chloride by 40.7 and 15.99%, respectively ($P< 0.05$). Fig. (3C) illustrates a significant increase in POX activity by 212.94 and 52.14%, for the two metals respectively ($P< 0.05$) in *Cx. pipiens* larvae relative to control.

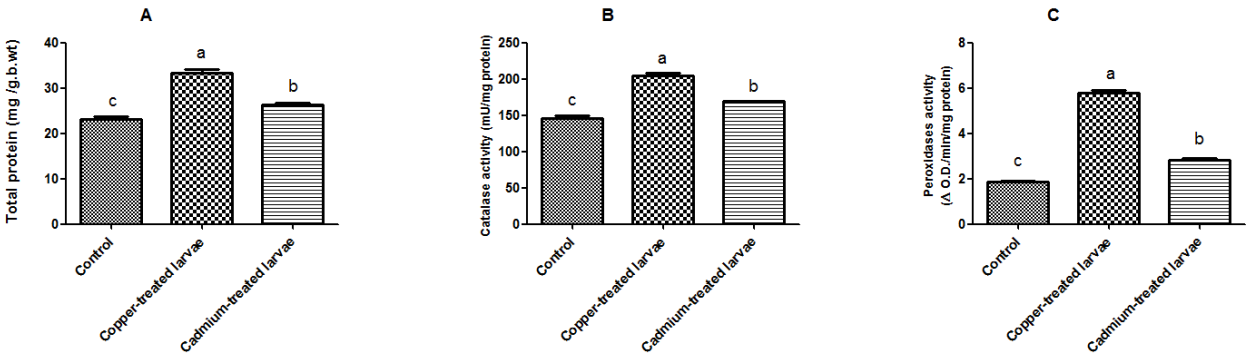


Fig. 3. Changes in **(A)** Total protein; **(B)** Catalase activity (CAT); **(C)** Peroxidase activity (POX) in the 3rd-instar larvae of *Cx. pipiens* 24h post-treatment with LC_{50} of copper chloride and cadmium chloride heavy metals. Means with different letters are significantly different (Tukey's test: $P< 0.05$)

3.3. Heavy metal effects on the activity of detoxifying enzymes

Treatment with copper chloride showed non-significant decrease in the GST activity of *Cx. pipiens* larvae relative to control by -8.26% ($P> 0.05$). Application of cadmium chloride significantly increased GST activity relative to control by 39.88% ($P< 0.05$) (Fig. 4A).

Data presented in Fig. (4B) indicate significant reduction in α-esterase activity in *Cx. pipiens* third-larval instar following exposure to copper chloride by -16.59% relative to control ($P< 0.05$). A significant increase was detected in larvae treated with cadmium chloride relative to control by 13.1% ($P< 0.05$).

Treatment with copper chloride insignificantly decreased β-esterase activity of *Cx. pipiens* larvae relative to control by -3.23% ($P> 0.05$). Treatment with cadmium chloride significantly increased β-esterase activity relative to control by 29.09% ($P< 0.05$) (Fig. 4C).

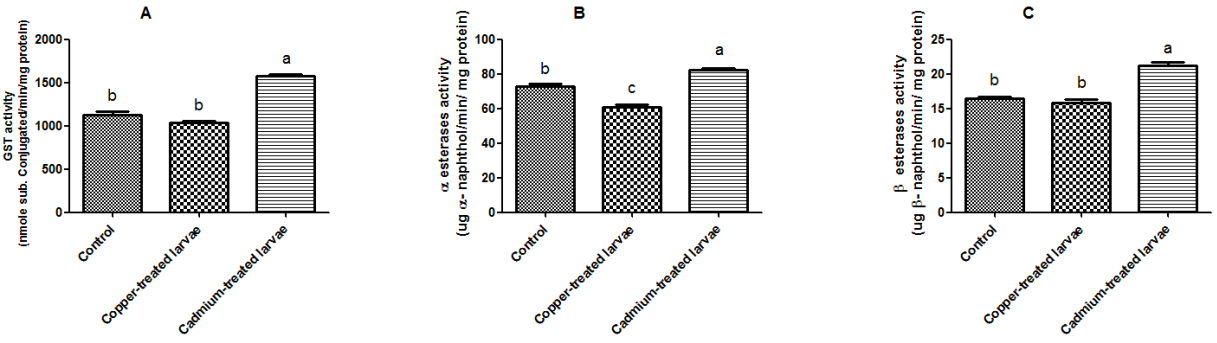


Fig. 4. Changes in **(A)** Glutathione S-transferase activity (GST)**; (B)** α-Esterase activity; **(C)** β-Esterase activity in the 3rd - instar larvae of *Cx. pipiens* 24 h post-treatment with LC_{50} of copper chloride and cadmium chloride heavy metals. Means with different letters are significantly different (Tukey's test: $P< 0.05$).

4. Genotoxicity in *Culex pipiens* larvae

Genotoxic effects were assessed by exposing *Cx. pipiens* third-larval instar to the LC_{50} of copper chloride and cadmium chloride, with DNA fragment migration analyzed via agarose gel-electrophoresis (Fig. 5A–C). After the treatment of larvae with the tested heavy metals, all parameters of the comet assay, viz., % of damage, tail length, DNA % in the tail, tail moment, and olive tail moment showed increases with varying degrees relative to the control (Fig. 6A-E). Exposure to copper chloride and cadmium chloride showed significant increase % of damage relative to control ($P< 0.05$) (Fig. 6A). Tail length (Fig. 6B) and tail moment (Fig. 6D) showed non-significant increase in the post-treatment of *Cx. pipiens* larvae to the two tested heavy metals relative to control ($P> 0.05$). DNA % in the tail (Fig. 6C) and olive tail moment (Fig. 6E) showed significant increase only in larvae treated with copper chloride relative to the control ($P< 0.05$).

