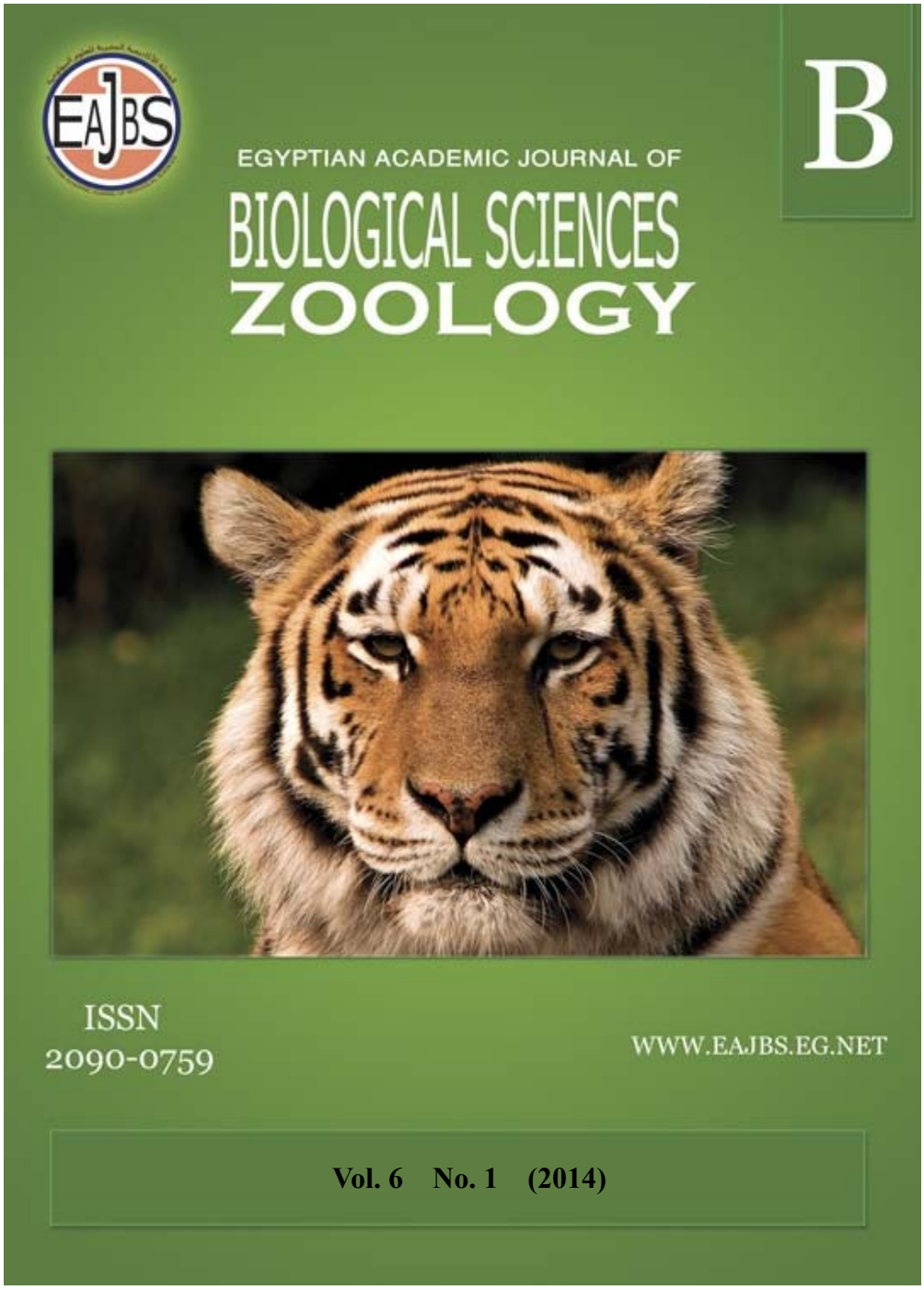


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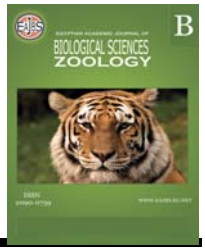


# Investigating the use of *Sphaeroma serratum* (Crustacea, Isopoda) as bio-indicator for heavy metals pollution in Lake Timsah, Suez Canal using alkaline comet assay technique

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## ABSTRACT

Pollution of the environment with heavy metals is a serious problem that is recognized in most countries in the world. Lake Timsah as an important lake in Suez Canal is highly polluted and receiving different sources of contaminations. However, attention and alarm thresholds of these parameters only concern the toxic effects of the polluting substances studied and do not take into consideration the question of chronic exposure at low doses of noxious chemicals. The physico-chemical parameters only provide snapshots of the condition of a water body and don't provide the integrative measure of overall health of any water body. On the other hand, crustaceans are used as bio-indicators in various aquatic systems in bio-accumulation experiments and in field studies. The crustacean isopod, *Sphaeroma serratum* which is found in large numbers at Lake Timsah was used for further investigation for detecting its DNA damages cause by the pollution. The comet assay technique which derives its name from the comet-like shape that cells with damaged DNA acquire was applied in this study in order to investigate on what level such poor water conditions in Lake Timsah can deform the DNA of its biota. It is recommended than other tests as it requires only a very small number of cells and these cells do not need to be undergoing active division. The present study confirmed the applicability of the alkaline comet assay technique as a sensitive tool for environmental monitoring. Also, it can be suggested that aquatic *S. serratum* which is a very tolerant species can resist pollution and can be used as a reliable bio-indicator of DNA damage.

**Keywords:** Comet Assay or Single cell gel electrophoresis, Comet Assay® kit, DNA damage, Biomontoring, *Sphaeroma serratum*.

## INTRODUCTION

Pollution of the environment with heavy metals is a serious problem that is recognized in most countries in the world (Characklis and Wiesner, 1997; Lee and Bang, 2000). During the last decades heavy metals have become common contaminants of aquatic and wetland environments throughout the world because of human activity and technological development (Cajaraville *et al.,* 2000).

