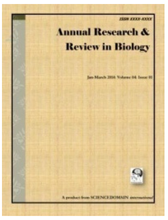

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Cypermethrin-Induces Oxidative Stress to the Freshwater Ciliate Model: *Paramecium tetraurelia*

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Authors' contributions

This work was carried out in collaboration between all authors. Author RA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MRD managed the analyses of the study. Authors MRD and HB followed and supervised this study. Author OM collected the data. Authors NG, HO and AA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Synthetic Pyrethroids are considered to be safe over other insecticides; however, data indicate that their use may pose risk to environmental biota, especially, aquatic organisms. Therefore, the aim of the present study was to investigate the effect of cypermethrin, a widely used insecticide and one of the most common contaminants in freshwater aquatic system on the oxidative stress biomarkers of the freshwater ciliate *Paramecium tetraurelia.*

After the treatment of paramecium cells with increasing concentrations of cypermethrin (0.05, 0.5,1, 2µg/l), we followed up the growth kinetics, generation time and the response percentage. Also, we studied the variation in biomarkers of stress such as: Proteins, GSH content, GST and CAT activities.

Our results showed a significant decrease in the proliferation of cell, we denote a difference of

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nearly 1,900 cells between the control cells and those treated with (2µg/l) at the fourth day of treatment. This finding is correlated by the decrease in generation number and velocity and the increase in generation time. Also, we noted an inhibition in the response percentage: It varies from (20%) to (54%) for 0.5µg/l and 2µg/l respectively.

The rate of total proteins increased in dose dependent manner and very highly significant for the tow highest concentrations (1 and 2µg/l).

The monitoring of biomarkers revealed a depletion in GSH content in a proportional and dose dependent manner (it is 7.34188758µmol/mg Pro for the control whereas it is 2.41682134µmol/mg Pro for 2µg/l) accompanied by an increase in the GST activity (we note an increase of the order of 1.62932472µM/min/mg Pro for the highest concentration compared to the control which is of the order of 0.59883133µM/min/mg Pro) .In parallel, a strong induction of the CAT activity was noted specially for the highest dose.

Keywords: Cypermethrin; Paramecium tetraurelia; growth; oxidative stress; GST; GSH; CAT.

1. INTRODUCTION

The long-term ecological hazard associated with the use of organochlorine, organophosphate and carbamate compounds propelled the introduction of new generation of pesticides with a lesser degree of persistence. As a consequence, the use of pyrethroids as insecticidal and anti-parasitic formulations has markedly increased as a viable substitute and currently accounts for over 30% of insecticides used globally [1]

Indeed, synthetic pyrethroids are synthetic chemical analogs and derivatives of pyrethrins, they represent the third largest class of chemical insecticides after organophosphates and chloronicotinyl insecticides [2]. The pyrethroids have been divided into two types (type I and type II) on the basis of their chemical structure and toxic manifestation. In fact, type I pyrethroids are those which lack α-cyano moiety and give rise to the tremor syndrome (T syndrome) while type II pyrethroids are those which contain α cyano moiety and cause choreoathetosis/ salivation (CS)syndrome [3]. Their general site of action is biological membranes by alteration of sodium transport but they also affect chloride and calcium channels [4].

Several studies have indicated that this class of insecticides is highly toxic to a number of non-traget organisms such as: Bees, fish and aquatic invertebrates [5-14]. For instance, these pesticides have been found to induce alterations in the hematological profiles of *Channa punctatus* and *Prochilodus lineatus* [15-16], reproduction and physiology of *Cyprinus carpio* [17] and *Atlantic salmon* [18]. Furthermore. [19] demonstrated that type II pyrethroids could increase SOD activity in zebrafish larvae after 8h exposure, which suggest that oxidative stress could be induced and played an important role in developmental toxicity in fish.