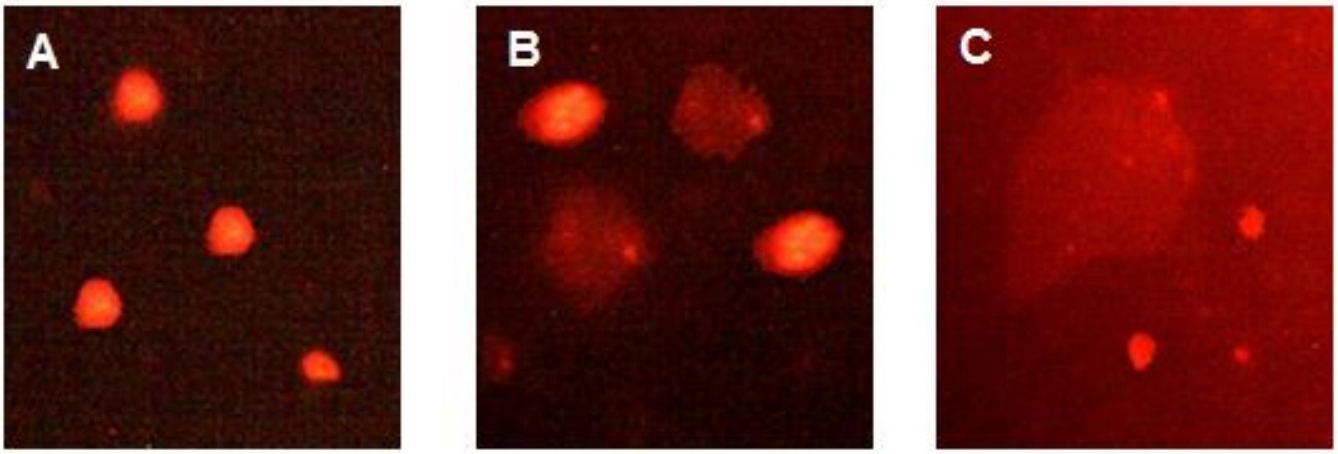


Fig. 5. Comet photomicrographs of DNA isolated from third-instar larvae of *Cx. pipiens* 24h post-treatment with LC_{50} of the two tested heavy metals. **(A)** Control; **(B)** Larvae treated with copper chloride; **(C)** Larvae treated with cadmium chloride

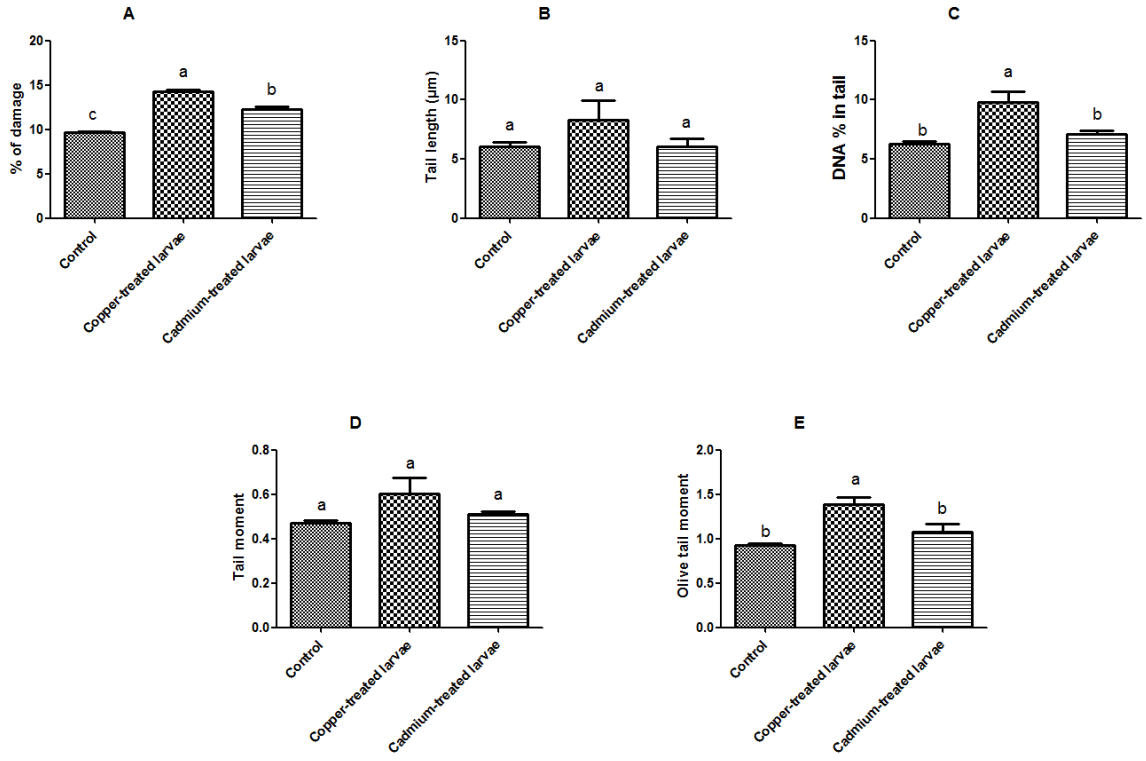


Fig. 6. Changes in comet assay parameters for genotoxicity in *Cx. pipiens* third-instar larvae 24h post-exposure to LC_{50} of copper chloride and cadmium chloride. **(A)** % of damage; **(B)** Tail length; **(C)** DNA % in tail; **(D)** Tail moment; **(E)** Olive tail moment. Means with different letters are significantly different (Tukey's test: $P< 0.05$)

5. Gene expression analyses of pollution-biomarker genes

Treatment of *Cx. pipiens* third-instar larvae with LC_{50} of copper chloride for 24h significantly decreased the relative expression of AChE (Fig. 7A) and CYP450 (Fig. 7B) but significantly up-regulated the relative expression of HSP (Fig. 7C), SOD (Fig. 7D) and MT (Fig. 7E) by 0.64, 0.15, 12.44, 1.51 and 1.84 folds, respectively, relative to the control ($P< 0.05$). Treatment with LC_{50} of cadmium chloride significantly decreased the relative expression of AChE (Fig. 7A) and CYP450 (Fig. 7B) but significantly up-regulated the relative expression of HSP (Fig. 7C), SOD (Fig. 7D) and MT (Fig. 7E) by 0.62, 0.76, 2.66, 8.54 and 1.62 folds, respectively, relative to the control ($P<0.05$).

