© 2015 IJSRSET | Volume 1 | Issue 6 | Print ISSN : 2395-1990 | Online ISSN : 2394-4099
Themed Section: Science and Technology

In Vivo Cytotoxicity Assessment of Thiram : Physiological and Biochemical Changes in *Paramecium sp.*

Meriem Djekoun*, Houria Berrebbah, Mohammed Reda Djebar
Department of Biology, Badji Mokhtar University, Laboratory of Cellular Toxicology, Annaba, Algeria

ABSTRACT

The freshwater protozoan ciliate *Paramecium sp.* was used in order to assess the potential cytotoxic effects of Thiram, a dithiocarbamates fungicide. To estimate the impact of this fungicide, cells were exposed to three concentrations (0.001, 0.002 and 0.003 mg/l) for 4 days. The respiratory metabolism and the antioxidant defence biomarkers (Catalase and reduced Glutathione) were assessed. Acute exposure caused marked decrease in number of cells with EC50 value estimated at 0.0028 mg/l. The obtained results concerning the cellular respiratory reveal a dose-dependent perturbation in exposed organisms. Also, the biomarkers assessed were sensitive to the presence of Thiram wich induced a strong antioxidative activities by stimulating the enzymatic activity of Catalase (CAT) and decreasing the rate of reduced Glutathione (GSH).

Keywords: *Paramecium sp.*, Thiram, Cytotoxicity, EC50, Biomarkers

I. INTRODUCTION

Modern methods of agriculture involve a very wide range of agrochemicals products. This can result in the dispersal of hazardous residues in soil, ground water and surface water **(Gimeno-Garcia *et al.*, 1996; Maksymiec, 1997)**. Such exogenous chemicals may cause irreversible environmental damages **(Fleeger *et al.*, 2003)** by reducing biodiversity and disrupting ecosystems. Phytosanitary products received enormous attention for their toxic effects on organisms **(Bonnemain and Dive, 1990; Robinson, 1998; Dewez *et al.*, 2005; De *Silva et al.*, 2006; Azzouz *et al.*, 2011; Xiyan *et al.*, 2013; Saib *et al.*, 2014; Amamra *et al.*, 2015 $_{a}$)**.

Among the various formulations of pesticides, Thiram is widely used in agriculture. This active ingredient (tetramethylthiuram disulphide) is a related Dithiocarbamate compound used to prevent crop damage in the field and to protect harvested crops from deterioration in storage or transport. It is also used as a seed protectant (*e.g.* small and large seeded vegetables, cereal grains and other seeds) and to protect turf from fungal diseases. In addition, Thiram is used as an animal repellent to protect crops from damage by rabbits, rodents, and deer.

Usage of non-targeted organisms in environmental toxicology is needed to understand the wide range of toxic effects caused by the pesticides on different organisms **(Wan *et al.*, 1994)**. The common model systems used in the research of the environmental toxicity include bacteria, algae, Daphnia, and zebrafish **(Dewez *et al.*, 2005; Lin and Janz, 2006; Sancho *et al.*, 2009; Sunil *et al.*, 2015)**. However, a variety of other organisms have been introduced successfully in toxicological investigations **(Miyoshi *et al.*, 2003)**, this is the case of protozoa. Free living fresh water protozoan ciliates are used as bioindicators of toxicity stress and chemical pollution mostly, especially in aquatic ecosystem **(Apostol, 1972; Rao *et al.*, 2006)**. The ciliated protozoan *Paramecium* is at the bottom foundation of the heterotrophic eukaryotic food chain. They are important bioindicators of environmental conditions, and changes due to anthropogenic activity on ecosystems, they also play a pivotal role as regulators of key ecosystem processes **(Finlay and Fenchel, 2004).**

