Potential Role of Mosquito Larvae *Culex Pipiens* as a Biological Indicator of Environmental Water Pollution in Egypt

Amira Afify
Entomology Department, Faculty of Science, Cairo University, Cairo, Egypt

Corresponding author email: ameraafify@yahoo.com

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Abstract River Nile represents the most important source of freshwater in Egypt. There are several factors lead to the water pollution in the River Nile System (main stream Nile, drains and canals). The water quality of River Nile was good despite high organic loads discharged from some of the drains and industrial activities. These were harmful both to human and stream ecosystem health. A biological approach to monitoring water quality incorporates use of stream organisms themselves as a basis for pollution detection. Fish and aquatic insects considered as bio-indicators of pollutant effects and help to investigate possible environmental problems. One of the recent biochemical techniques for detecting DNA damage as a result of DNA single strand breaks, alkali-labile sites, and cross-linking is the alkaline (pH>13) single cell gel SCG assay. In this study the comet assay measure the DNA damage in various stages of the mosquito *Culex pipiens* collected from two different polluted water streams (Nikla and Elmansoreyh). The DNA damage measured in 3rd, 4th larval instars, pupa, male and female adults. It is clear from the results obtained in this study that the genotoxicity of water pollution of two different polluted water streams (Nikla and Elmansoreyh) in *C. pipiens* was high in larval and pupal stages and this study affirmed the appropriateness of the comet assay as a sensitive tool for environmental monitoring. Additionally, it can be proposed that *C. pipiens* is a strong aquatic bioindicator of genotoxicity.

Keywords Alkaline comet assay; *Culex pipiens*; DNA damage; Water pollution

Background
River Nile represents the most important source of freshwater in Egypt. Pollution is the most dangerous problem facing different sources of water in Egypt (APRP, 2002). There are several factors lead to the water pollution in the River Nile System (main stream Nile, drains and canals) in the past few decades like new irrigated agriculture projects, the increment of population and other activities along the Nile (APRP, 2002). River Nile considered as a pool for pumping domestic, industrial and agricultural effluents due to receiving big quantities of domestic and agricultural wastes without natural cleaning. Previous studies on water concentrated on using different biological and chemical characters of River Nile to investigate the trophic and autotrophic state of the River (Ali et al., 2000). According to a detailed study undertaken in 2002 by a research team working for the Ministry of Water and Irrigation and USAID, the water quality of River Nile was good despite high organic loads discharged from some of the drains and industrial activities. The most contaminated water source was drainage canals (drains), particularly in all drains in Delta and some drains in Upper Egypt. Water pollutants classified according to their severity to public health and the environment into pathogenic microorganisms, followed by organic compounds, pesticides and heavy metals (APRP, 2002). These were harmful both to human and stream ecosystem health. In villages where the only available water is from irrigation canals, water is used for domestic purposes and dumped back into the drains. Villagers drinking polluted water have been affected with kidney and liver diseases (Land Center for Human Rights, 2005). The northeast Nile Delta region has a high incident rate of pancreatic cancer that is believed to be from high levels of heavy metals and organochlorine pesticides found in the soil and water. Exposure to cadmium may be from heavy metals and pesticides found in water (Soliman et al., 2006). A biological approach to monitoring water quality Incorporates use of stream organisms themselves as a basis for pollution detection. Fish and aquatic insects considered as bio-indicators of pollutant effects and help to investigate possible environmental problems and evaluation of environmental presence of substances potentially teratogenic and carcinogenic human beings (Matsumoto et al., 2006; Abdel-Gawad et al., 2011; Augustyniak et al., 2016). One of
the recent biochemical techniques for detecting DNA damage as a result of DNA single strand breaks, alkali-labile sites, and cross-linking is the alkaline (pH > 13) single cell gel SCG assay, including connection of an electrical current to cells, so DNA fragments move out of the nucleus giving head and tail to be known as comet assay (Singh et al., 1988; Klau-de et al., 1996; Singh and Stephens, 1996). Comet assay considered as one of the most important tests for genotoxicity determination of fish and aquatic insects after exposure to water pollutants, either in the environment or under experimental laboratory treatments (Minissi and Rizzoni, 1996; Lee and Steinert, 2003; Abdel-Gawad et al., 2011; Augustyniak et al., 2016). It considered as a rapid, sensitive, and inexpensive method to investigate DNA strand breaks in individual eukaryotic cells (McKelvey-Martin et al., 1993; Rojas et al., 1999; Koppen, 1999). As a result of single strand breaks, double strand breaks, oxidative base damage, alkali labile sites (primarily apurinic and a pyrimidinic sites), DNA cross-linking with DNA or protein and incomplete excision repair sites, and DNA cross links (Tice et al., 2000; Collins, 2004; Jehane et al., 2017). Eukaryotic organism and cell types have also been tested with this assay (Petras et al., 1995; Tice, 1995; Verschaeye and Gilles, 1995; Sasaki et al., 1997a, b). Some studies used small mammalian species, living in or close to polluted areas for detection of hazardous pollution (Farbairn et al., 1995; Petras et al., 1995; Tice, 1995; Baker et al., 1996; Salagovic et al., 1996; Ralph et al., 1997; Silva et al., 2000). Also, comet assay has been applied to cells of insects, including Shistocerca gregaria, Drosophila melanogaster, Curculio sikkimensis, mosquito larvae, Ephestia kuehniella and in grasshoppers Chorthippus brunneus (Siddique et al., 2005; Todoriki et al., 2006; Augustyniak et al., 2006; Yousef et al., 2010, Abdel-Gawad et al., 2011; Isabel and Maria, 2014; Pandir and Guven, 2014).