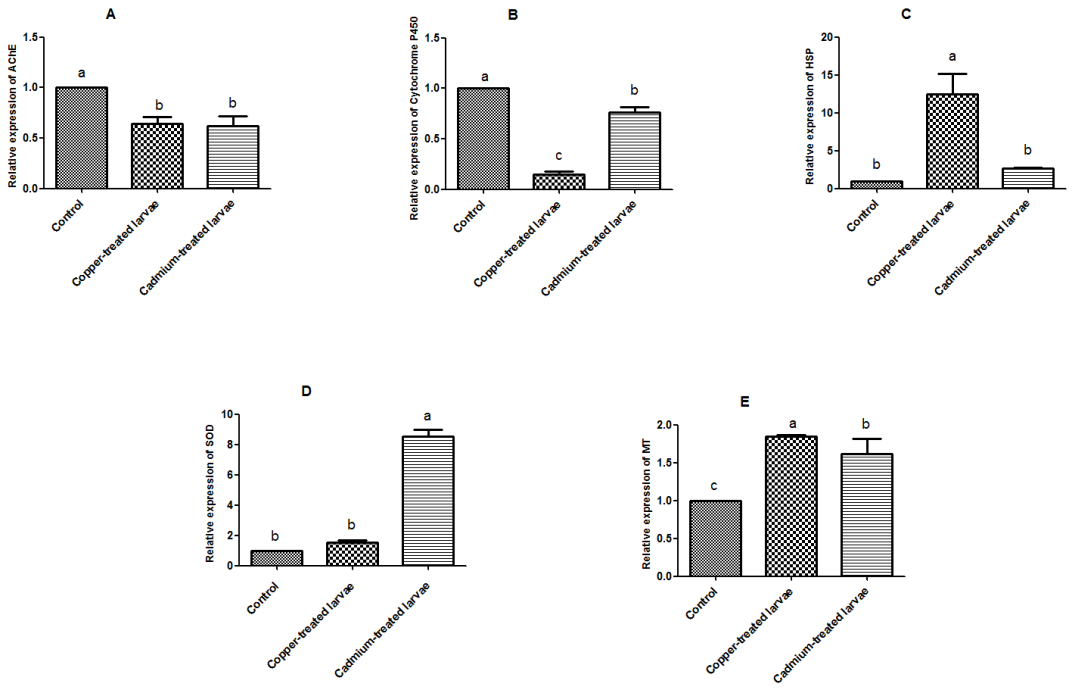


Fig. 7. Changes in the relative expression in the 3rd-instar larvae of *Cx. pipiens* 24 h post-treatment with LC_{50} of copper chloride and cadmium chloride. **(A)** Acetylcholinesterase (AChE); **(B)** Cytochrome P450 (CYP450); **(C)** Heat shock protein (HSP); **(D)** Superoxide dismutase (SOD); **(E)** Metallothionein (MT). Means with different letters are significantly different (Tukey's test: $P<0.05$)

DISCUSSION

Heavy metals represent a considerable risk to aquatic ecosystems and human health, rendering their widespread occurrence in water a significant environmental issue **(Ahmadijokani *et al.*, 2022)**. They are non-biodegradable and typically bioaccumulate within living organisms. Elevated metal concentrations in different organs of organisms serve as a critical indicator of metal pollution in aquatic environments **(Mona *et al.*, 2019)**. In recent decades, ecotoxicological investigations have evaluated anthropogenic environmental toxins by identifying organisms with deposited heavy metals. Insects serve as bioindicators to estimate the heavy metal accumulation in their ecosystems, especially when there is direct contact with the environment **(Chowdhury *et al.*, 2023)**.

The current findings indicated that copper and cadmium exposure greatly reduced the survival of *Cx. pipenis* larvae. As the concentration of the two heavy metals increased, the larval mortality also increased. Nonetheless, the current findings indicate that the toxic effects of the studied heavy metals differed significantly among the various metals when assessed against the larval stage. According to the LC_{50} values, copper exhibited the highest toxicity toward the larval stage, with cadmium following closely behind. These results align with those reported by **Oliver and Brooke (2018)**, who found

higher toxicity of copper than cadmium and lead against *Anopheles arabiensis.* **Hafez *et al.* (1999)** also observed a significant decrease in the survivorship of *Cx. pipiens* larvae with increasing cadmium concentration. **Salama (2002)** demonstrated that the mortality rate in *Cx. pipiens* larvae increased with increasing concentrations of pollutants, specifically Cd, Hg, and Pb. The toxicity of heavy metals was also documented in various insect species. **Pascoe *et al.* (1989)** observed that increased mortality rate was prevalent in 1st -instar of *Chironomus riparius* (Meigen) larvae when exposed to cadmium, with mortality rates rising in correlation with increased cadmium concentration. The LC_{50} value of copper for the heavy metals examined may align with those reported by **El-Sheikh *et al.* (2010)** for *Cx. pipiens* larvae (5.09ppm).

Accumulation of metals in organisms' tissues resulted in acute and chronic impacts like developmental abnormalities and growth aberration **(Sildanchandra & Crane, 2000)**. To understand the possible impacts of a heavy metal on living organisms, it is also necessary to evaluate the relation between environmental concentration and accumulation in organisms' tissue **(Krantzberg & Stokes, 1990)**. Aquatic organisms may be particularly susceptible as they could uptake copper from both water and diet **(Clearwater *et al.*, 2002)**. Copper might enter aquatic organisms' tissues through the chitinous exoskeleton, especially at the permeable respiratory surfaces through a passive diffusion process **(Simkiss & Taylor, 1989; Hare, 1992; Dutta *et al.*, 2010)**. The present finding showed an accumulation of copper and cadmium metals in the tissues of *Cx. pipiens* larvae. This result follows **Toto *et al.* (2024)**, who determined the accumulation of large quantities of heavy metals including Cd and Cu in the midgut tissues of *Cx. pipiens* larvae obtained from polluted water.

The present finding revealed that the application of copper chloride and cadmium chloride significantly increased the total protein content in the third-instar larvae of *Cx. pipiens*. This result is in line with the findings of **Hassan *et al.* (2011)**, who found that copper metal led to a significant increase in total protein content in *Cx. Pipiens*, while recording a notable reduction in the total protein of female ovaries tested with cadmium.

