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Cytotoxic Effect of Pesticide Malathion on Freshwater Ciliate, *Tetmemena* sp.

Ashish Choudhary[1], Jeeva Susan Abraham[1], S. Sripoorna[1], Manu Sankar[1],Vikas Ahlawat[1], Renu Gupta[2], Seema Makhija[1]*, Ravi Toteja[1]*
[1]Ciliate Biology Laboratory, Department of Zoology, Acharya Narendra Dev College, University of Delhi, Delhi, India; [2]Maitreyi College, University of Delhi, New Delhi
seemamakhija@andc.du.ac.in, ravitoteja@andc.du.ac.in

ABSTRACT

Pesticide malathion is extensively used in agriculture and also for mosquito eradication. It runs off into freshwater bodies thereby both unicellular and multicellular organisms get exposed to this pesticide. In the present investigation toxicity of malathion on freshwater ciliate *Tetmemena* sp. was evaluated. *Tetmemena* sp. is a cosmopolitan hypotrich ciliate found in most of the freshwater bodies in and around Delhi region. Morphological, behavioural and nuclear changes were observed in exposed cells suggesting malathion may have genotoxic effects. This study holds scope in making a microbiotest kit using ciliates as model organisms to detect pesticide levels in the aquatic ecosystems.

Keywords: Genotoxicity, Malathion, *Tetmemena* sp., Toxicity

INTRODUCTION

Freshwater ecosystems are frequently polluted by different toxicants like heavy metals and pesticides by anthropogenic activities (1, 2). In recent years, contamination of fresh water bodies like rivers and lakes by these toxicants is becoming a very serious problem (3). Yamuna River, flowing through Delhi, is one of the most-polluted rivers in the country. The water quality of the River is becoming very poor and has become a major threat to the flora and fauna of the River. Various contaminants in large scales are being continuously introduced into the River from the industrial discharge, waste water, drainage and agricultural run-off and thereby increasing the River toxicity (1, 2, 4, 5). Pesticides and heavy metals comprise one of the most hazardous groups due to their toxicity, accumulation and non-degradable nature. Presence of these contaminants above threshold levels causes adverse effects on human metabolism and health (2). One of the major pesticides that affect River Yamuna is malathion which is maximally present on the surface of the aquatic ecosystem. Malathion [S-1,2-bis(ethoxycarbonyl) ethyl O,O-dimethyl phosphorodithioate] is an organophosphate pesticide (Figure I) extensively used in developing countries like India. It is highly soluble in water and is commonly used pesticide in agriculture. It enters the food chain as agricultural run-off (6). Presence of malathion in untreated water leads to its oxidation into malaoxon and can cause serious health issues. Malaoxon is an active form of malathion which is a cholinesterase inhibitor.

It binds to cholinesterase irreversibly and can paralyze the organism. Malathion as such has low human toxicity but if ingested or absorbed into human body it metabolises into malaoxon. Exposure to high levels of malathion for short time periods can lead to skin and eye irritation, nausea, cramps and diarrhoea (7).

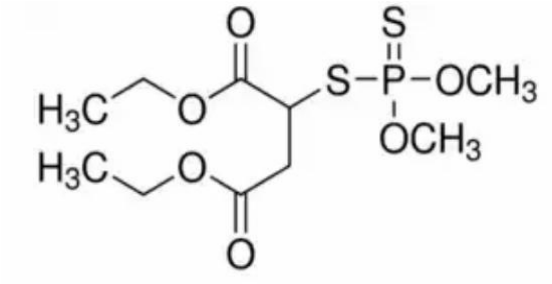


Figure-I: Chemical structure of pesticide malathion

Earlier investigations on the effect of pesticides such as carbofuran and delfin have been conducted on *Paramecium* and *Oxytricha* (8, 9, 10, 11, 12). The present investigation was conducted to assess the toxicity of pesticide malathion in freshwater ciliate *Tetmemena* sp., collected and isolated from Okhla Bird Sanctuary, Delhi. Toxicity can be determined *in vitro* under laboratory conditions using protozoans, which are considered to be the most suitable organisms. *Tetmemena* sp. is selected for this study because it is a cosmopolitan hypotrich ciliate found in most of the freshwater bodies in and around Delhi region. Also, *Tetmemena* cells are easy to culture *in vitro*, they are small in size, multiply through short cell cycles and their genetically homogenous cell population (clones) can be obtained over a short period of time. Ciliates are known to play an important role in the ecosystem and contribute a foremost role in the food web by transferring energy from lower to higher trophic level. They act as prey for higher organisms and as predators for bacteria (13). Also they show higher sensitivity towards pollutants as they lack cell wall (1, 14). In the present study, various aspects like cell mortality, morphological, behavioural and nuclear changes were evaluated in *Tetmemena* sp. to assess the toxicity of malathion.