Therefore, the present study aimed to evaluate the potentiality of mosquito *Culex pipiens* as bioindicator of environmental water pollution and to determine the genotoxic effect of environmental water pollution on the mosquito *culex pipiens* using comet assay in the field work.

1 Materials and Methods
1.1 Colonization of *Culex pipiens*
Control mosquitos samples were reared in the laboratory of mosquito at the Entomology department, Faculty of Science, Cairo, Egypt under controlled conditions. Mosquitos’ larvae and pupae were reared in plastic pans (25 x 30 x 15 cm) containing 2 litres of tap water and were provided with fish food as a diet. Then pupae were collected in plastic cups and transferred to wooden cages (30 x 30 x 30 cm) for adult emergence and adult were kept in these cages and were provided with sponge pieces soaked in 10% sucrose solution (Kasap and Demirhan, 1992).

1.2 Collection of *Culex pipiens* larvae and pupae
*Culex pipiens* larvae and pupae were collected by sweeping the water with D-framed net which was the most common method. The insects collected from two polluted water streams, River Nile mixed with sewage drainage (Elmansoreyah and Nikla, Giza Governorate). Then collected insects were transferred to the Laboratory of mosquito for identification and DNA damage analysis for larvae and pupae according to Saleh et al. (1992) and Fagr et al. (2011). The rest of pupae transferred to wooden cages for adult emergence and DNA damage analysis for male and female adults.

1.3 Sample preparation for alkaline Single Cell Gel (SCG) assay
The whole body of five mosquitos of different stages (3<sup>rd</sup> larval instar, 4<sup>th</sup> larval instar, and pupae, male and female adults) were minsined with 200 µl of PBS for each sample.