The cellular lipid peroxidation, enzymatic, and non-enzymatic antioxidants indicate the oxidative stress status of the organism and can be utilized to evaluate the environmental stress in aquatic organisms **(Vukašinović *et al.*, 2020)**. The oxidative stress, which is produced from increased cellular generation of reactive oxygen species (ROS), increases with exposure to environmental contamination **(Win *et al.*, 2018)**. Antioxidant enzymes, such as CAT and POX are the first defense line and can neutralize molecules that could become free radicals or cause more radicals to be triggered **(Ighodaro & Akinloye, 2018)**. Heavy metals exposure affects the oxidative equilibrium by boosting ROS generation and changing the activity of antioxidant enzymes **(Chen *et al.*, 2020)**. The current results indicated a significant increase in CAT level in *Cx. pipiens* larvae treated with copper and cadmium chloride. **Saleem and Afsheen (2022)** indicated that water pollution with heavy metals increased the CAT level in three species of water

striders. Conversely, **Toto *et al.* (2024)** found that the CAT activity was significantly decreased in *Cx. ppiens* larvae from heavy metal-polluted water.

The POX activity was also significantly increased in *Cx. pipiens* larvae treated with copper chloride and cadmium chloride. This finding aligns with the results of **El-Saad *et al.* (2017)**, who found a declining level of GPx activity in *Apis mellifera* inhabiting a contaminated environment. Additionally, **Mese *et al.* (2022)** found a reduction in *G. mellonella*'s GPx level in all treated groups that were exposed to Cu and Zn.

Our results showed an insignificant reduction in GST, α-esterase, and β-esterase activities in *Cx. pipiens* larvae treated with copper chloride, while treatment with cadmium chloride illustrated significant increases in their activities. These findings align with the results of **Yang *et al.* (2023)**, who observed that GST level in *Protohermes costalis* was increased by cadmium exposure. Mechanisms of defense against oxidative stress after exposure to metal pollutants likely benefit adult mosquitoes in environments polluted with insecticides because it unintentionally increases the expression of specific insecticide resistance phenotypes through the increase of GSTs **(Oliver & Brooke, 2016)**. Results by **Oliver and Brooke (2018)** stated that the activity of GST was significantly reduced in *Anopheles arabiensis* females after exposure to different metals (cadmium, copper, and lead). On the other side, there was no significant change in α-esterase or β-esterase activities.

The strand breaks in DNA caused by various genotoxic agents have been studied in numerous aquatic animals using comet assay, demonstrating reliability and sensitivity as a biomarker **(Park & Choi, 2009)**. **Yousef *et al.* (2019)** noticed a direct link between increased DNA damage and heavy metal exposure and ROS generation. Our findings indicated that treatment with copper and cadmium chloride showed a significant increase in the percentage of DNA damage, and DNA % in tail and olive tail moment showed significant increase only in larvae treated with copper chloride. These results is in accordance with **Toto *et al.* (2024)**, who found a notable elevation in DNA damage in *C. pipiens* larvae obtained from polluted site. **Lee *et al.* (2006)** demonstrated that cadmium exhibits cytotoxic effects on *Chironomus tentans* larvae.

In the current investigation, exposure of *Cx. pipiens* 3rd-instar larvae to LC_{50} of copper chloride and cadmium chloride significantly decreased the relative expression of AChE. Inhibition of acetylcholinesterase may result in post-synaptic cholinergic receptor hyperstimulation, leading to physiological dysfunction **(Tilton *et al.*, 2011)**. Results about the action mechanism of AChE inhibition in insects due to metal exposure remain ambiguous. It was assessed that metals may inhibit AChE in both vertebrates and invertebrates **(Frasco *et al.*, 2005)**. **Amer *et al.* (2022)** indicated that AChE concentration in *Cx. pipiens* larvae were elevated in both control and low copper conditions, but they significantly decreased under high concentrations**.**

All functions of CYP450s in the detoxification process are well known, also their charge in the oxidative metabolism of a wide range of endogenous and exogenous chemicals in insects **(Monostory & Dvorak, 2011)**. Our results indicated that exposure of *Cx. pipiens*3rd -instar larvae to LC_{50} of copper chloride and cadmium chloride significantly decreased the relative expression of CYP450. This result is compatible with the outcome of **Toto *et al.* (2024)**, who found that CYP450 activity in the polluted *Cx. pipiens* larval group was at a lower level than the reference larvae. In a previous study by **Musasia *et al.* (2013)**, lead and cadmium exposures significantly downregulated the expression of CYP450 genes in *Anopheles gambiae*. In contrast, **Bernabò *et al.* (2017)** stated an elevation of CYP450 enzymatic activity in *Chironomus riparius* larvae exposed to copper.

According to physiological and environmental factors, the role of the HSP70 protein in insects varies across species or within the same species **(King & MacRae, 2015)**. It is present in early instars of insects, supporting them in defeating unfavorable environments **(Kamel & Mahmoud, 2018)**. The HSH70 protein may protect cells from metal-induced chromosomal aberrations by promoting cell cycle regulation and decreasing genomic instability **(Barnes *et al.*, 2002)**. The expression of HSP70 is enhanced for cellular protection during stressors brought by temperature, pathogenic infections, and exposure to heavy metals. Generally, it is expressed at low levels in normal states **(Song *et al.*, 2006)**. It also prevents the aggregation of degraded proteins, resulting in intense damage in the stressed cells **(Azam *et al.*, 2017)**. Our results revealed that exposure of *Cx. pipiens* 3rd -instar larvae to LC_{50} of copper chloride and cadmium chloride increased the relative expression of heat shock protein (HSP). This aligns with **Toto *et al.* (2024)**, who demonstrated that *Cx. pipiens* larvae with the largest level of heavy metal accumulation showed a considerable increase in HSP expression compared with the reference group. Elevation in HSP70 expression levels in insects due to heavy metal pollution has been reported in other studies **(Braeckman *et al.*, 1997a, b; Joshi & Tiwari, 2000; Kafel *et* al., 2012; Doğanlar *et al.*, 2014; El-Samad *et al.*, 2021)**.

In our investigation, heavy metal exposure increased the relative expression of SOD in *Cx. pipiens* 3rd -instar larvae. This result is aligned with **Islam *et al.* (2019)**, who observed higher SOD activity in muga silkworms *Antheraea assamensis* after exposure to heavy metals contamination. In contrast, **Azam *et al.* (2017)** 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. The midgut tissues of *Cx. pipiens* larvae taken from heavy metals polluted site showed a significant decrease in SOD activity **(Toto *et al.*, 2024)**.