METHODOLOGY

The collection site is Okhla Bird Sanctuary (28.5700° N, 77.3023° E) which is a bird sanctuary at the Okhla barrage over River Yamuna. The site is located at the point where the River enters Uttar Pradesh. The most prominent feature of the sanctuary is the large lake created by damming the River between Okhla village to the West and Gautam Budh Nagar to the East (Figure II). Spreading over 4 square kilometres, the areas around the sanctuary are mainly thorny scrub, grassland and a wetland that was formed as a result of creation of the Okhla Barrage. The sediment in the wetland consists of organic debris and fine sand. There is an extensive growth of water hyacinths on the banks and also inside the wetland.

Figure-II: Map of Delhi showing Okhla bird sanctuary, Delhi, India, the site of sample collection for the present study. Source: Google Maps

Water sampling

Fresh water samples were regularly collected throughout the year (Figure III). The water samples were passed through a 120 µm Nytex mesh to filter and collect ciliates as filtrate. Mixed planktonic cultures were initially grown at room temperature with addition of fresh boiled cabbage pieces to promote bacterial growth which served as food.

Figure-III: Photographs of the collection site, Okhla bird sanctuary.

In vitro culturing of Tetmemena sp.

Identification of *Tetmemena* sp. was done *in vivo* from the mixed cultures under stereoscopic microscope. Genus-level identifications were made according to Berger (1999)[15]. Clonal cultures of *Tetmemena* sp. were raised and maintained in the laboratory at 22-23 °C in Pringsheims medium (0.85 mM $Ca(NO_3)_2$. $4H_2O$, 0.35 mM KCl, 0.08 mM

$MgSO_4.7H_2O$) and 0.11 mM $Na_2HPO_4.2H_2O$) (16). Boiled cabbage was added to the medium to promote bacterial growth which served as food source for the cells.

Toxicity assay for malathion

Toxicity assay for malathion (Sigma Aldrich, catalogue no. 36143-100MG, molecular weight: 330.36 g/mol) was carried out to determine tolerance limits of *Tetmemena* sp. Initially, cells were exposed to various doses of malathion for 24 hr to determine the percent survival. 20 cells from the stock cultures were exposed to a range of concentrations from 0.033 ppm to 33 ppm. Each treatment was carried out in triplicate and was performed without adding fresh food source to the medium. Appropriate control experiments (without malathion) with same number of cells were performed simultaneously. After 24 hr period, the cells were counted in order to determine percent survival and mortality rate under different concentrations of malathion. Cells were counted under a Magnüs stereoscopic microscope at 20-40X magnification and 24 hr-LC_{50} value was calculated from semi log plot of percentage inhibition against pesticide concentration.

Morphological, behavioural and nuclear changes

The cells treated with malathion for 24 hr were evaluated for morphological, behavioural and nuclear changes. Morphological and behavioural changes were monitored by observing the cells under microscope at 40X. Cell size was determined with the help of Scope Image Software.
Nuclear changes were studied by Feulgen reaction. Cells were fixed in Carnoy's fixative (4 parts methanol and 1 part glacial acetic acid) for 20 minutes. After fixation, cells were hydrolysed in 1N HCl at 60 ºC for 7 minutes and stained with Schiff's reagent for 30 min (17, 18). Stained cells were observed under microscope for detecting number, shape and size of macronuclei and micronuclei of *Tetmemena* sp. in control and malathion treated cells.

DNA isolation

Genomic DNA of *Tetmemena* sp. was isolated using Qiagen Kit (Catalogue no. 69504) according to manufacturer protocol. One day starved cultures of both control and malathion treated (LC_{50} dose 0.22 ppm) cells were separately pelleted down by centrifugation (500 g, 5 min). Eluted DNA was stored at 4°C. 50 µl of the isolated DNA from the unexposed cells (control) was subjected to malathion treatment (0.22 ppm) for 24 hr to check for the effect of malathion on DNA.