Heavy metals are considered the most hazardous contaminant in the environment due to their persistence and accumulation in water, sediments and in tissues of the living organisms; this is by bio-concentration and bio-magnifications (Chaphekar, 1999; Dembitsky, 2003). Increasing attention has been given during the last decade to the protection of marine and freshwater aquatic environment against pollution, both nationally and internationally. The aquatic environment is often the ultimate recipient of anthropogenic contaminants. Some of the heavy metals such as lead and cadmium are toxic to living organisms even at low concentrations because they can be strongly accumulated through the food web in aquatic systems (El-Shenawy *et al*., 2006; Farahmandkia *et al*., 2010). As known, pollutants affect organisms at various levels of biological organization, from molecular to community levels (Theodorakis *et al*., 2000). The first step in monitoring water quality is to measure physico-chemical parameters. However, attention and alarm thresholds of these parameters only concern the toxic effects of the polluting substances studied and do not take into consideration the question of chronic exposure at low doses of noxious chemicals, frequently present in complex mixtures (Pellacani *et al*., 2006).

Physiological, morphological and genetic changes in certain organisms have been recognized to be related to particular environmental stressors and can also be used as indicators of adverse conditions. Furthermore, aquatic organisms tend to bio-accumulate pollutants, especially metals, from the surrounding environment or from food sources and these are also important bio-monitoring devices (Phillips and Rainbow, 1993; Kotzé *et al*., 1999). The monitoring of metals in an aquatic system is not only important for indicators of temporal and spatial extent of metal accumulation in a system, but also for organism health and the potential impact on human health (fish consumed) (Heath *et al*., 2004). The central aim of water quality analysis is to evaluate the impact of past, current or future anthropogenic stresses on aquatic ecosystems. Biological monitoring assays, on the other hand, could effectively define risks for the environment and man (Ohe *et al*., 2004). Although the physico-chemical parameters only provide snapshots of the condition of a water body and don't provide the integrative measure of overall health of any water body, the biological measurements provide an integrated, comprehensive assessment of the health of any water body overtime.

At least, there are three major sources responsible for the heavy metal contamination in Lake Timsah (area of interest) namely; agricultural drainage, industrial drainage and municipal (sewage) effluent. In the last three decades the aquatic ecosystem of Lake Timsah recorded hazardous levels of pollutant of various forms, heavy metals concentrations in the water sediments and marine fauna (Attwa, 1997; El-Moselhy, 2006; Gabr and Gab-Alla, 2008). While pesticides and polycyclic and aromatic hydrocarbons were investigated by Mostafa (2002).

Crustaceans are frequently used as bio-indicators in various aquatic systems in bio-accumulation experiments and in field studies. They have great advantages compared to other groups. They are an important link in food chains of virtually every inland water body converting phytoplankton/submerged plants, bacteria, fungi and

decaying organic matter into animal tissue that can be used by larger animals (Canton and Adema, 1978). The small sized crustacean, *Sphaeroma serratum* holds an important position in the aquatic food chain, responds to many pollutants, easy to culture and has short life cycle; thus, are considered suitable species for aquatic bio-monitoring (Lee *et al*., 2000). It occurs in a wide variety of habitats and it is very tolerant to desiccating conditions and can tolerate polluted water (Holdich and Tolba, 1985). Fortunately, *S. serratum* was found in large numbers at Lake Timsah, Egypt which considered as much polluted lake receiving different sources of pollution (receives toxicants and hydrocarbons accidentally discharged from ships passing through the Suez Canal) and exhibiting different degrees of water quality (Abdel-Hamed *et al*., 1991).

Bio-monitoring evaluations are required to be quick, relatively inexpensive, sensitive, and reproducible (Belpaeme *et al*., 1998). Belpaeme *et al*. (1998) concluded that the simplicity and sensitivity of the comet assay make it an adequate test system for bio-monitoring of chronic low level exposure. The comet assay is better than other tests (chromosome aberration, micronucleus assay and sister chromatid exchange tests) because it requires only a very small number of cells and these cells do not need to be undergoing active division, which means that any tissue can be evaluated (Fairbairn *et al*., 1995; Tice *et al*., 2000; Lee and Steinert, 2003; Speit and Hartmann, 2005). Other assays have been developed to detect DNA strand breaks at the time of tissue preparation, i.e. "snapshot of DNA strand breaks". However, protocols and experimental conditions have to be chosen carefully. For bio-monitoring, there are important advantages to standardizing comet assay protocols and analysis methods. Recommendations for conducting the comet assay for this purpose have evolved from several workshops and working groups (Speit *et al*., 1996; Albertini, 2000; Tice *et al*., 2000) Considerations of various statistical approaches are also available (Duez *et al*., 2003; Verde, 2006).

The present study aims at evaluate the usefulness of the alkaline comet assay as a bio-monitoring tool affected by different sources of pollutants and detect the deformation which happened in the biota due to the pollution.

## MATERIAL AND METHODS

### Sampling stations of study area

Lake Timsah is a small and shallow lake, lies on the Suez Canal at mid way between Port Said and Suez. It lies between 30° 23' and 30° 36' N latitude and 30° 16' and 32° 21' E longitude. The region can be distinctly divided into three basins: Lake Timsah, the western lagoon and the Suez Canal pathway (Fig. 1).


Fig.1: Lake Timsah, Ismailia, Egypt

Lake Timsah plays an important role in most of the activities in Ismailia city, such as tourism, fisheries, navigation, etc.

The lake has nearly a triangular shape with elongated sides extending roughly East-West. It has a surface area of about 8 $km^2$ and with an average depth of 11 meters and a volume of about 90 million cubic meters of water (El-Sharkawy, 2012). It is significantly polluted; the sources of pollution are essentially the raw sewage from the city network, industrial pollution from shore-line workshops, domestic sewage from unconnected areas adjoining the shore, agricultural drainage water, and possibly marine pollution (El-Moselhy and Yassien, 2005). Samples collected during winter season from two stations through the lake which subjected to different sources of pollution of Lake imsah as follows: El-Tarsana station (30º 33′ 31.592" N, 32º 17′ 31.562" E) which received industrial wastes from shore-line workshops (Plate 1A) and El-Taween station (30º 33′ 04.740" N, 32º 17′ 56. 783" E) which was polluted by domestic sewage from unconnected areas adjoining the shore (Plate 1B). The following experiments were performed in Environmental Lab., Tohoku University, Sendai, Japan.