Our results illustrated that heavy metal treatment increased the relative expression of MT in *Cx. pipiens* 3rd -instar larvae. Our finding is consistent with **Toto *et al.* (2024)**, who demonstrated that the midgut tissues of *Cx. pipiens* larvae, from heavy metal polluted site, had higher MT concentrations than those from the reference site. The higher

MT production is the mechanism for metal tolerance, as reported in *Chironomus javanus* fourth-instar larvae **(Somparn *et al.*, 2015)**.

CONCLUSION

This study concluded that the third-instar larvae of *Cx. pipiens* can bioaccumulate heavy metals present in their aquatic habitats since their tissues were recorded to be highly sensitive to heavy metal pollution resulting in biochemical, genotoxicity, and molecular alteration. Consequently, *Cx. pipiens* larvae can be considered an effective bioindicator for heavy metal pollution in the aquatic environment.

ETHICAL APPROVAL

All experiments in the present study received approval from the Research Ethics Committee of the Faculty of Science, South Valley University, Qena governorate, Egypt. (Code No. 009/02/24).

ACKNOWLEDGMENTS

The authors would like to thank Prof. Shaymaa H. Mahmoud, Zoology Department, Faculty of Science, Menoufia University, Shebin El-Kom, Egypt for her keen interest, support and help, and acknowledge partial financial support from the Zoology Department, Faculty of Science, South Valley University and Zoology Department, Faculty of Science, Suez University.

REFERENCES

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. J. Econ. Entomol., 18: 265–267.

Aebi, H. (1984). Catalase *in vitro*. Methods Enzymol., 105: 121–126. https://doi.org/10.1016/s0076-6879(84)05016-3.

Ahmadijokani, F.; Molavi, H.; Peyghambari, A.; Shojaei, A.; Rezakazemi, M.; Aminabhavi, T. M. and Arjmand, M. (2022). Metal-organic frameworks and electrospinning: A happy marriage for wastewater treatment. J. Environ. Manage., 316: 115214. **https://doi.org/10.1002/adfm.202207723**

Akhtar, N.; Syakir Ishak, M. I.; Bhawani, S. A. and Umar, K. (2021). Various natural and anthropogenic factors responsible for water quality degradation: a review. Water, 13(19): 2660. https://doi.org/10.3390/w13192660

Amer, N. R.; Lawler, S. P.; Zohdy, N. M.; Younes, A.; ElSayed, W. M.; Wos, G. and Connon, R. E. (2022). Copper exposure affects anti-predatory behaviour and acetylcholinesterase levels in *Culex pipiens* (Diptera, Culicidae). Insects, 13(12): 1151. https://doi.org/10.3390/insects13121151

Azam, I.; Afsheen, S.; Sarwar, M. K.; Zia, A. and Bhatti, A. R. (2017). Effect of heavy metals on antioxidant enzymes in *Oxya hyla hyla* (Orthoptera: Acrididae). Pakistan Entomol., 39(2): 37-44.

Aziz, K. H. H.; Mustafa, F. S.; Omer, K. M.; Hama, S.; Hamarawf, R. F. and Rahman, K. O. (2023). Heavy metal pollution in the aquatic environment: efficient and low-cost removal approaches to eliminate their toxicity: a review. RSC Adv., 13(26): 17595-610. https://doi.org/10.1039/D3RA00723E

Barnes, J. A.; Collins, B. W.; Dix, D. J. and Allen, J. W. (2002). Effects of heat shock protein 70 (Hsp70) on arsenite-induced genotoxicity. Environ. Mol. Mutagen., 40(4): 236–42. https://doi.org/10.1002/em.10116

Bernabò, P.; Gaglio, M.; Bellamoli, F.; Viero, G. and Lencioni, V. (2017). DNA damage and translational response during detoxification from copper exposure in a wild population of *Chironomus riparius*. Chemosphere, 173: 235-44. https://doi.org/10.1016/j.chemosphere.2017.01.052

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254. https://doi.org/10.1006/abio.1976.9999

Braeckman, B.; Raes, H. and Van Hoye, D. (1997a). Heavy-metal toxicity in an insect cell line. Effects of cadmium chloride, mercuric chloride and methylmercuric chloride on cell viability and proliferation in *Aedes albopictus* cells. Cell Biol. Toxicol., 13(6): 389–97. https://doi.org/10.1023/A:1007425925726

Braeckman, B.; Simoens, C.; Rzeznik, U. and Raes, H. (1997b). Effect of sublethal doses of cadmium, inorganic mercury and methylmercury on the cell morphology of an insect cell line (*Aedes albopictus*, C6/36). Cell Biol. Int., 21(12): 823–32. https://doi.org/10.1006/cbir.1998.0194

Ceschi-Bertoli, L.; Vidal, F. A. P.; Balsamo, P. J. and Abdalla, F. C. (2020). Comet assay protocol for *Bombus atratus* fat body and pericardial cells (Hymenoptera, bombini) at a safe concentration of mercury. Chemosphere, 261: 127752. https://doi.org/10.1016/j.chemosphere.2020.127752

Chen, J.; Wang, J. -W. and Shu, Y. –H. (2020). Review on the effects of heavy metal pollution on herbivorous insects. J. Appl. Ecol., 31(5): 1773-82. https://doi.org/10.13287/j.1001-9332.202005.035

Chowdhury, S.; Dubey, V. K.; Choudhury, S.; Das, A.; Jeengar, D. and Sujatha, B. (2023). Insects as bioindicator: a hidden gem for environmental monitoring. Front. Environ. Sci., 11. https://doi.org/10.3389/fenvs.2023.1146052

Clearwater, S. J; Farag, A. M. and Meyer, J. S. (2002). Bioavailability and toxicity of dietborne copper and zinc to fish. Comp. Biochem. Physiol. C Toxicol., 132: 269-313. 10.1016/s1532-0456(02)00078-9

Dalzochio, T.; Rodrigues, G. Z. P.; Petry, I. E.; Gehlen, G. and da Silva, L. B. (2016). The use of biomarkers to assess the health of aquatic ecosystems in Brazil: a review. Int. Aquat. Res., 8: 283- 98. https://doi.org/10.1007/s40071-016-0147-9

Delhaye, J.; Aletti, C.; Glaizot, O. and Christe, P. (2016). Exposure of the mosquito vector *Culex pipiens* to the malaria parasite *Plasmodium relictum*: effect of infected blood intake on immune and antioxidant defences, fecundity and survival. Parasit. Vectors, 9: 1-12. https://doi.org/10.1186/s13071-016-1905-7