Agarose gel electrophoresis

DNA analysis was carried out on 1 % agarose gel. The DNA samples isolated from control cells, cells treated with malathion and isolated DNA from control cells treated with malathion were loaded along with the marker (*Eco*RI and *Hin*dIII double digest lambda DNA ladder) on to agarose gel. The gel was run at a constant voltage of 80 V till the bromophenol dye reaches $3/4^{th}$ of gel. The gel was stained with ethidium bromide (10 mg/ml). After about 30 min, the stained gel was visualized under UV transilluminator and photographed.

RESULTS

Morphological charactersitics of Tetmemena sp.

Tetmemena sp. has a rigid oval body, lacks cortical granules and is dorso-ventrally flattened with average body length to width ratio of 3:1 (Figure IV). As observed under stereoscopic microscope, it moves rapidly with restless hasty turns and the cells are found mostly clustered near the piece of cabbage. Healthy cells are often found swimming in the upper layer of the medium.

Figure-IV**:** Photomicrograph of a live cell of *Tetmemena* sp. Scale bar represents 20 µm.

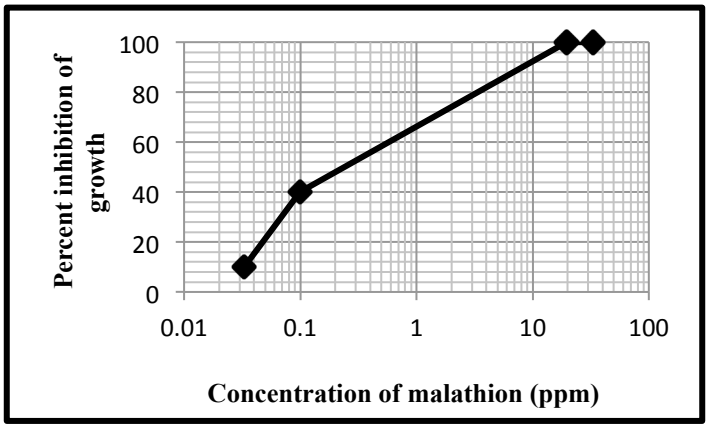
Toxicity assay for malathion

The toxicity assay was performed to observe the lethal effect of malathion on the growth of *Tetmemena sp*. Percent survival of *Tetmemena* cells in presence of various doses of malathion for 24 hr is described in Table I. 24 hr-LC_{50} value was found to be 0.22 ppm (Figure V). The overall response shows considerable increase in % mortality of *Tetmemena.*

Table-I: Sensitivity of *Tetmemena* sp. to malathion determined by 24 hr test method. *Tetmemena* cells were exposed to different concentrations of malathion for 24 hr. After 24 hr percent inhibition of growth in relation to control was calculated.

S. No.	Percent mortality	Dose (ppm)
1.	0% (Control)	0
2.	10% (LC_{10})	0.033
3.	50% (LC_{50})	0.22
4.	90% (LC_{90})	8
5.	MIC (Minimum inhibitory concentration)	20

Figure-V**:** *In vitro* growth inhibition of *Tetmemena* sp. in response to various concentrations of malathion for 24 hr period.



Morphological, behavioural and nuclear changes

Morphological characters (size and shape of the cell), behavioural characters (cell movement) and nuclear changes (number, size and shape of macronucleus and micronucleus) were evaluated in the presence and absence of malathion. Several morphological and behavioural changes were observed in cells exposed to varying doses of malathion (Table II and Figure VI).