Plate 1: Sampling stations. A) El-Tarsana station; B) El-Taween station.

**Water and biota sampling**

**Water samples**

Water samples were collected seasonally from all six stations to determine the concentrations of heavy metals. The filtered water samples were analyzed by mixing 25 ml. of water with 1 ml. of concentrated $HNO_3$ according to the method of APHA (1989). They were heated to concentrate the volume to 5 ml. and then the samples were diluted with distilled water to 25 ml. The heavy metal concentrations (Cd, Co, Cu, Fe, Mn, Ni, Pb and Zn) in water were measured by Plasma Optical Emission-Mass Spectrometer (POEMS III). Results were expressed as milligram per liter (ppm).

**Biota sample**

*Sphaeroma serratum* (Isopoda, Sphaeromatidae) is a mottled grey-brown isopod reaching 1 cm. in length. Its body is broadly oval and has a smooth dorsal surface. It rolls into a ball when disturbed. Samples were found inter-tidally under stones and pebbles near the shore as well as on the shore. They were collected by using small brush and washing the submerged stones randomly chosen by the reach of hand and rinsed with lake water. Sampling time was adjusted according to the life cycle of Isopoda. The specimens were then transported to laboratory and preserved in absolute alcohol at 4ºC until performing DNA extraction and comet assay experiments (Baratti *et al*., 2005).

**DNA extraction, primers, PCR amplification, and sequencing**

*Sphaeroma serratum* DNA was extracted from muscle tissue of complete specimens using DNeasy® 96 blood and tissue kit (Qiagen®, Netheland, Spin-Column Protocol) following its manufactures protocol for animal tissues. After extraction, DNA

concentration was quantified with ND-1000 spectrophotometer (NanoDrop Technologies).

Both region mitochondrial DNA (cytochrome oxidase I) and Histon (Histon3) were amplified by specific primers for crustaceans, isopods; for mtDNA: mtd10 (mtDNA_COI, TTGTTTTTTGGTCATCCAGAAGT) (Roehrdanz, 1993) and Florence (mtDNA_COI, CCTAAAAAATGTTGAGGGAA) (Gopurenko*et al*., 1999; Bratti *et al*., 2005), for Histon3: HistonH3_1 (H3_1, ATGGCTCGTAC CAAGCAGACVGC) and HistonH3_2 (ATATCCTTRGGCATRATRGTGAC) (Colgan *et al*., 2000). PCR was performed in a 10µl reaction volume with 1-100 ng template DNA, 0.25 U Taq DNA polymerase (TaKaRa), 1.0 pM for both primers, each combination, 1xPCR buffer (TaKaRa), 3mM $MgCl_2$, 0.4mM dNTP (TaKaRa) and add $H_2O$ up to 10µl. PCR cycling conditions were 5 min at 94°C, followed by 30 cycles of 30s at 94°C, 30s at 45-60 °C, 1 min at 72°C and a final extraction step of 5 min at 68°C and reactants were stored at 4°C using VeritiTM96-Well thermal cycler (Applied Biosystems). Annealing temperatures were changed based on presence of PCR products. If we could not confirm PCR products by the electrophoresis, the temperature were decreased. The PCR products were cleaned up by GENECLEAN kit (Molecular Products). The PCR products were analyzed using an ABI 3730XI DNA Sequencer (Applied Biosystems) from both directions of forward and reverse commissioned to Hokkaido System Science Co., Lud. The data were inspected and aligned by MEGA 5.2 (Tamura *et al*., 2011) and searched the closest sequences by Basic Local Alignment Search Tool (BLAST).

**Alkaline Comet Assay**

The comet assay was performed using Trevigen's Comet Assay kit® (Catalog # 4250-050-K) following the manufacturer's instructions. Trevigen's Comet Assay®, or single cell gel electrophoresis assay, provides a simple and effective method for evaluating DNA damage in cells and introduces a cost-effective, ready-to-use specially designed microscope slide that allows direct application of cell samples and the use of Trevigen's Comet Slide® shortens assay time and allows the rapid and reliable analysis of large numbers of samples. It provides several significant advantages over traditional slides for use in the comet assay: cost-effectiveness, reliability, simplicity and a standardized assay platform (Lemay and Wood, 1999).

**Tissue preparation**

Cell preparations for the comet assay were made from whole animal collected from both stations. Following determination, organisms were washed with bi-distilled water (in order to remove bacteria, protozoa and algae), collected in plastic tubes and weighed, and were placed in Eppendorf tubes. Suspension of cells was prepared by crushing the animals by a homogenizer due to its hard shell. In this assay, cells were immobilized in a bed of low melting point agarose, on a Trevigen Comet Slide®. Following gentle cell lysis, samples were treated with alkali to unwind and denature the DNA and hydrolyze sites of DNA damage. The samples were then submitted to electrophoresis and staining with a fluorescent DNA intercalating dye (SYBR® Green).

**Control cells**

Trevigen's Comet Assay® Control Cells (Catalog # 4256-010-CC) for alkaline electrophoresis were performed to monitor assay conditions and verify reproducibility between separate runs. When performing alkaline electrophoresis, the four control cell populations show incremental increases in percent DNA in the tail. The healthy control cell population (CCO) was treated with Etoposide under various condition to increase the amount of damage in populations CC1, CC2 and CC3 respectively. These

cryopreserved controls are designed to act as controls to standardize and compare alkaline electrophoresis methods between individual users and laboratories.

**Image analysis**

Slides were visualized by an Olympus epifluorescence microscopy (Olympus, Japan) fitted with filter set (excitation 520 nm, emission 590 nm). Quantitative and statistical data can readily be generated by analysis of the results using one of several commercially available image analysis software packages (Comet assay IV) which calculate head length, head intensity, tail length, percent DNA in the tail, tail moment (i.e. the tail length multiplied by the per cent tail DNA/100, where tail length is defined as comet extent minus head extent) and tail migration.

The amount of DNA damage in cells was estimated from comet tail length as the extent of migration of the genetic material. A significant increase in comet tail length indicating DNA damage was observed at all concentrations compared with control ($p < 0.05$). The mean comet tail length showed a concentration-related and time-dependent increase (Farghaly and Abo-Zeid, 2010).