Cypermethrin (CYP), the alpha-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl)-cyclopropane-carboxylic acid is the most widely used type II pyrethroid insecticide, it is commonly used in urban and agricultural environments [20].

Cypermethrin is very highly toxic to fish and aquatic invertebrates. Many studies reported that this compound is metabolized and eliminated significantly more slowly by aquatic organisms than by mammals or birds [21].

The environmental contaminants affect aquatic ecosystems by inducing oxidative damage as a sensitive and specific biomarker and causing cell death, via the enhancement of intracellular reactive oxygen species (ROS) and perturbation of antioxidant efficiency [22].

Cypermethrin produces drastic effects on both invertebrates [23] and vertebrates [24,25] reported its potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish *Danio rerio*. As well, [20] indicated the behavioral morphological deformities and the induction of biomarkers of oxidative damage due to sublethal concentration of cypermethrin on tadpoles of *D. melanostictus*. Also, experiments conducted with *Ceriodaphnia dubia* showed the increase of the toxic effect with increasing concentrations and exposure time [26]. Moreover, *Brachionus calyciflorus* and *Thamnocephalus platyurus* have, also, demonstrated high sensitivity to permethrin, resmethrin and cypermethrin [27].

The use of *Paramecium* species as a model of survey has been reported by several authors in some disciplines; in genetic, because its sequencing genome is well known, researchers used *Paramecium tetraurelia* for genetic analyses, gene expression and mutation [28,29]. In physiology, paramecia is used in general for studying the role, the function and the cell organization [30]. In ecotoxicology: *Paramecium* species were used to study environmental qualities and toxic effects of industrial, domestic and agricultural chemicals [31-38]. Further, the unicellular ciliate facilitates the study of physiological process and cytotoxicity of pollutants, that's why, they are well suited to being included in the increasing panel of organismic systems that could meet the 3Rs (aimed at Reducing, Refining and Replacing tests on vertebrate organisms in toxicological studies) and sensitive to such environmental compounds. This sensitivity is due to their simple eukaryotic single-cell and organism organization which exposes their receptors to external environment, making them respond to environmental stimuli [39]. Moreover, their easy culture and maintenance and their short cell-cycle provide results in a short time. For all these reasons, ciliates, especially *Paramecium* species, have been exploited as excellent tools for environmental biomonitoring, either as bioindicators of pollution or bioassays to evaluate the effect of toxic compounds [40-43].

Thus, the present work was carried to investigate the cytotoxicity of cypermethrin at different sub lethal concentrations on population growth and some biomarkers of oxidative stress of the ciliated protozoan *Paramecium tetraurelia.*

2. MATERIALS AND METHODS

2.1 Test Organisms

The biological model used in our study is a unicellular microorganism *Paramecium tetraurelia*.

2.2. Test Chemical

The insecticide used for our experiments is cypermethrin (Fig. 1) that belongs to the chemical family of pyrethroids type II.

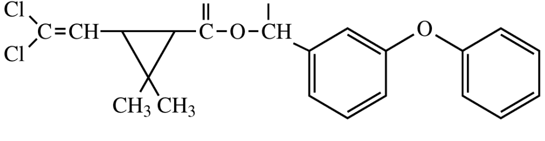

Fig. 1. Chemical structure of cypermethrin

2.3 Treatment

The habitual culture of *Paramecium tetraurelia* was done in the culture medium described by [35] at Ph 6.5 and 28±2℃.
Cells were transplanted each three days for keeping the youthful state of culture [35,36,38].

Paramecium tetraurelia were incubated with the tested insecticide concentrations in aliquots of 10ml, the retained concentrations were 0.05, 0.5, 1 and 2µg/l.

Two modes of treatment have been adopted: For growth kinetics, the insecticide treatment was performed before the transplantation of Paramecium cells (at t=0). For the enzymatic assays, the treatment was carried at the end of the exponential growth phase (t=96H) [44].