Doğanlar, Z. B.; Doğanlar, O. and Tabakc¸ıoğlu, K. (2014). Genotoxic effects of heavy metal mixture in *Drosophila melanogaster*: Expressions of heat shock proteins, RAPD profiles and mitochondrial DNA Sequence. Water Air Soil Pollut., 225(9): 1-14. https://doi.org/10.1007/s11270-014-2104-9

Dua, V. K.; Kumar, A.; Pandey, A. C. and Kumar, S. (2013). Insecticidal and genotoxic activity of *Psoralea corylifolia* Linn. (Fabaceae) against *Culex quinquefasciatus* Say, 1823. Parasit. Vectors, 6(1): 1-8. https://doi.org/10.1186/1756-3305-6-30

Dutta, A.; Kumari, S.; Smita, A. and Dutta, S. (2010). Bioaccumulation of copper and lead in *Chironomus* Sp.(Diptera: Chironomidae) at different temperature under laboratory. The Bioscan, 2: 313–322.

El-Gendy, A. H.; Augustyniak, M.; Toto, N. A.; Al Farraj, S. and El-Samad, L. M. (2020). Oxidative stress parameters, DNA damage and expression of HSP70 and MT in midgut of *Trachyderma hispida* (Forskål, 1775) (Coleoptera: Tenebrionidae) from a textile industry area. Environ. Pollut., 267: 115661. https://doi.org/10.1016/j.envpol.2020.115661

El-Saad, A. M. A.; Kheirallah, D. A. and El-Samad, L. M. (2017). Biochemical and histological biomarkers in the midgut of *Apis mellifera* from polluted environment at Beheira Governorate, Egypt. Environ. Sci. Pollut. Res., 24: 3181-93. https://doi.org/10.1007/s11356-016-8059-1

El-Samad, L. M.; El-Ashram, S.; Kheirallah, D. A.; Abdul-Aziz, K. K.; Toto, N. A. and Mokhamer, E. H. M. (2021). Relative gene expression, micronuclei formation, and ultrastructure alterations induced by heavy metal contamination in *Pimelia latreillei* (Coleoptera: Tenebrionidae) in an urban-industrial area of Alexandria, Egypt. Plos One, 16(6):e0253238. https://doi.org/10.1371/journal.pone.0253238

El-Samad, L. M.; Radwan, E. H.; Mokhamer, E. H. M. and Bakr, N. R. (2019). Aquatic beetles *Cercyon unipunctatus* as bioindicators of pollution in Lake Edku and Mariut, Egypt. Environ. Sci. Pollut. Res. Int., 26(7): 6557-64. https://doi.org/10.1007/s11356-018-4016-5

El-Sheikh, E. S. M.; Fouda, M. A.; Hassan, M. I.; Abd-Elghaphar, A. E. A. and Hasaballah, A. I. (2010). Toxicological effects of some heavy metal ions on *Culex pipiens* L.(Diptera: Culicidae). Egypt. Acad. J. Biol. Sci., F Toxicol. Pest Control, 2(1): 63-76. https://doi.org/10.21608/EAJBSF.2010.17465

Finney, D.J. (1971). Probit analysis (3 rd ed.). Cambridge University Press, Cambridge, UK.

Frasco, M. F.; Fournier, D.; Carvalho, F. and Guilhermino, L. (2005). Do metals inhibit acetylcholinesterase (AchE)? Implementation of assay conditions for the use of AchE activity as a biomarker of metal toxicity. Biomarkers, 10: 360–375. https://doi.org/10.1080/13547500500264660

Habig, W. H.; Pabst, M. J.; Jakoby, W. B. (1974). Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8

Hafez, G. A.; Aly, S. A. and El-Ebiarie, A. S. (1999). Effect of some Heavy metals alone and combined with bacteria on the survivorship of *Culex pipiens* larvae. J. Egypt. Ger. Soc. Zool., 30: 61-75.

Hammerschmidt, R.; Nuckles, F. and Kuc, J. (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrchum lagenarium*. Physiol. Plant Pathol., 20: 73–82. https://doi.org/10.1016/0048-4059(82)90025-X

Han, J. and Lee, K. -W. (2021). Identification and response of cytochrome P450 genes in the brackish water flea *Diaphanosoma celebensis* after exposure to benzo[α]pyrene and heavy metals. Mol. Biol. Rep., 48: 657-64. https://doi.org/10.1007/s11033-020-06113-y

Harbach, R. E. (1985). Pictorial keys to the genera of mosquitoes, sub-genera of *Culex* and the species of *Culex* (*Culex*) occurring in south-western Asia and Egypt, with a note on the sub-generic placement of *Culex deserticola* (Diptera: Culicidae). Mosquito Systematics, 17(2): 83-107.

Hare, L. (1992). Aquatic insects and trace metals: bioavailability, bioaccumulation and toxicity. Crit. Rev. Toxicol., 22: 327 -369. https://doi.org/10.3109/10408449209146312

Hassan, M. I.; Fouda, M. A.; El-Sheikh, T. M.; Abd-Elghaphar, A. A. and Hasaballah, A. I. (2011). Electrophoretic study of ovarian protein and amino acid in the mosquito *Culex pipiens* L.(Diptera: Clucidae) as influenced by some heavy metal ions. Egypt. J. Biomed. Sci., 36: 1-14.

Hu, X.; Fu, W.; Yang, X.; Mu, Y.; Gu, W. and Zhang, M. (2019). Effects of cadmium on fecundity and defence ability of *Drosophila melanogaster*. Ecotoxicol. Environ. Saf., 171: 871-7. https://doi.org/10.1016/j.ecoenv.2019.01.029

Ighodaro, O. and Akinloye, O. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alex. J. Med., 54(4): 287-93. https://doi.org/10.1016/j.ajme.2017.09.001

Islam, S. J.; Manna, P.; Unni, B. and Kailta, J. (2019). Higher concentrations of heavy metals impair antioxidant defense mechanism and growth response of muga silkworm, *Antheraea assamensis* (Lepidoptera: Saturniidae). J. Entomol. Zool. Stud., 7(2): 715-24.