1.4 Detection of DNA damage using alkaline SCG assay
Alkaline single cell gel comet assay (pH 13) used as a biochemical technique for DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites, and cross-linking detection (Singh et al., 1988). DNA damage were analysed in the whole body cells of *C. pipiens* to evaluate the genotoxic effects of water pollution. 20 µL of the minsend tissue solution from the pool of 5 insects were centrifuged at 1000 rpm for 10 min. Isolated hemocytes were immediately suspended in cooled 50 µL Ringer solution and kept on ice. 10 µL of isolated cells were mixed with 90 µL of 1% low melting point agarose (LMPA), and placed on a microscope slides, pre-coated with 1.5% normal melting point agarose (NMA). A cover slip was added, and then slides were...
immediately placed on ice. After agarose solidified, cover slips were removed, and slides were immersed in a lyses buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.25 M NaOH, 1% TritonX-100, and 10% dimethylsulfoxide (DMSO), pH 10.0) for 24 h at 4°C. After lysis, slides were placed in a horizontal gel electrophoresis tank and DNA was allowed to unwind for 20 min in electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13). Electrophoresis was carried out at 24 V and 270 mA, at 4°C, for 20 min. Then the slides were neutralized in 0.4 M Tris–HCl (pH 7.4), fixed with methanol and allowed to dry overnight at room temperature before staining with ethidium bromide (2 µg/mL). Comets were analyzed with Axio fluorescence microscope (Carl Zeiss, Germany) with an excitation filter of 524 nm and a barrier filter of 605 nm. Three replicates were prepared and each of them consisted of a pool of 5 individuals.

1.5 Evaluation of DNA Damage
DNA damage was visualized with fluorochrome stain of DNA with fluorescent microscope and a 40 X objective (depending on the size of the cells being scored). A Komet analysis system 4.0 developed by Kinetic Imaging, LTD (Liverpool, UK) linked to a CCD camera was used to measure the length of DNA migration (Tail length) (TL), and the percentage of migrated DNA (DNA %). To distinguish between populations of cells differing in size nuclear diameter was measured. Finally, the program calculated tail moment. Each cell was scored visually as belonging to either one of the five specific damage stages based on the relative intensity of the head and tail fluorescence (from undamaged DNA stage 0 to maximal damaged DNA, stage D). Undamaged DNA stage 0 has no tail, damaged DNA stage A has a tail length equal to or shorter than the length of head diameter, damaged DNA stage B has a tail length 1.1–3.5 times longer than the head diameter, damaged cell stage C has a tail length greater than 3.5 times the head diameter, damaged DNA stage D has no “head” since all DNA migrated to the tail according to Fagr et al. (2011). 50-100 randomly selected cells are analyzed per sample (at least 25 cells per slide and 3 slides per treatment were evaluated).

1.6 Statistical analysis
Comet assay data parameters (tail length, % DNA, and tail moment) were done using one-way ANOVA. Data from three replicas of each group were analysed, using SPSS software (version 15; SPSS, Chicago, IL).

2 Results
2.1 Single-cell gel electrophoresis (comet assay)
Typical DNA damage of body cells of *Culex pipiens* collected from Elmansoreyah and Nikla water streams can be seen in Figure 1. Body cells of the control showed almost rounded nuclei (Figure 1 A). In the body cells of the collected mosquitos from polluted water, nuclei with a clear tail like extension were observed indicating that the body cells of the insect were damaged and DNA strand breaks had occurred (Figure 1 B, C, D and E). DNA damage of the body cells of different stages of *C. pipiens* collected from studied polluted water streams was analyzed quantitatively by comet assay and expressed as tail length (TL), DNA % and tail moment (TM) (Figure 2; Figure 3; Figure 4).

![Figure 1](image1) Different cell damage stages in the comet assay in *Culex pipiens*

The damage of body cells DNA expressed as TL and DNA% under the effect of water pollution of Elmansoreyah and Nikla water streams, analysed by the comet assay. It was found that Elmansoreyah and Nikla water streams pollution caused a significant increase in the values of TL, in the body cells of different stages (Figure 2). The prominent increase in values of TL in response to pollution of Nikla water stream was observed in the 3rd larval
instar, pupae and male stages but in Elmansoreya water stream mainly in the pupal stage. Generally by using T-test analysis resulted that the 2 different polluted water streams under study caused a significant increase in the results of TL in the body cells of different stages of *C. pipiens* comparing to control results. There is no significant difference in TL data between Elmansoreyah and Nikla water streams 4th instar and pupa stage, but TL data is significantly higher in Nikla 3rd instar and male stage than Elmansoreyah data and the reverse in the female stage.