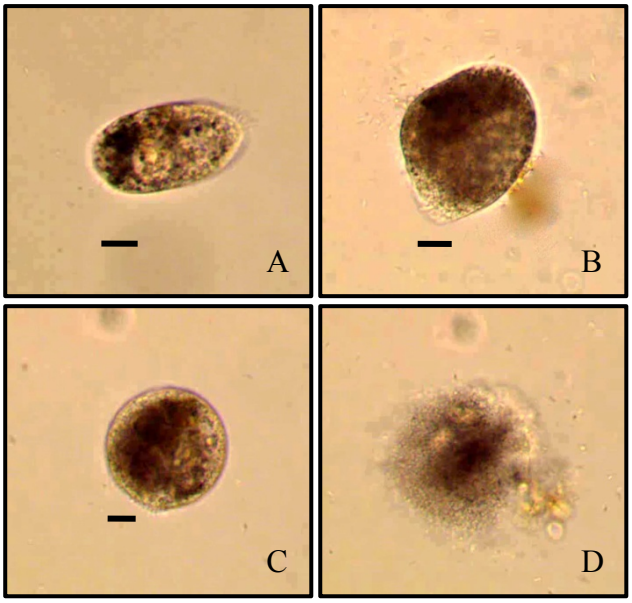


Figure-VI: *Tetmemena* sp. cells showing morphological deformities (A, B, C) and cell lysis leading to release of cytoplasmic contents (D) due to malathion treatment (0.22 ppm) after 24 hr observation at 40X magnification. Scale bar represents 20 µm.

Tetmemena sp. in the absence of malathion (control) has two types of nuclei i.e. macronucleus and micronucleus. Feulgen staining of *Tetmenena* sp. reveals that it has 2 macronuclei and 2-4 micronuclei (Figure VII, A). Both type of nuclei contain full complement of genes that bear the hereditary information of the organism. Macronucleus are oval in shape and regulates cellular metabolism. Micronuclei are smaller and round in shape. It is germinal i.e. responsible for tranfer of genetic information during sexual reproduction (conjugation). Cells treated with 24 hr-LC_{50} dose showed distortion and

shrinkage in the macronucleus (Table III) while micronucleus was not visible in most of the observed cells (Figure VII, B).

Effect of malathion on genomic DNA of Tetmemena sp.

The genomic DNA of treated cells was severely affected which can be clearly identified by comparing with the control DNA. The macronuclear and micronuclear DNA underwent fragmentation due to which smear was observed in the malathion treated cells. Along with these two DNA samples (control and malathion treated), DNA isolated from control cells was incubated in malathion (0.22 ppm for 24 hr), but no change was observed in this sample (Figure VIII).

Table-II: Effect of malathion on morphology and behaviour of *Tetmemena* cells after exposure to 0.22 ppm (LC_{50} dose) and 20 ppm (MIC).

Dose	Morphology and Behaviour	Cell size
Control (No treatment)	Rapid movement Shape rigid Intact cell membrane Maximum cells were swimming on the surface of the medium	Body length : 120 µm Body width : 40 µm Length:Width ratio 3:1
LC_{50} (0.22 ppm)	0-20 hr, no significant change visible in cell morphology. Cell movement slowed down.	No significant change
	After 21 hr, cell size decreased along the longitudinal axis. Cell movement slowed down and maximum cells were settled at the bottom of the Petri dish.	Body length : 75.84 Body width : 37.78 (Figure VI, A) Length:Width ratio 2:1
	Ater 22 hr, cell shape deformed and cytoplasm become dark may be due to deposition of insecticde withing the cytoplasm.	Body length : 81.53 Body width : 62.52 (Figure VI, B) Length:Width ratio 1:1
	After 23 hr, cells started rounding off which lead to rupturing of cell membrane and dispersion of cellular components in the surrounding media (cell death).	Body length : 60.97 Body width : 53.78 (Figure VI, C) Length:Width ratio 1:1
MIC (20 ppm)	**After 3 hr (since no cell survived till 24 hr):** Cell movement almost stopped Cell cytoplasm become dark may be due to deposition of insecticde withing the cytoplasm. Cells rounded off and ruptured releasing the cell contents.	------

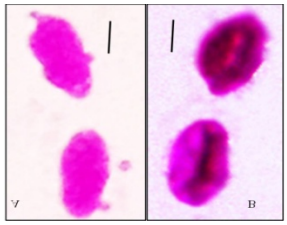


Figure-VII: Feulgen stained macronuclei and micronuclei of *Tetmemena* sp. control (A) and treated with malathion (B). Observations at 40X magnification. Scale bar represents 20 µm.

Table-III. Effect of malathion on macronuclear size of *Tetmemena* sp.