Joshi, A. and Tiwari, P. K. (2000). Chromosomal responses of blowfly *Lucilia cuprina* to heat and heavy metal stress. Genetica, 109(3): 211–8. https://doi.org/10.1023/a:1017541901690

Kabata-Pendias, A. (2000). Trace elements in soils and plants. (CRC Press: Boca Raton, Florida). https://doi.org/10.1201/b10158

Kafel, A.; Nowak, A.; Bembenek, J.; Szczygieł, J.; Nakonieczny, M. and Swiergosz-Kowalewska, R. (2012). The localisation of HSP70 and oxidative stress indices in heads of *Spodoptera exigua* larvae in a cadmium-exposed population. Ecotoxicol. Environ. Saf., 78: 22–7. https://doi.org/10.1016/j.ecoenv.2011.10.024

Kamel, A. and Mahmoud, S. (2018). Molecular characterisation and expression of heat shock protein gene (HSP70) in *Tribolium castaneum* adults under different environmental stressors. Afr. Entomol., 26(2): 495– 506. https://doi.org/10.4001/003.026.0495

King, A. M. and MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. Annu. Rev. Entomol., 60: 59-75. https://doi.org/10.1146/annurev-ento-011613-162107

Krantzberg, G. and Stokes, P. M. (1990). Metal concentrations and tissues distribution in larvae of *Chironomus* with reference to x-ray microprobe analysis. Arch. Environ. Contam. Toxicol. 19:84–93. https://doi.org/10.1007/BF01059816

Lai, H.; Su, S.; Guo, H. and Chen, Z. (2011). Heavy metals contaminated soils and phytoremediation strategies in Taiwan. In: Pascucci, S., (ed). Soil contamination. Croatia: Intech Open: 107-26. http://dx.doi.org/10.5772/24230

Lee, S. E.; Yoo, D. H.; Son, J. and Cho, K. (2006). Proteomic evaluation of cadmium toxicity on the midge *Chironomus riparius* Meigen larvae. Proteomics, 6: 945–957. https://doi.org/10.1002/pmic.200401349

Lekfeldt, J. D. S.; Holm, P. E.; Kjaergaard, C. and Magid, J. (2017). Heavy metal leaching as afected by long-time organic waste fertilizer application. J. Environ. Qual., 46: 871–8. https://doi.org/10.2134/jeq2016.11.0458

Liaqat, I.; Virk, N. and Ali, N. M. (2023). Recent advances in evaluating insects as bioindicators of heavy metal poolution. In: Almayyahi, B. A. (ed). Heavy metals Recent Adavance. Intench Open: 1-17. http://dx.doi.org/10.5772/intechopen.110212

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25: 402–408. https://doi.org/10.1006/meth.2001.1262

Loring, D. H. and Rantala, R. T. (1992). Manual for the geochemical analyses of marine sediments and suspended particulate matter. Earth Sci. Rev., 32(4):235-83. https://doi.org/10.1016/0012-8252(92)90001-A

Mebane, C. A.; Schmidt, T. S.; Miller, J. L. and Balistrieri, L. S. (2020). Bioaccumulation and toxicity of cadmium, copper, nickel, and zinc and their

mixtures to aquatic insect communities. Environ. Toxicol. Chem., 39(4): 812-33. https://doi.org/10.1002/etc.4663

Mese, Y.; Tuncsoy, B. and Ozalp, P. (2022). Effects of Cu, Zn and their mixtures on bioaccumulation and antioxidant enzyme activities in *Galleria mellonella* L. (Lepidoptera: Pyralidae). Ecotoxicol., 31(4): 649-56. https://doi.org/10.1007/s10646-022-02531-9

Midway, S.; Robertson, M.; Flinn, S.; Kaller, M. (2020). Comparing multiple comparisons: practical guidance for choosing the best multiple comparisons test. PeerJ 8, e10387. https://doi.org/10.7717/peerj.10387

Mireji, P. O.; Keating, J.; Hassanali, A.; Mbogo, C. M.; Nyambaka, H.; Kahindi, S. and Beier, J. C. (2008). Heavy metals in mosquito larval habitats in urban kisumu and Malindi, Kenya and their impact. Ecotox. Environ. Safe., 70: 147-53. https://doi.org/10.1016/j.ecoenv.2007.03.012

Mona, M. H.; El-Naggar, H. A.; El-Gayar, E. E.; Masood, M. F. and Mohamed, E. N. E. (2019). Effect of human activities on biodiversity in Nabq Protected Area, South Sinai, Egypt. Egy. J. Aqua. Res., 45: 33-43. https://doi.org/10.1016/j.ejar.2018.12.001

Monostory, K. and Dvorak, Z. (2011). Steroid regulation of drug-metabolizing cytochromes P450. Curr. Drug Metab., 12(2): 154-72. https://doi.org/10.2174/138920011795016854

Musasia, F. K.; Isaac, A. O.; Masiga, D. K.; Omedo, I. A.; Mwakubambanya, R. and Ochieng, R. (2013). Sex specific induction of CYP6 cytochrome P450 genes in cadmium and lead tolerant *Anopheles gambiae*. Malar. J., 12: 1-5. https://doi.org/10.1186/1475-2875-12-97

Nebbak, A.; Almeras, L.; Parola, P. and Bitam, I. (2022). Mosquito vectors (Diptera: Culicidae) and mosquito-borne diseases in North Africa. Insects, 13(10): 962. https://doi.org/10.3390/insects13100962

Oliver, S. V. and Brooke, B. D. (2016). The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors *Anopheles arabiensis* and *Anopheles funestus.* PLoS One, 11 (3): e0151049. https://doi.org/10.1371/journal.pone.0151049 PMID: 26964046

Oliver, S. V. and Brooke, B. D. (2018). The effect of metal pollution on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). PloS One, 13(2): e0192551. https://doi.org/10.1371/journal.pone.0192551

Park, S. Y. and Choi, J. (2009). Genotoxic effects of nonylphenol and bisphenol a exposure in aquatic biomonitoring species: Freshwater crustacean, *Daphniamagna*, and aquatic midge, *Chironomusriparius*. Bull. Environ. Contam. Toxicol., 83(4): 463–468. https://doi.org/10.1007/s00128-009-9745-1

Pascoe, D.; Williams, K. A. and Green, D. W. J. (1989). Chronic toxicity of cadmium to *Chironomus riparius* Meigen- effects upon larval development and adult emergence. Hydrobiologia, 175: 109-115. https://doi.org/10.1007/BF00765121

Salama (2002). Bioaccumulation and effects of certain heavy metal ions in *Culex pipiens* L. (Diptera: Culicidae). M. Sc. Thesis, Faculty of Science, Al-Azhar University (Girls), Cairo, Egypt.