The comet assay was carried out under alkaline conditions, basically as described by Singh *et al.* (1988). In control and polluted samples, digital images were scanned into an image analyzer (Comet analyzer software IV) to determine the length of DNA migration (Comet tail length) due to genotoxicity. Head length, head intensity, tail length, tail intensity, tail moment, and tail migration were measured. The tail length is the distance from the comet head to the last visible signal in the tail. The percentage of DNA in the tail is calculated from the fraction of DNA in the tail divided by the amount of DNA in the nucleus multiplied by 100. The tail moment is the product of the amount of DNA in the tail and mean distance of migration in the tail (Olive *et al.,* 1990).

**Determination of heavy metals in isopods' tissue**

*Sphaeroma serratum* at tissue level was analyzed and used as bio-indicator for pollution by the heavy metals concentrations. The heavy metals concentrations (Cd, Co, Fe, Ni, Pb and Zn) in digested isopods were measured by using flam atomic absorption spectrophotometer (FAAS), Environmental Lab., Tohoku University, Sendai, Japan. Each sample was analyzed in triplicate to ensure accuracy and precession for the analytical procedure. The results for metal concentration were expressed as micrograms per gram ($\mu g / g^{-1}$) of dry weight.

**Statistical analysis of data**

The data of comet assay were analyzed for homogeneity of variance using the general linear model procedure of statistical analysis system, SPSS software, version 17 (SPSS, 1999). Variable means for treatments indicating significant differences in the ANOVA were compared and the significances were indicated using Duncan multiple range tests (Duncan, 1955). The significance of the differences was evaluated using the one way ANOVA by comparing comet %, tail length and tail moment of samples. All values are expressed as mean $\pm$ S.E. of six replicates in one representative experiment. The Person's rank correlation coefficient ($r$) was used for determining associations between heavy metals in *Sphaeroma serratim* tissue and heavy metals in collected water and between heavy metals in *S. serratum* tissue and comet assay parameters.

## RESULTS

**Biota (*Sphaeroma serratum*)**

**Seasonal abundance**

Results on the seasonal abundance of the collected species *Sphaeroma serratum*

from the two sites of collections along Lake Timsah are showed in Table (1). The trend of seasonal abundances in both sites is almost coincidence with increasing in summer and decreasing in spring. The abundance in El-Tarsana is lower than El-Taween through all season.

Table 1: Seasonal abundance of *Sphaeroma serratum* (Crustacea, Isopoda, Sphaermatidae) at sites of collection during the study period from winter 2010/2011 and autumn 2011.

| Seasons | Sites of collection | |
|---|---|---|
| | El-Tarsana | El-Taawen |
| Winter | 85.000 ± 2.887 | 100.000 ± 0.000 |
| Spring | 14.667± 0.333 | 25.000 ± 2.887 |
| Summer | 150.000 ± 28.868 | 250.000 ± 28.868 |
| Autumn | 41.667 ± 4.410 | 100.000 ± 0.000 |

X= mean number; S.E. = standard error.

## DNA extraction and sequencing

DNA concentrations of *Sphaeroma* sp. were measured by spectrophotometer Nanodrop (ND1000). They were 15.9 ng/µl of dsDNA in El-Tarsana station and 10.2 ng/µl of dsDNA in El-Taween station. After extraction of DNA, Basic Local Alignment Search Tool (BLAST) search ensured our DNA sequencing is from *Sphaeroma serratum* as showed by taxonomy.

## *Sphaeroma serratum* sequence

TCATCCAGAAGTATATATTTTGATTCTTCCCGCTTTCGGGATAATCTCGCACATTGTGAGCCAA
GAGTCCGGGAAGAAAGAGGCGTTCGGGGCGTTGGGGATGATCTATGCTATAGCCGCGATCGG
GGGATTGGGGTTCGTGGTATGAGCCCATCATATATTTACGGTAGGAATGGACGTAGATACTCG
AGCTTACTTTACTTCCGCGACTATAATTATCGCAATCCCGACGGGGATTAAAGTGTTTAGGTGA
TTGAGAACTTTGCACGGGGCATGCCTAAGGTTTAGGCCTTCCTTGCTGTGGGCTCTCGGATTT
GTTTTCTTGTTTACGGTTGGAGGGCTAACGGGAGTGGTGCTAGCCAACTCTTCCGTTGATATT
ATTTTACACGATACCTACTACGTGGTCGCTCATTTTCATTACGTGTTGTCAATGGGGGCGGTAT
TCGCTATCTTCGGCGGAGTGGCCCATTGGTTCCCTCTCTTTACAGGGTTGACTTTAAATTCCA
AATGATTGAGTGCGCAGTTCGTGGCTATATTTGTGGGCGTAAATTTAACTTTCTTCCCTCAACA
TTTTTTAGG.

## Alkaline comet assay technique

The comet assay was carried out under alkaline conditions, basically as described by Singh *et al.* (1988). Alkaline CometAssay® Control Cell population (cat # 4256-010-CC) showed examples of comet tails for each population (Plate 2, A-D) and the alkaline comet assay of *S. serratum* from the collected stations with automated images that were scanned into an image analyzer (comet analyzer software IV) (Plates 3 & 4). The head length, head intensity %, tail length, tail intensity %, tail moment and tail migration of the control groups and collected specimens from the two stations are presented in Table (2).

The results in Table (2) and plates (2 A-D; 3 & 4) showed that the means of head lengths of all the tested samples from the two stations were less than that of the control groups. The head intensities of the samples (% of DNA in the head) were less than that of the control groups CCO and CC1. However, the mean head intensity of the samples which were collected from El-Tarsana (29.970 ± 1.073) was significantly lower than that of those collected from El-Taween (62.989 ± 3.198) and each of them is significantly higher than that of control group CC3 (22.235).

As shown in Table (2), the samples of *S. serratum* (whole mount) which were collected from El-Tarsana station showed a significant increase in the tail length (171.175 ± 55.333), tail intensity (70.562 ± 9.924), tail moment (49.798 ± 3.246) and tail migration (171.175 ± 55.333) compared to the control groups CCO, CC1 and CC2. However all these values were significantly lower than those of the CC3.