Figure 2 Comet Tail length (TL) data of body cells from different stages of *C. pipiens* collected from Elmansoreya and Nikla water streams
Note: The same number in each group means there is no significant between them

Figure 3 Comet DNA% data of body cells from different stages of *C. pipiens* collected from Elmansoreyah and Nikla water streams
Note: The same number in each group means there is no significant between them
Figure 4 Comet Tail Moment (TM) data of body cells from different stages of *C. pipiens* collected from Elmansoreya and Nikla water streams

Note: The same number in each group means there is no significant between them.

As shown in Figure 3 DNA damage of the body cells presented by DNA % values of different stages of *C. pipense* of Nikla water stream was higher than of Elmansoreya water stream but the both were significantly higher than of the control insects. By using ANOVA, there is no significant difference in the DNA% data between 3rd larval instar and pupae in response to pollution of Nikla water stream but these data significantly higher than the rest of stages while in Elmansoreya water stream, the DNA% data were significantly increase in 4th instar, pupa and male stages than 3rd instar and female stage.

It was found that the 2 different polluted water streams (Nikla and Elmansoreyah) caused a significant increase in the values of TM in the body cells of different stages of *C. pipiens* than in control values using T-test analysis. By using ANOVA, polluted water of Nikla water stream caused a significant increase in the TM data in the pupal body cells than 3rd larval instar (Figure 4). There is a lower significance increase in TM data of the 4th instar, pupae and male stages Elmansoreyah water stream. Generally, there is insignificant increase of TM data of the adult stage (male and female) arises from pupae of Nikla and Elmansoreyah water streams.

3 Discussions

3.1 The statistical analysis of DNA damage

One way ANOVA for tail % DNA values of different stages of *C. pipense* of Nikla water stream was significantly higher than of the control insects (P < 0.05). The level of DNA in the tail region (tail % DNA) was thought to be the most suitable standard for measuring DNA damage. The results demonstrated higher tail moment in aquatic insects gathered from 2 diverse polluted water streams (Nikla and Elmansoreyah) than the control and (Best and Ross, 1977) likewise got comparable results by examination of change of tail moment revealed significant differences between infected and control fish and the comparative results were gotten by Abd-Allah et al. (1999) who utilized the Comet as a basic and quick technique by which DNA damage can be shown as a function of the tail moment. Furthermore, TM has the upside of considering damage communicated as a short tail with a high division of DNA or a long tail with a low division of DNA (White and Rasmussen, 1998). According to Duez et al. (2003) and Pereira et al. (2010), the lower significance increase in TM data of the 4th instar, pupae and male stages Elmansoreyah water stream may reflect the cells with DNA cross-linking lesions.
From the above results, the tail moment more responsive than tail length, and these results agreed with data obtained by Pereira et al. (2010). White and Rasmussen, (1998) showed that TM has the advantage of considering damage expressed as a long tail with a low fraction of high fraction of DNA or a short tail with a highly fragmented DNA. There are another study revealed that tail length and tail moment can provide similar results (Duez et al., 2003).

The high rates of comet cells of different stages of mosquitos might be due to the River might be blended with genotoxic materials from residential waste and farming spillover that contain pesticides and composts. Intense genotoxins generally found in the local squanders, which are known to be available in human sterile outpourings found in municipal discharges (White and Rasmussen, 1998). Additionally, it can be seen from the results there is a level of DNA damage in control mosquito samples that the previous studies likewise expressed that any normal cell commonly contains a specific extent of single strands in its DNA, the after effects of either unconstrained damage or DNA breakage important to DNA synthesis (Koppen 1999).