	Control	**LC_{50} dose (0. 22ppm) (24 hr)**	**Number of cells**
Macronucleus 1 Length	21.91µm	16.83µm	5
Macronucleus 1 Width	8.82µm	13.79µm	5
Macronucleus 2 Length	22.09µm	20.90µm	5
Macronucleus 2 Width	8.80µm	11.44µm	5

Lane 1: *Eco*RI/ *Hind*III double digest lambda DNA marker
Lane 2: Genomic DNA of *Tetmemena* sp. (Control cells)
Lane 3: Genomic DNA of *Tetmemena* sp. [cells treated with malathion (24 hr-LC_{50} dose)]
Lane 4: Genomic DNA from control cells treated with malathion (24 hr-LC_{50} dose)

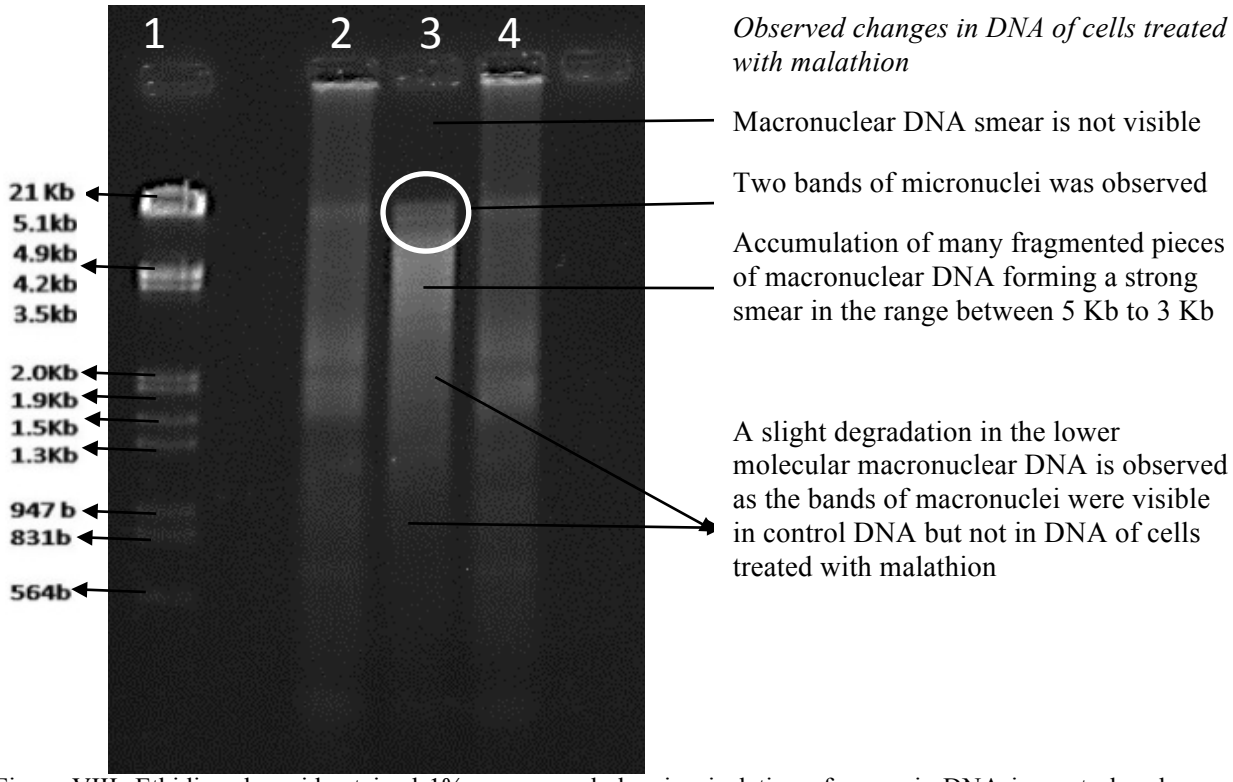


Figure-VIII: Ethidium bromide stained 1% agarose gel showing isolation of genomic DNA in control and malathion (24 hr-LC_{50} dose) treated cells of *Tetmemena* sp.