Table 2: Results of the alkaline comet assay on *Sphaeroma serratum* collected from Lake Timsah in winter 2010/2011. Statistical evaluation was made using Student's *t test* for independent samples.

| Comet Assay Parameters | Stations | El-Tarsana | | El-Taween | |
|---|---|---|---|---|---|
| | Controls | | % of damage | Controls | % of damage |
| Head Length | CCO | 136.187 | -85.194* | 136.187 | -85.387* |
| | CC1 | 101.035 | -80.045 | 101.035 | -80.304* |
| | CC2 | 82.244 | -75.485 | 82.244 | -75.804* |
| | CC3 | 102.012 | -80.236 | 102.012 | -80.492* |
| | Sample X ± SD | 20.162±3.779 | | 19.900±2.694 | |
| Head Intensity% | CCO | 78.846 | -61.989 | 78.846 | -20.111 |
| | CC1 | 71.024 | -57.803 | 71.024 | -11.313 |
| | CC2 | 32.506 | -7.802 | 32.506 | +93.777* |
| | CC3 | 22.235 | +34.787 | 22.235 | +183.288* |
| | Sample X ± SD | 29.970 ± 1.073 | | 62.989 ± 3.198 | |
| Tail Length | CCO | 107.381 | +59.409 | 107.381 | -67.278 |
| | CC1 | 124.464 | +37.530 | 124.464 | -71.769 |
| | CC2 | 159.729 | +7.166 | 159.729 | -78.002* |
| | CC3 | 259.174 | -33.954 | 259.174 | -86.443* |
| | Sample X ± SD | 171.175 ± 55.333 | | 35.137 ± 12.248 | |
| Tail Intensity | CCO | 21.154 | +233.563 | 21.154 | +78.463 |
| | CC1 | 28.976 | +143.519 | 28.976 | +30.287 |
| | CC2 | 67.494 | +4.546 | 67.494 | -44.066 |
| | CC3 | 77.765 | -9.263 | 77.765 | -51.454* |
| | Sample X ± SD | 70.562 ± 9.924 | | 37.752 ± 3.482 | |
| Tail Moment | CCO | 8.601 | +478.979 | 8.601 | -19.451 |
| | CC1 | 12.741 | +290.848 | 12.741 | -45.624 |
| | CC2 | 42.716 | +16.579 | 42.716 | -83.781* |
| | CC3 | 123.297 | -59.611 | 123.297 | -94.381* |
| | Sample X ± SD | 49.798 ± 3.246 | | 6.928 ± 1.970 | |
| Tail Migration | CCO | 39.292 | +308.287 | 39.292 | -32.941 |
| | CC1 | 37.946 | +116.948 | 37.946 | -64.367 |
| | CC2 | 118.607 | +35.257 | 118.607 | -77.785 |
| | CC3 | 208.172 | -22.937 | 208.172 | -87.343* |
| | Sample X ± SD | 171.175 ± 55.333 | | 26.349 ± 1.377 | |

* Significant difference (Duncan, $p < 0.05$), significantly increased as compared to sample N.

For the samples collected from El-Taween, the means of tail length (35.137±12.248), tail moment (6.928±1.970) and tail migration (26.349±1.377) were significantly lower than those of all control groups CCO (107.381, 8.601), CC1 (124.464, 12.741), CC2 (159.729, 42.716) and CC3 (259.174, 123.297). On the other

hand the mean tail intensity (35.137 ± 12.248) was significantly higher than that in control groups CCO (21.154) and CC1 (28.976) and lower than that in control groups CC2 (67.494) and CC3 (77.765) (Table 2).

In general, the mean values of tail length, tail intensity, tail moment, and tail migration were significantly higher in the samples collected from El-Tarsana station compared to those from El-Taween station while the mean value of head intensity was significantly higher in samples of El-Taween compared to those of El-Tarsana station.

**Determination of heavy metals**

**Heavy metals in water**

The concentrations of eight selected heavy metals (Cd, Co, Cu, Fe, Mn, Ni, Pb and Zn) in water for the two collected stations through Lake Timsah from spring 2010 to winter 2011 are summarized in Table (3). Heavy metal concentrations of collected water in both stations were in the following order Fe>Co>Zn>Ni>Mn>Cu. In El-Tarsana station, the concentrations of heavy metals were higher than those in El-Taween station. From the results, winter season showed high values of iron, cobalt, nickel and lead as follow Fe>Co>Ni>Pb in El-Taween station and Fe>Co>Pb>Ni in El-Tarsana station and low values of cadmium (Cd), zinc (Zn), copper (Cu) and manganese (Mn), respectively as follows Cd<Zn<Cu<Mn in both stations.

Table 3: Heavy metals concentration of water from El-Tarsana and El-Taween stations in winter Heavy metal concentrations (mean ± S.E) of raw water was expressed as (mg/l) from winter 2011 at Lake Timsah, Egypt.

| Heavy metals | N | Sites of collection | |
|---|---|---|---|
| | | El-Tarsana | El-Tawaan |
| Cd | 3 | 0.003±0.002 | 0.002±0.001 |
| Co | 3 | 2.333±0.018 | 1.930±0.012 |
| Cu | 3 | 0.083±0.037 | 0.079±0.036 |
| Fe | 3 | 2.650±0.001 | 2.319±0.002 |
| Mn | 3 | 0.230±0.001 | 0.190±0.001 |
| Ni | 3 | 1.407±0.004 | 1.210±0.001 |
| Pb | 3 | 0.011±0.001 | 0.003±0.001 |
| Zn | 3 | 1.720±0.001 | 1.408±0.004 |

∗Significant different ($P < 0.05$) as compared to the water concentration of each metal at the respective date of collection.

**Heavy metals in *Sphaeroma serratum* tissues**

The concentrations of six selected heavy metals (Cd, Co, Fe, Ni, Pb and Zn) in *S. serratum* tissue that collected from El-Tarsana and El-Taween stations during winter season were analyzed and summarized in Table (4). The accumulations of heavy metal in *S. serratum* tissue from El-Tarsana station were in the following order Fe>Zn>Pb>Ni>Co. In El-Taween station, heavy metal concentrations in specimens were in the following order Fe>Zn>Ni>Co. Cd and Pb levels were remarkably low.