Paramecium sp. is an organism of choice on cytotoxicity studies. Its short generation time compared to invertebrates and the fact that it can be grown quickly in a laboratory culture medium are especially advantageous for studying the action of xenobiotics on several generations of cells. This ciliate has been used to determine the effects of different xenobiotics **(Rehman *et al.* 2008; Amanchi and Bhagavathi, 2009; Rouabhi, 2011; Benbouzid *et al*., 2015)**. In addition, Thiram was shown to be a very toxic substance when tested on the ciliate protozoan *Colpidium campylum* **(Bonnemain and Dive, 1990)** and the crustacean *Gammarus pulex* **(Bluzat *et al*., 1982)**. Some reports have been described the mutagenicity of thiram on *Salmonella typhimurium* **(Franekic *et al*., 1994).**

In this context, the purpose of this study is to provide the cytotoxic effects under acute exposure in *Paramecium sp*. to sublethal concentrations of Thiram. Respiratory metabolism and monitoring of some biomarkers were performed to evaluate a possible oxidative stress, so we followed up the variations of enzymatic activity of Catalase (CAT) and the rate of reduced Glutathione (GSH).

II. METHODS AND MATERIAL

A. Test Chemical

Thiram fungicide (Thiramchim, Sipcam Inagra) used in the experiments is wettable powder and 80% pure. Stock solutions were prepared by dissolving the toxicant in distilled water.

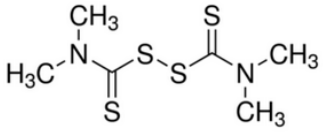

Fig. 1 Chemical structure of Thiram

B. Test organisms

The biological material used in our work is a single-celled microorganism whose culture is obtained by simply soaking hay and lettuce in rainwater. The preparation is left in a warm place (20 °C) dark and airy.

C. Sublethal toxicity study

Preliminary toxicity test were conducted in our laboratory in order to determine sublethal concentrations. The experiments were performed according to **Azzouz *et al.* (2011)**. Paramecium culture was exposed to a range of concentrations: 0.001, 0.002 and 0.003 mg/l. Each test concentration was replicated 3 times. The culture was done at 28 ± 2° C in test tubes using 10 ml of the culture medium. The growth kinetics study was realized by the daily cell counting, under optic microscope using grooved blade. The count was repeated at least three times for each repetition.

D. Response percentage

After 96h of exposure, changes in growth rates were examined. The number of living cells was counted and a percentage response was determinate according to the following formula:

$$Response\ percentage = \left[N_C - N_E / N_C \right] \times 100$$

Where N_C is the number control cells, N_E is the number of treated cells.

E. EC50 determination

The median effective concentration is estimated using the software REGtox® **(Vindimian *et al.*, 1983)**.

F. Polarographic study

The monitoring of the respiratory metabolism is carried out through an oxygen electrode (HANSENTECH) according to **Djebar and Djebar (2000)** adapted at the Laboratory of Cellular Toxicology. Oxygen consumption signals are visualized in spot shape on the computer screen.

G. Biochemical assays

Catalase activity (CAT) was measured according to the method of **Regoli and Principato (1995)** by following the decrease of absorbance every 15 seconds for one minute at 240 nm due to H_2O_2 consumption. For the assessment of enzymatic activity of Catalase, the samples are homogenized in phosphate buffer 1 ml (0.1M, pH7.5) using an ultrasonic crusher (SONICS, Vibra cell). The homogenate thus obtained was centrifuged at 15 000 rpm/min for 10 minutes and the supernatant recovered serves as enzyme source. The

reaction is initiated by the addition of hydrogen peroxide (500 mM, 30 V). Reading is done in a spectrophotometer (JENWAY, 6300) and the results of CAT activity are expressed in µmol H_2O_2 per minute per mg of protein.

The rate of glutathione (GSH) is quantified according to the method of **Weckberker and Cory (1989)**, based on the colorimetric measurement of the 2-nitro-5 mercapturic acid at 412 nm. The samples are homogenized in 1 ml EDTA (0.02 M, pH 9.6). 0.2 ml of SSA are added to 0.8 ml of the homogenate prepared beforehand. The mixture was centrifuged at 1000 rpm / min for 5 min. An aliquot of 500 µl of the supernatant is added to 1 ml Tris / EDTA buffer. The amount of glutathione is expressed in µmol / mg protein.