Saleem, R. and Afsheen, S. (2022). Analysis of antioxidants in water striders (Hemiptera: Gerridae) as bioindicator of water pollution. Braz. J. Biol., 84. https://doi.org/10.1590/1519-6984.258106

Schwartz, G. G.; Il'yaova, D. and Ivanova, A. (2003). Urinary cadmium, impaired fasting glucose, and diabetes in the NHANES III. Diabetes Care, 26(2): 468-470. https://doi.org/10.2337/diacare.26.2.468

Sildanchandra, W. and Crane, M. (2000). Influence of sexual dimorphism in *Chironomus riparius* Meigen on toxic effects of cadmium. Environ. Toxicol. Chem., 19: 2309–2313. https://doi.org/10.1002/etc.5620190921

Simkiss, K. and Taylor, M. G. (1989). Convergence of cellular systems of metal detoxification. Mar. Environ. Res. 28: 211–214. https://doi.org/https://doi.org/10.1016/0141-1136(89)90227-4

Singh, J. and Kalamdhad, A. S. (2011). Effects of heavy metals on soil, plants, human health and aquatic life. Int. J. Res. Chem. Environ., 1: 15-21

Singh, N. P.; McCoy, M. T.; Tice, R. R. and Schneider, E. L. (1988). A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res., 175: 184-91. https://doi.org/10.1016/0014-4827(88)90265-0

Somparn, A.; Iwai, C. and Noller, B. (2015). Potential use of acetylcholinesterase, glutathione-S-transferase and metallothionein for assessment of contaminated sediment in tropical chironomid, *Chironomus javanus*. J. Environ. Biol., 36(6):1355.

Song, L.; Wu, L.; Ni, D.; Chang, Y.; Xu, W. and Xing, K. (2006). The cDNA cloning and mRNA expression of heat shock protein 70 genes in the haemocytes of bay scallop (*Argopecten irradians*, Lamarck 1819) responding to bacteria challenge and naphthalin stress. Fish Shellfish Immunol., 21(4): 335-45. https://doi.org/10.1016/j.fsi.2005.12.011

Sowa, G. and Skalski, T. (2019). Effects of chronic metal exposure on the morphology of beetles species representing different ecological niches. Bull. Environ. Contam. Toxicol., 102: 191–7. https://doi.org/10.1007/s00128-018-02532-7

Tariq, S. R.; Shafq, M. and Chotana, G. A. (2016). Distribution of heavy metals in the soils associated with the commonly used pesticides in cotton fields. Scientifca, 2016(1): 7575239. https://doi.org/10.1155/2016/7575239

Tian, M.; Liu, B.; Hu, H.; Li, X.; Guo, Q.; Zou, F.; Liu, X.; Hu, M.; Guo, J.; Ma, L.; Zhou, D.; Sun, Y.; Shen, B. and Zhu, C. (2016). MiR-285 targets P450

(CYP6N23) to regulate pyrethroid resistance in *Culex pipiens pallens*. Parasitol. Res., 115: 4511–4517. https://doi.org/10.1007/s00436-016-5238-4

Tilton, F. A.; Bammler, T. K. and Gallagher, E. P. (2011). Swimming impairment and acetylcholinesterase inhibition in zebrafish exposed to copper or chlorpyrifos separately, or as mixtures. Comp. Biochem. Physiol. C Toxicol. Pharmacol., 153: 9–16. https://doi.org/10.1016/j.cbpc.2010.07.008

Toto, N. A.; Khattab, S. A.; El-Abbassy, S. A. and El-Saidy, S. A. (2024). The possibility of using *Culex pipiens* (Diptera: Culicidae) larvae as a bioindicator of water pollution in Burullus Lake, Egypt. J. Med. Life Sci., 6(2): 144-164. https://doi.org/ 10.21608/JMALS.2024.351977

Van Asperen, K. (1962). A study of house fly esterase by means of sensitive colorimetric method. J. Insect Physiol. 8, 401–416. https://doi.org/10.1016/0022-1910(62)90074-4

Vukašinović, E. L.; Čelić, T. V.; Kojić, D.; Franeta, F.; Milić, S. and Ninkov, J. (2020). The effect of long term exposure to cadmium on *Ostrinia nubilalis* growth, development, survival rate and oxidative status. Chemosphere, 243: 125375. https://doi.org/10.1016/j.chemosphere.2019.125375

WHO (2005). Guidelines for laboratory and field testing of mosquito larvicides. World Health Organization. https://apps.who.int/iris/handle/10665/69101.

Win, M. S.; Tian, Z.; Zhao, H.; Xiao, K.; Peng, J. and Shang, Y. (2018). Atmospheric HULIS and its ability to mediate the reactive oxygen species (ROS): a review. J. Environ. Sci., 71: 13-31. https://doi.org/10.1016/j.jes.2017.12.004

Yang, J.; Huang, X.; Wen, F.; Huang, X.; Liu, Z. and Zhang, Y. (2023). Characterization and expression analysis of glutathione S-transferase genes from an aquatic predator *Protohermes costalis* (Megaloptera: Corydalidae) on exposure to cadmium. J. Asia. Pac. Entomol., 26(2): 102061. https://doi.org/10.1016/j.aspen.2023.102061

You, H. J.; Lee, K. J. and Jeong, H. G. (2002). Overexpression of human metallothionein-III prevents hydrogen peroxide-induced oxidative stress in human fibroblasts. FEBS Lett., 521(1-3): 175-9. https://doi.org/10.1016/s0014-5793(02)02870-3

Yousef, H. A.; Abdelfattah, E. A. and Augustyniak, M. (2019). Antioxidant enzyme activity in responses to environmentally induced oxidative stress in the 5th instar nymphs of *Aiolopus thalassinus* (Orthoptera: Acrididae). Environ. Sci. Pollut. Res., 26: 3823-33. https://doi.org/10.1007/s11356-018-3756-6

Zaynab, M.; Al-Yahyai, R.; Ameen, A.; Sharif, Y.; Ali, L.; Fatima, M.; Khan, K. A. and Li, S. (2022): Health and environmental effects of Heavy metals. J. King Saud Univ. Sci., 34, 101653. http://dx.doi.org/10.1016/j.jksus.2021.101653