Person's correlation coefficients showing principal interactions in explaining data variation between bio-accumulation of heavy metals in biotic tissues and heavy metals in water (Table 5) and between bio-accumulation of heavy metals in biotic tissues and comet assay parameters (Table 6). This correlation could be positive or negative according to the station and parameters. In El-Tarsana, tail length, tail intensity %, tail moment and tail migration were positively correlated with heavy metals while head length and head intensity % were negatively correlated with them station. On the other hand, in El-Taween station, head intensity %, tail length and tail migration were

negatively correlated with heavy metals while, head length and tail intensity % were positively correlated with them (Table 6).

Table 4: Mean heavy metal concentration in *S. serratum* (Crustacea, Isopoda, Sphaermatidae) tissue from El-Tarsana and El-Taween stations in winter 2011.

| Heavy metals | Concentrations (X±S.D) | |
|---|---|---|
| | El-Tarsana Station | El-Tawaan Station |
| **Cd** | $0.000^{*} \pm 0.000$ | $0.000^{*} \pm 0.000$ |
| **Co** | $0.0004^{*} \pm 0.0002$ | $0.0002^{*} \pm 0.0001$ |
| **Fe** | $0.883 \pm 0.002$ | $0.858 \pm 0.0006$ |
| **Ni** | $0.002^{*} \pm 0.0002$ | $0.001^{*} \pm 0.0002$ |
| **Pb** | $0.003^{*} \pm 0.0002$ | $0.000^{*} \pm 0.000$ |
| **Zn** | $0.060 \pm 0.003$ | $0.020 \pm 0.0002$ |

Heavy metal concentration (X=mean ± S.D. = standard deviation) of Isopoda was expressed ($\mu g.g^{-1}$ dry weight ± standard deviation) during winter 2011 at Lake Timsah, Egypt. *Significant difference (Duncan, $p < 0.005$) as compared to the tissue concentration of each metal at the respective date of collection.

Table 5: Person correlation coefficient (r) between heavy metals in water and the accumulation of heavy metals in *S. serratum* tissue from El-Tarsana and El-Taween stations.

| Heavy metals in water | Heavy metals in tissue | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | El-Tarsana station | | | | | El-Taween station | | | | |
| | Co | Fe | Ni | Pb | Zn | Co | Fe | Ni | Pb | Zn |
| **Cd** | 1.000** | 0.866 | 0.961 | 1.000** | 0.982 | 0.996 | 0.996 | 0.971 | - | 0.996 |
| **Co** | 0.999* | 0.841 | 0.946 | 0.999* | 0.972 | 0.994 | 0.994 | 0.904 | - | 0.994 |
| **Cu** | 0.945 | 0.982 | 0.999* | 0.945 | 0.990 | 0.996 | 0.996 | 0.971 | - | 0.996 |
| **Fe** | 0.945 | 0.982 | 0.999* | 0.945 | 0.990 | 0.945 | 0.945 | 1.000** | - | 0.945 |
| **Mn** | 1.000** | 0.866 | 0.961 | 1.000** | 0.982 | 0.996 | 0.996 | 0.971 | - | 0.996 |
| **Ni** | 0.918 | 0.993 | 0.992 | 0.918 | 0.976 | 0.954 | 0.954 | 0.803 | - | 0.954 |
| **Pb** | 0.866 | 1.000** | 0.971 | 0.866 | 0.945 | 1.000** | 1.000** | 0.945 | - | 1.000** |
| **Zn** | 0.870 | 1.000** | 0.972 | 0.870 | 0.947 | 0.982 | 0.982 | 0.866 | - | 0.982 |

*Correlation is significant at the 0.01 level
** Correlation is significant at the 0.001 level

Table 6: Person correlation coefficient (*r*) between comet assay parameters and the accumulation of heavy metals in *S. serratum* tissue from El-Tarsana and El-Taween stations.

| Comet Assay Parameters | Heavy metals in tissue | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | El-Tarsana station | | | | | | El-Taween station | | | | | |
| | Cd | Co | Fe | Ni | Pb | Zn | Cd | Co | Fe | Ni | Pb | Zn |
| Head length | - | -0.997* | -0.902 | -0.979 | -0.997* | -0.994 | - | +0.588 | +0.588 | +0.820 | - | +0.588 |
| Head length | - | -1.000* | -0.851 | -0.952 | -1.000* | -0.976 | - | -0.631 | -0.631 | -0.850 | - | -0.631 |
| Tail length | - | +0.933 | +0.988 | +0.996 | +0.933 | +0.984 | - | -0.676 | -0.676 | -0.880 | - | -0.676 |
| Tail intensity % | - | +1.000** | +0.873 | +0.965 | +1.000** | +0.985 | - | +0.301 | +0.301 | +0.597 | - | +0.301 |
| Tail moment | - | +0.986 | +0.936 | +0.993 | +0.986 | +1.000* | - | -0.101 | -0.101 | +0.230 | - | -0.101 |
| Tail migration | - | +0.938 | +0.985 | +0.997* | +0.938 | +0.987 | - | -0.769 | -0.769 | -0.936 | - | -0.769 |

*Correlation is significant at the 0.01 level

** Correlation is significant at the 0.001 level.

## DISCUSSION

The comet assay, also known as the single cell gel electrophoresis (SCGE) assay, is a rapid, simple, visual and sensitive technique for detecting and analyzing DNA strand breakage in a variety of organs and various mammalian cells. It allows any viable eukaryote cells to be analyzed. For these reasons, the comet assay is now widely used in researches of bio-monitoring and DNA damage processes to routine assessments of genotoxicity. The resulting images were subsequently named "Comet" because of their appearance and their total length was considered directly related to the DNA damage. From that moment a range of applications of the Comet assay have been used in investigations of the physiochemical behavior of DNA, through studies of cellular responses of DNA damage, to bio-monitoring of human population (Hassan and Mazhar, 2010).

The comet assay provides an advantage over other strand break assays because measurements are made on individual cells. Scoring these cells on slides provides an independent measure of the toxicity of a test compound (Fairbairn *et al*., 1995; Speit *et al*., 1996).