H. Data analysis and statistics

The basic statistics (means and standard errors) of the measured parameters were estimated.

Statistical comparisons were made using Minitab 16. The significance of differences between samples was determined by using the analysis of variance (ANOVA) Student's t-test, where p value less than 0.05 was considered to be significant.

III. RESULTS AND DISCUSSION

Microorganisms have a high surface area-to-volume ratio because of their small size and therefore provide a large contact area that can interact with pollutants in the surrounding environment **(Ledin, 2000)**. Free living fresh water protozoan ciliates like Paramecia are the most commonly used ciliated and they are considered as excellent bioindicators of toxicity stress and chemical pollution. Thereby, in this work we used *Paramecium sp.*, as a model cell to study the impact of Thiram, a widely used fungicide, on reproduction and stress antioxidant systems.

Paramecium cells exposed to Thiram undergone a series of physiological and biochemical changes.

A. Population growth and response percentage

The acute toxicity test on *Paramecium sp.* with increasing concentrations was performed during four days.

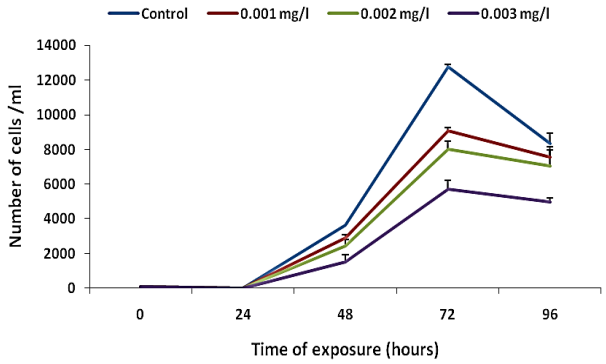

Figure 1 : Effect of Thiram on the growth of *Paramecium sp.* (T 28 ºC pH: 6.5). Each value represents the mean of three independent assays ± standard error ($P < 0.001$).

The results given in figure 1 represent the effect of Thiram on the growth of paramecium cells in function of time. The selected concentrations inhibited the population growth in a dose-dependent manner especially for the highest concentration. The statistical analysis revealed a significant difference ($P < 0.001$). Indeed, the number of cells is reduced by half at the end of the experiment.

The response percentage confirms the results obtained in the growth kinetics (Figure 2).

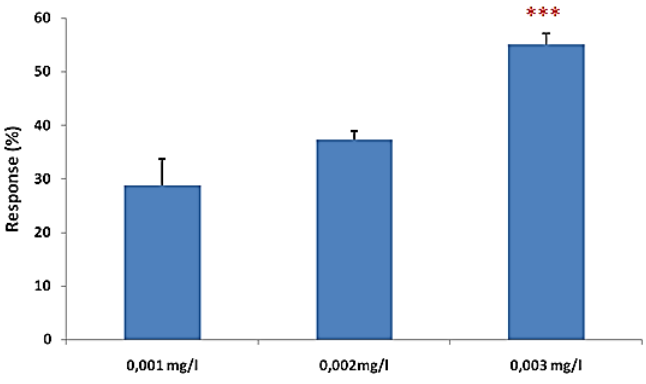

Figure 2 : Evolution of response percentage in *Paramecium sp.* exposed to increasing concentrations of Thiram. Each value represents the mean of three independent assays ± standard error (*** $P < 0.001$).

The results show a positive and significant percentage of inhibition of about 55% in cells treated with Thiram at the concentration of 0.003 mg/l.

B. EC50 determination

The estimated EC50 for the freshwater protozoan, Paramecium sp. exposed to Thiram was 0.028 mg/l (Figure 3).