DISCUSSION

The cytotoxicity of insecticides and pesticides has been evaluated in various experimental model organisms but due to ciliate sensitivity to environmental alterations they have been proposed as biological indicators of water pollution (19, 20). *Tetmemena* sp. used in the present study was found to be widely distributed in fresh water samples collected from Delhi region. The observed 24 hr-LC_{50} dose in the present investigation was found to be 0.22 ppm for *Tetmemena* sp. The 24 hr-LC_{50} of ciliate species used in the present investigation is comparable to that reported for *Daphnia* (21) suggesting *Tetmemena* to be

a model organism for the evaluation of toxicity assays and can possibly replace higher organisms like fish and mammals (22, 23)

In the present investigation, malathion was found to cause morphological, behavioural and nuclear changes in *Tetmemena* cells. Malathion treated cells (0.22 ppm, 24 hr-LC 50) showed distortion in cell shape, disturbed cell movement from, rounding of the cells and finally leading to disruption of the cell membrane and release of cellular contents leading to cell death. Similar results have been reported in other ciliates exposed to other pesticides and heavy metals (24, 25). Changes in morphology and cell movement may serve good biomarkers to evaluate the toxicity of pesticides in ciliates.

The pesticide malathion had also shown to interfere with the macronuclei and micronuclei of the ciliate *Tetmemena* sp. leading to chromatin condensation and decrease in size along the longitudinal axis of the macronuclei as reported earlier in *Paramecium* (12). In malathion treated cells, micronuclei was not observed which could be due to shearing of micronuclear DNA suggesting genotoxicity of malathion. Interestingly, there was no shearing of DNA when DNA was directly incubated with malathion. This interesting fact can be explained by the observation that malathion becomes more toxic when it is converted to malaoxon in biological systems (26).

Therefore, it is hypothesised that when live cells were treated with malathion in the medium, it gets converted to malaoxon producing the observed alterations, but when DNA was directly incubated with malathion for 24 hr, malathion was not converted to malaoxon. Therefore, it can be concluded that malathion is not a direct DNA damaging agent but it becomes genotoxic once it is converted to malaoxon.

The increasing concentration of malathion in River Yamuna, Delhi seems alarming (27). Present investigation shows that malathion brings about morphological, behavioural and nuclear changes at very low concentrations of pesticide malathion. Further, it should also be noted that changes accumulated in natural ecosystems over a period of time due to exposure to such pesticides and insecticides can alter the morphology and molecular identification of a species leading to evolution of an altogether new species with new characteristics or even complete elimination of that particular species from that ecosystem.

CONCLUSIONS

From the experiments performed, various changes in the morphology, behaviour and nuclear changes on exposure to increasing concentrations of malathion to *Tetmemena* sp. confirms the cytotoxicity and genotoxicity of malathion to this ciliate. The present study holds scope to use ciliates such as *Tetmemena* sp. to develop a microbiotest kit wherein we can isolate *Tetmemena* sp. from any given water sample and study its morphology, behaviour and nuclei to check if the water sample is polluted with pesticides or not. Also, we can introduce *Tetmemena* sp. to any polluted water sample and observe the above mentioned changes in the cells and confirm the level of pollution by comparing with the present experimental data.