This work aims to evaluate the usefulness of the alkaline comet assay as a bio-monitoring tool at the level of aquatic macro-invertebrate communities to observe the deformation which happened in the biota due to the pollution by using alkaline comet assay and to show at which degree the isopod *S. serratum* can resist pollution, and can be used as bio-indicator.

Isopods have a great quantity of living forms adapted to life either in aquatic (marine, brackish, or even freshwater) media or on land. However, the composition of the isopod fauna is substantially changed from low latitudes to high and from shallow depths to great (Kussakin, 1979). During the present study *S. serratum* (Crustacea,

Isopoda) was the most abundant aquatic macro-invertebrate even in the most polluted stations of the lake and this agrees Holdich and Tolba (1985) and Tolba *et al*. (1995) who stated that the isopod *S. serratum* has a wide geographical distribution and a wide range of habitats-even those which are grossly polluted.

Assessment of DNA damage is considered important in toxicity testing. The occurrence of DNA damage in marine organisms may be relevant due to the fact that DNA strands may be broken due to the interaction with free radicals, organic and inorganic contaminants, heavy metals, etc., and the formation of adduct (Pisoni *et al*., 2005).

Following electrophoresis, the presence of DNA strand breaks allows fragments of DNA to move from the nucleoid core towards the anode, thus resulting in (Comet) formation (Singh *et al*., 1988). The amount of DNA breakage in a cell in the comet assay was estimated from the migration extent (tail length) of the genetic material in the direction of anode (Singh *et al*., 1988). Furthermore, the percentage of DNA in the tail (tail intensity) has been shown to be proportional to the frequency of DNA strand breaks (Olive *et al*., 1990). Tail moment is a simple descriptor calculated by the computerized image analysis system considering both the migration tail length as well as the fraction of DNA migrated in the tail (Villarini, 1998; Lee and Steinert, 2003).

The isopod *S. serratum* collected from El-Taween station showed a lower degree of DNA damage compared with that collected from El-Tarsana station. During the present study the comet assay showed significant increase in, tail length, tail intensity, tail moment and tail migration of *S. serratum* collected from El-Tarsana station compared to the control groups and to those collected from El-Taween station. This was related to the significant increase of DNA damage in *S. serratum* of El-Tarsana station compared to those from El-Taween station.

**Using of Sphaeroma serratum as bio-indicator for heavy metals pollution in Lake Timsah**

Environmental pollution due to heavy metals represents a serious problem because of its high toxicity and of the bio-accumulation ability of these agents (Guecheva *et al*., 2001; Pruski and Dixon, 2002; Lee and Steinert, 2003). Heavy metals, such as copper, zinc, lead, mercury and cadmium are among the most dangerous and abundant inorganic environmental pollutants, arising from industrial discharges and mining practices (Nriagu and Pacyna, 1988). They enter aquatic systems via natural and anthropogenic sources, including industrial, agricultural and mining activities. The aquatic environment is more susceptible to the harmful effects of heavy metal pollution because aquatic organisms are in close and prolonged contact with soluble metals. Moreover, unlike toxic organic compounds, metals cannot be degraded but undergo bioaccumulation through food chain (Hickey *et al*., 1995; Kong *et al*., 1995; Cohen *et al*., 1996).

During the present study, Iron (Fe) was the predominant toxicant in both El-Tarsana and El-Taween stations which are the most polluted stations. Fe is accumulated in the tissue of specimens in both stations with high percentage because of its highest concentration in the water of El-Tarsana (2.650 mg/l) and the water of El-Taween (2.319mg/l). According to Ipinmoroti and Oshodi (1993) when concentrations of iron exceed 0.1 mg/L, iron precipitates on exposure to air, decreasing pond clarity and encouraging iron bacteria, which affects the flavor of both fish and water. Levels greater than 0.3 mg/L can cause staining on buildings and sidewalks when the water is used for irrigation. On the other hand the present study reported an increase in Cd concentration during summer which was related to the increase of

salinity. According to Zirino and Yamamoto (1972), the increase in salinity increases the interaction between Cd and chloride ions. The opposite happened particularly for Cd. Our data also reported a decrease in Cd concentration during winter.

Bryant and Kuropat (1980) have pointed out that metal toxicity to marine crustaceans, including isopods, varies with environmental conditions such as temperature, salinity, and metal type, thus the tested species should be monitored seasonally in order to assess the changes in its sensitivity under various conditions.

The isopod *S. serratum* was reported to be very tolerant to desiccating conditions and can tolerate brackish water (Abdel-Hamed *et al*., 1991). The presence of high densities of *S. serratum* in the most polluted stations during the present study was related also to the presence of high amount of food and nutrients and the lack of competitors and predators in these stations. According to Barbary (1987) the amount of diatoms and filaments algae which could be used as a food source for such isopods and others in Lake Timsah are very high.

The relatively lower density of *S. serratum* in El-Tarsana station compared to that in El-Taween station during the present study was related to the significant high concentrations of heavy metals in El-Tarsana station compared to that in El-Taween. Such high concentration of heavy metals in the water led them to accumulate in the tissue of the tested isopods collected from El-Tarsana more than those in El-Taween. This resulted in a relatively high damage of DNA in the isopods collected from El-Tarsana compared to those of El-Taween. Such damage was observed by alkaline comet assay. Similar to our results, Prato *et al*. (2006) compared the sensitivity of 2 species of isopods, *Sphaeroma serratum* and *Idotea baltica* to three reference toxicants (Copper, Cadmium and Mercury) and found that *S. serratum*, didn't show an elevated sensitivity toward the metals tested and seemed the most tolerant species.

In conclusion, the comet assay had adequate sensitivity to detect the differences in the levels of DNA damage among the isopods species studied. The DNA damage observed in the isopod *S. serratum* of El-Tarsana station was related to the significant increase in metal concentrations in this station, which in turn led to significant increase in metals accumulation in the tissues of the samples. These results suggest the high tolerance of the isopod *S. serratum* to heavy metal pollution and the usefulness of comet assay to detect DNA damage resulting from such pollution.