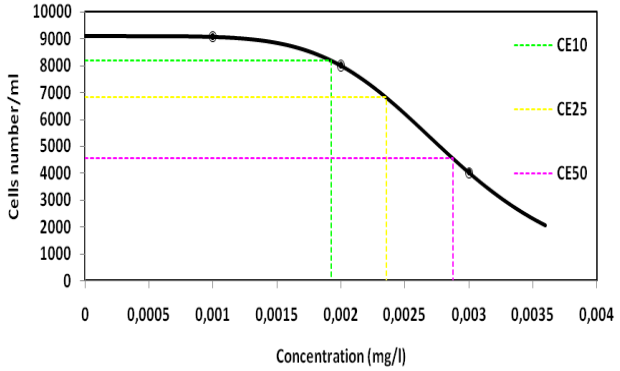

Figure 3 : Median Effective Inhibitory Concentration (EC50 expressed in mg/l) of Thiram using growth of *Paramecium sp.*

The growth curve of *Paramecium* gave a clear gradual decrease in the number of cells. Thiram seemed toxic for the tested protozoan (Figure 2-3); this is supported by equal to 0.0028 mg/l. Our results can be compared with previous reports on the toxicity of several chemical classes of pesticides **(Rehman *et al.*, 2008; Amanchi and Hussain, 2010; Benbouzid *et al.*, 2012; Djekoun *et al.*, 2015)**.

C. Respiratory Metabolism

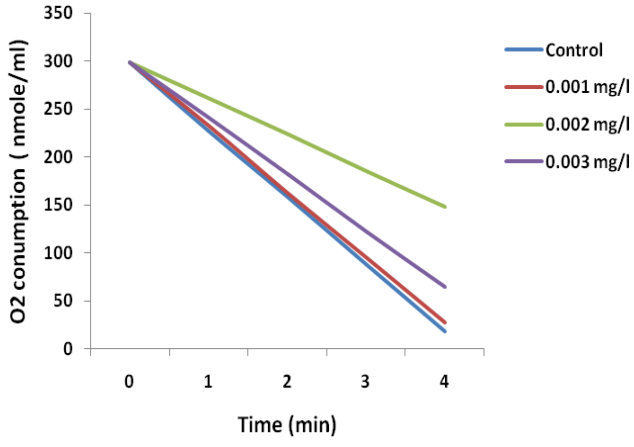

Figure 4: Evolution of respiratory metabolism of *Paramecium sp*. exposed to Thiram.

Finding in figure 4 shows that the sublethal exposure disrupts cellular respiration in the studied model. Indeed we noticed a decrease in oxygen consumption for the concentrations 0.001 and 0.003 but the amount of consumed O_2 increased for the concentrations 0.002 mg/l with an oxygen consumption of 148.58 nmol/ml after the 4th minute in comparison to the control. Similar observations have been reported when paramecia were exposed to other chemical products **(Rouabhi *et al.* 2006; Benbouzid *et al.* 2015)**. Indeed, during the xenobiotics metabolization, cellular defense system can contribute to the formation of reactive oxygen intermediates that are known to be disruptive of the respiratory chain.

D. In vivo effects of Thiram on response of antioxidant defence markers

It is suggested that biomarkers can be used to devise rapid, effective screening assays, which can complement other testing techniques **(Mountassif *et al.*, 2007)**.

The exposure of *Paramecium sp.* to increasing concentrations of Thiram shows that this fungicide exerted a strong disturbance on the antioxidant system. The results concerning the variations of CAT activity and the rate of GSH are represented in table 1. We note that the CAT activity reaches its maximum (0.300 µmol/min/mg pro) at the concentration of 0.001 mg/l. The statistical analysis revealed a significant difference ($P \leq 0.05$) compared to control. However, the activity of this enzyme was less important at the highest concentration.

Increased activities of CAT have been reported in several aquatic species **(Dewez *et al.*, 2005; Clasen *et al.*, 2014; Djekoun *et al.*, 2015)**. This enzyme is considered as an important and sensitive biomarker of oxidative stress, revealing biological effects on the redox status of the marine organisms. The removal of H_2O_2 is an important strategy of aquatic organisms against oxidative stress (**Regoli *et al.*, 2002a, b)**.