3.2 Genotoxicity and DNA repair

Generally, there is insignificant increase of TM data of the adult stage (male and female) arises from pupae of Nikla and Elmansoreyah water streams. These results may be due to DNA repair occurred which differ from the study of Yousef et al. (2010) that there were a higher DNA damage in the mature adults with respect to the long period of feeding of *Shistocerca gregaria* on clover treated with CdCl₂ and PbCl₂ reflects the absence of repair mechanism. But there are many studies used the comet assay to observe DNA repair in irradiated cells (Isabel and Maria, 2014) and in different cells of *Drosophila* after exposure to different genotoxic agents (Bilbao et al., 2002; Mukhopadhyay et al., 2004; Siddique et al., 2005b, 2013; García-Sar et al., 2012; Sharma et al., 2012; Mishra et al., 2014; Gaivão et al., 2014; Isabel and Maria, 2014).

The principle point when outlining the first comet test convention in *Drosophila* was to build up an apparatus to study DNA repair in vivo in substantial cells (Bilbao et al., 2002). Consequently, many (but not all) of the works completed with this measure in *Drosophila* were meant to think about genotoxicity or potentially DNA repair in physical cells in vivo. Notwithstanding its utilization in the examine configuration, utilizing model genotoxic operators, and efficient and deficient repair strains (Bilbao et al., 2002), brain cells, used to show in vivo comet repair measure (García-Sar et al., 2012). Recently, brain cells have been utilized to actualize the in vitro comet repair measure in Drosophila, to have the capacity to quantitate DNA repair exercises in vitro (Gaivão et al., 2014). Also, hemocytes and midgut cells have been utilized to consider oxidative DNA damage (Sharma et al., 2012); likewise, some genotoxic compounds were examined in various repair conditions, with the in vivo comet repair test (Mishra et al., 2014; Isabel and Maria, 2014).

3.3 Environmental biomonitoring using alkaline comet assay

Sasaki et al. (1997) proposed that the determination of genotoxicity because of environmental contamination of water should be conducted with the water as a whole and not specifically for each (contaminating) component and that the comet would be a suitable test for this sort of monitoring. The information on genotoxicity in aquatic insects and fish in various oceanic sources in River Nile exhibited the low quality of that condition which might be because of the high sewage release, human activities and the stagnant water movement (Van et al., 2003). Water pollution may influence DNA of fish and actuate hereditary modifications that can be utilized as markers of DNA changes in ecological contamination (Jha, 2008). Different aquatic and terrestrial invertebrates have been utilized for genotoxicity examines utilizing the Comet assay which have likewise been reviewed (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003; Martins et al., 2013; de Lapuente et al., 2015). Cells from haemolymph and different tissues have been utilized for ecogenotoxicity considers utilizing the Comet assay. The Comet assay has been utilized to evaluate the degree of DNA damage at polluted areas in contrast with control areas in the environment or in the laboratory, it has been considered as a technique to measure the effects of pollutants and DNA damage mechanism (Labieniec and Gabryelak, 2006; Alok and Diana, 2016). Our results for *C. pipiens* indicate that pollution-induced DNA damage is detectable by the SCG assay, and confirm the viability of this method in the environmental biomonitoring. The study of animals in their habitat and without the need to
sacrifice them provides an ideal approach for environmental evaluation (Silva et al., 2000). Van et al. (2003) showed that the assay is easy, generally modest and simple to perform. Fish biomarker might be valuable instruments in a few stages of the hazard evaluation process: impact, presentation and danger appraisal, chance portrayal or grouping and observing the natural nature of amphibian environments. Augustyniak et al. (2016) anticipate that the Comet assay will be utilized as a part of natural hazard appraisals and to enhance our comprehension of numerous critical wonders of insect life, for example, metamorphosis, shedding, and diapause. The utilization of this strategy to contemplate species that are of key significance to humans, for example, useful insects and pests, has all the earmarks of being profoundly likely and exceptionally encouraging. The utilization of the Comet measure for DNA soundness testing in insects will in all likelihood quickly increment later on.

4 Conclusions

Therefore, it could be concluded that, the genotoxicity of water pollution of two different polluted water streams (Nikla and Elmansoreyh) in C. p. pipiens was high in larval and pupal stages and this study affirmed the appropriateness of the comet assay as a sensitive tool for environmental monitoring. Additionally, it can be proposed that C. p. pipiens is a strong aquatic bioindicator of genotoxicity.

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