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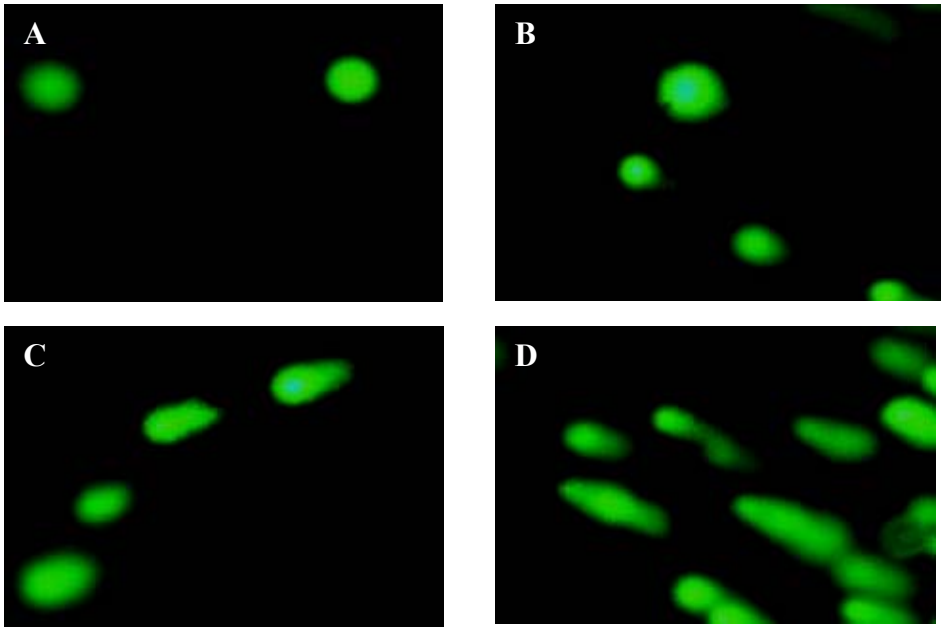


Plate 2: Alkaline CometAssay® Control Cell population.
A) CCO, B) CC1, C) CC2, D) CC3.

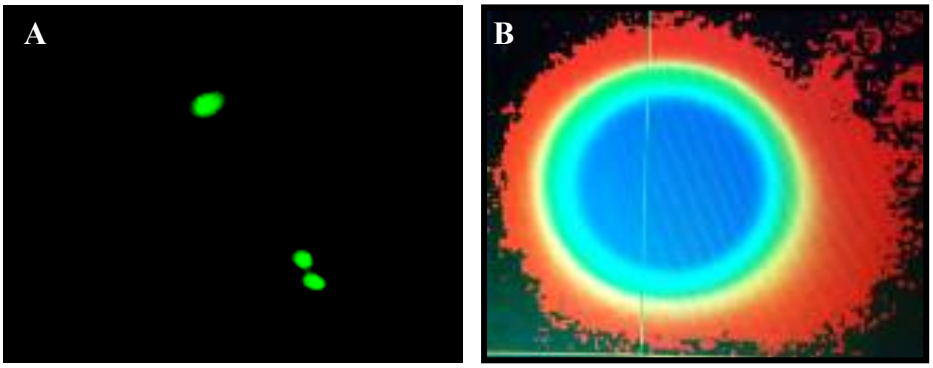


Plate 3: Photograph image of *S. serratum* from El-Tarsana station subjected to single gel electrophoresis (alkaline comet) and subsequently analyzed by image analysis system , Tail moment = 49.798 ± 3.24.

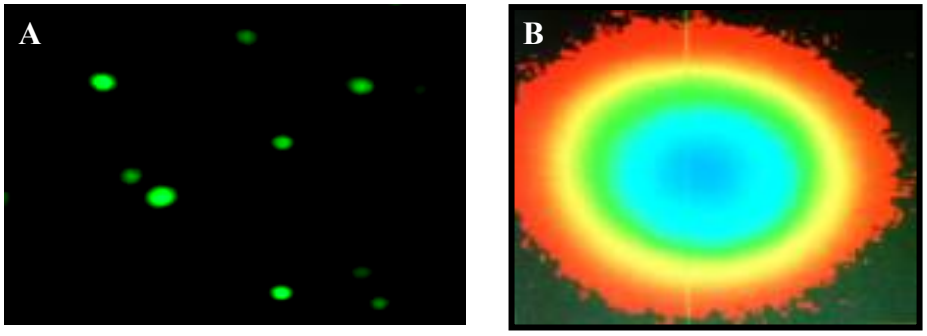


Plate 4: Photograph image of *S. serratum* from El-Taween station subjected to single gel electrophoresis (alkaline comet) and subsequently analyzed by image analysis system , Tail moment = 6.928 ± 1.970.

## ARABIC SUMMARY

**التحقيق فى استخدام سفيروما سيراتم (قشريات:ايزوبودا) كمؤشر حيوي لتلوث المعادن الثقيلة فى بحيرة التمساح- قناة السويس باستخدام تقنية مقايسة المذنب القلوية**

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تقنية مقايسة المذنب (comet assay) و التى أستمدت أسمها من خلال ظهور شكل المذنب برأسه و ذيله دليلا على حدوث تشوه فى الحمض النووى DNA فى هذه الدراسة بغرض معرفة الى أى مدى ستؤثر طبيعة مياه بحيرة التمساح الفقيرة على تشوه الحمض النووى للأنواع الكائنة بها. و تعتمد هذه التقنية على قياس المسافة التى تقطعها شظايا الحمض النووى بعد عملية التحليل الكهربائى و قياس حجم الذيل المتكون مما يعكس مدى التلف الواقع على الحمض النووى. تعد تقنية مقايسة المذنب أفضل من غيرها لأنها تحتاج الى عدد قليل من الخلايا بمعنى أن أى نوع من الأنسجة يمكن ان يتم تقيمه كما أنها وسيلة حساسة للغاية لتحديد الضرر الواقع فى المادة الوراثية. من خلال هذه الدراسة تم التأكيد على براعة إستخدام تقنية مقايسة المذنب خاصة النوع القلوى ( Alkaline) comet assay كأداة حساسة للتقييم البيئى و كذلك الدراسة الحالية طبقت على إستخدام (سفيروما سيراتم) كدليل بيولوجى موثوق فيه على التلوث و تشوه الحمض النووى و أن هذا النوع يستطيع مقاومه التلوث.