Thiram Exposure induces an important dose-dependent decrease in GSH content ($P \leq 0.05$). At the highest concentration (0.003 mg/l) the GSH rate is of the order of 0.190 µmol/mg pro while it is 0.274 µmol/mg pro for the control (Table1).

Table 1: Variations in Catalase activity and GSH content in *paramecium sp.* Exposed to increasing concentrations of Thiram

Parameter	Control	0.001 mg/l	0.002 mg/l	0.003 mg/l
CAT (µmol/min/mg proteins)	0.125±0.03	0.300±0.05*	0.263±0.06 ns	0.232±0.05 ns
GSH (µmol/mg proteins)	0.229±0.03	0.279±0.006 ns	0.232±0.007 ns	0.190±0.01*

Values are given as means of three replicates. ns: statically non-significant differences, * Significantly different from the control.

Our data for decreases in reduced Glutathione may reflect its utilization in countering the prevailing oxidative stress under the influence of reactive oxygen species (ROS) generated from Thiram oxidative stress. Indeed, this tripeptide is a vital protective antioxidant against oxidative stress, which plays a key role in the capture and sequestration of free radicals but also acts as a substrate for the regeneration of other essential antioxidants **(Milter *et al.*, 2004; Foyer and Noctor, 2005; Bashandy *et al.*, 2010)**. The diminution in GSH levels might be an adaptive response of the cells to counter the increase of oxidative stress (**Yonar *et al.*, 2014; Liu *et al.*, 2015)**.

IV.CONCLUSION

After considering the results of the present study, it is to be concluded that Thiram is cytotoxic at the tested concentrations and *Paramecium sp.* is an excellent alternative model to study the mode of action of pesticides. Results clearly demonstrated that sublethal concentrations of Thiram cause changes in the biochemical parameters like Catalase activity and the rate of reduced Glutathione.

V. REFERENCES

[1] Amamra R., Djebar M.R., Grara N., Moumeni O., Otmani H., Alayat A., and Berrebbah H. 2015. Cypermethrin-Induces Oxidative Stress to the Freshwater Ciliate Model: Paramecium tetraurelia. Ann. Res. & Rev. Biol. 5(5): 385-399.

[2] Amanchi N. and Bhagavathi M. 2009. Comparative study on cytotoxicity of delfin insecticide using two vital protozoan ciliates Paramecium caudatum and Oxytricha fallax. Asian J. Exp. Sci. 23: 55-60.

[3] Amanchi N.R. and Hussain M.M. 2010. Cytotoxicity assessment of monocrotophos in Paramecium caudatum and Oxytricha fallax. J. Environ. Biol. 31(5): 603-607.

[4] Apostol S., 1973. A Bioassay of Toxicity Using Protozoa in the Study of Aquatic Environment Pollution and Its Prevention. Environ. Res. 6: 365-372 .

[5] Azzouz Z., Berrebbah H., Djebar M.R. 2011. Optimization of Paramecium tetraurelia growth kinetics and its sensitivity to combined effects of azoxystrobin and cyproconazole. African J. Microbiol. Res. 5(20): 3243-3250.

[6] Bashandy T., Guilleminot J., Vernoux T., Caparros-Ruiz D., Ljung K., Meyer Y., Reichheld J.P. 2010. Interplay between the NADP-linked thioredoxin and glutathione systems in Arabidopsis auxin signaling. Plant Cell. 22: 376-391.

[7] Benbouzid H., Berrebbah H., Berredjem M., Djebar M.R. 2012. Toxic effects of phophoramidate on Paramecium sp. With special emphasis on respiratory metabolism, growth and generation time. Toxicol. Environ. Chem. 94 (3): 557-565.

[8] Benbouzid H., Berrebbah H., Djebar M.R. 2015. Toxicity of the Chlorfenapyr: Growth inhibition and induction of oxidative stress on a freshwater protozoan: Paramecium sp. Adv. Environ. Biol. 9(3): 281-285.