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REFERENCES

1. Amanchi, N. R. (2010) A low cost microbiotest for screening behavioural and exotoxicological responses of *Paramecium caudatum* and *Oxytricha fallax* to azadirachtin. *Advances in Applied Science Research* 1(2): 124–131.
2. Peters, K., Bundschuh, M., and Schäfer, R. B. (2013) Review on the effect of toxicants on freshwater ecosystem functions. *Environmental Pollution* 180: 324–329.
3. Twagilimana, L., Bohatier, J., Groliere, C. A., Bonnemoy, F., and Sargos, D. (1998) A new low-cost microbiotest with the protozoan *Spirostomum teres*: Culture conditions and assessment of sensitivity of the ciliate to 14 pure chemicals. *Ecotoxicology and Environmental Safety* 41: 231–244.
4. Gupta, P. K. (2004) Pesticide exposure- Indian scene. *Toxicology* 198(1–3): 83–90.
5. Malik, D., Singh, S., Thakur, J., Singh, R. J., Kaur, A., and Nijhawan, S. (2014) Heavy metal pollution of the Yamuna River: An introspection. *International Journal of Current Microbiology and Applied Sciences* 3(10): 856–863.
6. Nair, A. S., and Pradeep, T. (2007) Extraction of chlorpyrifos and malathion from water by water nanoparticles. *Journal of Nanoscience and Nanotechnology* 7:
   Newhart, K. L. (2006) Environmental Fate of Malathion. California Department of Pesticide Regulation. http://www.cdpr.ca.gov/docs/emon/pubs/fatememo/efate_malathion. pdf
7. Daniz, Y., Mustafa, C., Serkan, Y., Fatma, U. and Huseyin, A. (2006) Clastogenicity of the fungicide afugan in cultured human lymphocyte. *Mutation Research* 604: 53–59.
8. Fenech, M. and Crott, J. (2002) Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient in human lymphocytes- evidence for breakage- fusion- bridge cycle in the cytokinesis-block micronucleus assay. *Mutation Research* 504: 131–136.
9. Geeta, M. and Fulekar, M. H. (2008) Bioremediation of pesticides in surface soil treatment unit using microbial consortia. *African Journal of Environmental Science and Technology* 2: 36–45.
10. Wild, D. (2006) Mutagenicity studies on organophosphorus insecticides. *Mutation Research* 32: 133–150.
11. Amanchi, N. and Bhagavathi, M. (2009) Comparative study on cytotoxicity of Delfin insecticide using two vital protozoan ciliates *Paramecium caudatam* and *Oxytricha fallax. Asian Journal of Experimental Sciences* 23(1): 55–60.
12. Dopheide, A., Lear, G., Stott, R. and Lewis, G. (2009) Relative diversity and community structure of ciliates in stream biofilms according to molecular and microscopy methods. In American Society for Microbiology, *Applied Environmental Microbiology* 1–37.
13. Gutiérrez, J. C., Martín-González, Díaz, S., Amaro, F., Ortega, R., Gallego, A. and de Lucas, M. P. (2008) In S. E. Brown, & W.C. Welton, *Heavy Metal Pollution* (pp. 1-44. New York: Nova Science Publishers, Inc.
14. Berger, H. (1999) Monograph of the Oxytrichidae (Ciliophora, Hypotrichia). *Monographie Biologicae*, Kluwer Academic Publishers, Dordrecht, Boston, London 78: 501–605.
15. Chapman-Andresen, C. (1958) Pinocytosis of inorganic salts by *Amoeba proteus* (*Chaos diffluens*). *Comptes rendus des travaux du Laboratoire Carlsberg. Série chimique* 31: 77–92.
16. Chieco, P. and Derenzini, M. (1999) The Feulgen reaction 75 years on. *Histochemistry and Cell Biology* 111: 345–358.
17. Feulgen, R. and Rossenbeck, H. (1924) Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* 135: 203–248.
18. Darcy, P., Kelly, J. P., Leonard, B. E. and Henry, J. A. (2002) The effect of lofepramine and other related agents on the motility of *Tetrahymena pyriformis*. *Toxicolology Letters* 128: 207–214.
19. Dias, N., Renato, A. M. and Nelson, L. (2003) Morphological and physiological changes in *Tetrahymena pyriformis* for the in vitro cyotoxicity assessment of Triton X-100. *Toxicology In Vitro* 17: 357–366.
20. Rassoulzadean, M. and Akyurtlakli, N. (2002) An investigation on the toxic effects of malathion (Organophosphate insecticide) on *Daphnia magna* Straus, 1820 (Crustacea, Cladocera). *Turkish Journal of Zoology* 26: 349–355.
21. Choudhary, N., Goyal, R. and Joshi, S. C. (2008) Effect of malathion on reproductive system of male rats. *Journal of Environmental Biology* 29(2): 259–262.

22. Magar, R. S. and Bias, U. E. (2013) Histopathological Impact of malathion on the ovary of fresh water fish *Channa punctatus* 2(3): 59–61.
23. Amanchi, N. R. and Hussain, M. M. (2008) Cytotoxic effects of delfin insecticide *(Bacillus thuringiensis*) on cell behaviour, phagocytosis, contractile vacuole activity and macronucleus in a protozoan ciliate *Paramecium caudatam. African Journal of Biotechnology* 7(15): 2637–2643.
24. Pudpong, S. and Chantangsi, C. (2015) Effects of four heavy metals on cell morphology and survival rate of ciliate *Bresslauides* sp. *Tropical Natural History* 15(2):117–125.
25. Edwards, D. (2006) Reregistration Eligibility Decision for Malathion. *US Environmental Protection Agency- Prevention, Pesticides and Toxic substances EPA 738-R-06-030 journal*: 9.
26. Aleem, A. and Malik, A. (2005) Genotoxicity of the Yamuna River water at Okhla (Delhi), India. *Ecotoxicology and Environmental Safety* 61: 404–412