[9] Bluzat R., Jonot O., Seugi J. 1982. Toxicité aigue d'un fongicide, le thirame (dithiocarbamate), chez le crustacé amphipode d'eau douce Gammarus pulex. Environ. Pollut. 29, 225-233.

[10] Bonnemain H. and Dive D. 1990. Studies on Synergistic Toxic Effects of Copper and Dithiocarbamate Pesticides with the Ciliate Protozoan Colpidium campylurn (Stokes). Ecotox. Environ. Safe. 19: 320-326.

[11] Clasen B., Leitemperger J., Murussi C., Pretto A., Menezes C., Dalabona F., Marchezan E., Bohrer Adaime M., Zanella R., Loro V.L. 2014. Carbofuran promotes biochemical changes in carp exposed to ricefield and laboratory conditions. Ecotox. Environ. Safe. 101: 77-82.

[12] De Silva H.J., Samarawickrema N.A., Wickremasinghe A.R. 2006. Toxicity due to organophosphorus compounds: what about chronic exposure? Trans. R. Soc Trop Med Hyg. 100: 803-806.

[13] Dewez D., Geoffroy L., Vernet G. Popovic R. 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga Scenedesmus obliquus. Aquat. Toxicol. 74: 150-159.

[14] Djebar H. and Djebar M.R. 2000. Bioénergétique, Les mitochondries végétales. Revue des sciences et technologie ; Synthèse 2000. Publication de l'Université de Annaba. Edition Végarol: 103.

[15] Djekoun M., Berrebah H. and Djebar M.R. 2015. Growth kinetics, monitoring of biomarkers and respiratory metabolism to evaluate the combined effects of fludioxonil and difenoconazole in a freshwater ciliated protist: Paramecium sp. Der Pharma Chemica. 7(11): 197-204.

[16] Finlay B.J. and Fenchel T. 2004 Cosmopolitan metapopulations of free-living eukaryotes. Protist. 155: 237-244.

[17] Fleeger, J.W., Carman, K.R., Nisbet, R.M., 2003. Indirect effects of contaminants in aquatic ecosystems. Sci. Total Environ. 317: 207-233.

[18] Foyer C. and Noctor G. 2005. Oxidant and antioxidant signalling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell. Environ. 28:1056-1071.

[19] Franekic J., Bratulic N., Pavlica M., Papes D. 1994. Genotoxicity of dithiocarbamates and their metabolites. Mutat. Res. 325: 65–74.

[20] Gimeno-Garcia E., Andreu V., Boluda R. 1996. Heavy metals inci-dence in the application of inorganic fertilizers and pesticides to rice farming soils. Environ. Pollut. 92 : 19-25.

[21] Ledin M., 2000. Accumulation of metals by microorganisms-processes and importance for soil systems. Earth-Sci. Rev. 51: 1-31.

[22] Lin L.L., Janz D.M. 2006. Effects of binary mixtures of xenoestrogens on gonadal developement and reroduction in zebrafish. Aquat. Toxicol. 80: 382-395.

[23] Liu L., Zhu B., Gong Y., Liu G., Wang G., 2015. Neurotoxic effect of triazophos on gold fish (Carassius auratus) and tissue specific antioxidant responses. Ecotoxicol. Environ. Saf. 116: 68-75.

[24] Maksymiec W., 1997. Effect of copper in higher plants. Photosynthetica. 34: 321-342.

[25] Mittler R., Vanderauwera S., Gollery M., Van Breusegem F. 2004. Reactive oxygen gene network of plants. Trends Plant. Sci. 9: 490-498.

[26] Miyoshi N., Kawano T., Tanaka M., Kadono T., Kosaka T., Kunimoto M., Takahashi T., Hosoya H. 2003. Use of paramecium species in bioassays for environmental risk management: determination of IC50 values for water pollutants, J. Health Sci. 49: 429-435.

[27] Mountassif D., Kabine M., Manar R., Bourhim N., Zaroual Z. Latruffe N., El Kebbaj M.S. 2007. Physiological, morphological and metabolic changes in Tetrahymena pyriformis for the in vivo cytotoxicity assessment of metallic pollution: Impact on D-b-hydroxybutyrate dehydrogenase. Ecol. Ind. 7: 882-894.

[28] Rao J.V., Srikanth K., Arepalli S.K., Gunda V.G., Toxic effects of acephate on Paramecium caudatum with special emphasis on morphology, behaviour, and generation time, Pest. Biochem. Physiol. 86: 131-137.

[29] Regoli F and Principato G. 1995. Glutathione, glutathione-dependant and antioxidant enzymes in mussel Mytilus galloprovincialis exposed to metals under field and laboratory conditions: implication for the biomarkers. Aquat. Toxicol. 31: 143-164.

[30] Regoli F., Gorbi S., Frenzilli G., Nigro M., Corsi I., Focardi S., Winston G.W., 2002a. Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Mar. Environ. Res. 54: 419–423.

[31] Regoli F., Pellegrini D., Winston G.W., Gorbi S., Giuliani S., Virno-Lamberti C., Bompadre S., 2002b. Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (Mullus barbatus). Mar. Pollut. Bull. 44: 912-922.

[32] Rehman A., Shuja R. and Shakoori A.R. 2008. Tolerance of Two Ciliates, Stylonychia mytilusand, Paramecium caudatum, Isolated from

Industrial Effluents to an Organophosphate, Endosulfan and their Potential use in Bioremediation of Insecticide-contaminated Wastewater. Pakistan J. Zool.40(4): 255-261.

[33] Robinson P.W. 1998. The toxicity of pesticides and organics to mysid shrimps can be predicted from Daphnia spp. Toxicity data. Wat. Res. 33 (6): 1545-1549.

[34] Rouabhi R, Berrebbah H, Djebar MR. 2006. Toxicity evaluation of flucycloxuron and diflubenzuron on the cellular model, Paramecium sp. Afr. J. Biotechnol. 5 (1): 45-48.

[35] Rouabhi R. 2011. Influence of Nickel on Growth, Movement Speed, Respiratory Metabolism, and Biochemical Parameters of Paramecium sp. WebMed Central. 2046-1690.

[36] Saib, A., Berrebbah H., Berredjem M., Djebar M. R. 2014. Cytotoxic study of three derivatives amidophosphonates on alternative cellular model: Paramecium tetraurelia. Toxicol. Res. 3: 395-399.

[37] Sancho E., Villarroel M.J., Andreu E., Ferrando M.D. 2009. Disturbances in energy metabolism of Daphnia magna after exposure to tebuconazole. Chemosphere. 74: 1171-1178.

[38] Sunil S., Rashi G., Shilpi S. 2015. Effects of chemical and biological pesticides on plant growth parameters and rhizospheric bacterial community structure in Vigna radiate. J. Hazard. Mater. 291: 102-110.

[39] Vindimian E., Robaut C. and Fillion G. 1983. A method for cooperative and non cooperative binding studies using non linear regression analysis on a microcomputer. J. Appl. Biochem. 5: 261-268.

[40] Wan M.T., Watts R.G., Moul D.J. 1994. Impact of chemigation on selected non-target aquatic organisms in cranberry bogs of British Columbia, Bull. Environ. Contam. Toxicol. 53: 828-835.

[41] Wechbeker G, Cory J.G. 1988. Ribonucleotide reductase activity and growth of glutathione depleted mouse leukemia L1210 cells in vitro. Cancer let. 40: 257-264.

[42] Xiyan M., Sen P., Xingze S., Jiejun G., Jiayi C., Xiaofeng C., Xuefeng L., Chengju W. 2013. Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. Environ. Pollut. 175 : 147-157.

[43] Yonar S., Ural, M., Silici, S., Yonar, M., 2014. Malathion-induced changes in the haematological profile, the immune response, and the oxidative/antioxidant status of Cyprinus carpio carpio: protective role of propolis. Ecotoxicol. Environ. Saf. 102: 202-209.