2.4 Parameter Measurement

2.4.1 Growth kinetic

For growth experiments, the culture was done at 28℃ in test tube using 10ml of culture medium. For each tube we added 13 cells of paramecia. The growth kinetic study was realized by the daily cell counting after fixation with lugol under optic microscope type LEICA DM 1000.

Based on the data, the number, the time and the velocity of generation were calculated by the following formula:

$$N = (\log Nt - \log N0)/ \log 2$$

$$k = n/t$$

$$g = 1/k$$

Where n is the number of generation, Nt is the population in time t, N0 is the initial number of cells, k is velocity of generation and g is the generation time.

2.4.2 Response percentage

The response percentage was calculated to evaluate the toxicity of xenobiotics via the inhibition of cell growth after 96H of exposure.

Positive values indicate an inhibition of growth while negative values indicate a stimulation of growth [45].

The assessment of the response percentage is calculated according to the following formula:

$$\text{Response (\%)} = \frac{(Nc-Ne)}{Nc} \times 100$$

Where Nc is the number control cells, Ne is the number of treated cells.

2.4.3 Total protein estimation

Total protein is determined by the method of [46]. It is a colorimetric method using BSA as standard. The absorbance is measured at a wavelength of 595nm using spectrophotometer type Jenway 3600.

2.4.4 Estimation of Glutathione (GSH) content

GSH content was quantified using the method of [47]. Cells are mixed in 1ml EDTA (0,02M). 0,2ml of ASS was added to 0,8ml of homogenate. After agitation, the homogenate was centrifuged. The assay mixture contains 1ml tris/EDTA buffer (0.02M, pH 9,6), 0.025ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and the paramecium sample. The reaction was monitored at 412nm and the amount of GSH was expressed as µmol/mg of proteins.

2.4.5 Determination of Glutathione S-transferase (GST) activity

The GST activity was measured according to the method of [48]. The homogenization of samples was done in 1ml of phosphate buffer (0,1M, pH 6) and centrifuged (14000rpm/30min). The final reaction contain 1,2ml CDNB (1mM)/GSH (5mM) and the sample. The absorbance was measured spectrophotometrically at 340nm. The result was expressed as µmol/min/mg of proteins.

2.4.6 Determination of Catalase (CAT) activity

The CAT activity was determined spectrophotometrically at 240nm by calculating the rate of degradation of H_2O_2 [49]. Samples are mixed in 1ml of phosphate buffer then centrifuged at 15000g. At 0,025ml of supernatant we added 0,75ml of phosphate buffer and H_2O_2. The result was expressed as µmol/min/mg of proteins.

2.5 Statistical Analysis

The obtained results are represented by the average ± Standard Error. Statistical analysis of data is performed using Minitab student t-test.

3. RESULTS

3.1 Effect of Cypermethrin on Growth Kinetic

The growth kinetic provides information about the toxic effect of a specific substance. Fig. 2 represents the effect of cypermethrin on the variation of paramecium cells number (control and treated) versus time.

Different chosen concentrations inhibited the population growth in a dose-dependent manner especially for the highest concentration that inhibits strongly after 4 days of treatment. Indeed, we denote a difference of nearly 1,900 cells between the control cells and those treated with this concentration (2µg/l) at the fourth day of treatment.

The proliferation of *Paramecium tetraurelia* was significantly affected by the action of cypermethrin as the generation number and concentration of cypermethrin are inversely proportional (Table 1). The generation time gradually increased with the increase of Cypermethrin concentrations.

The decrease in the velocity of generation compared to the control shows the negative response of *Paramecium tetraurelia* to the increasing concentrations of cypermethrin (Fig. 3). Result revealed that insecticide has slowed the generation velocity of exposed paramecia in a dose dependent manner.

3.2 Response Percentage

The results obtained concerning the response percentage confirm those of kinetics growth.

We denote that the inhibitory effect was dose-dependent and proportional to the increasing concentrations (Fig. 4).

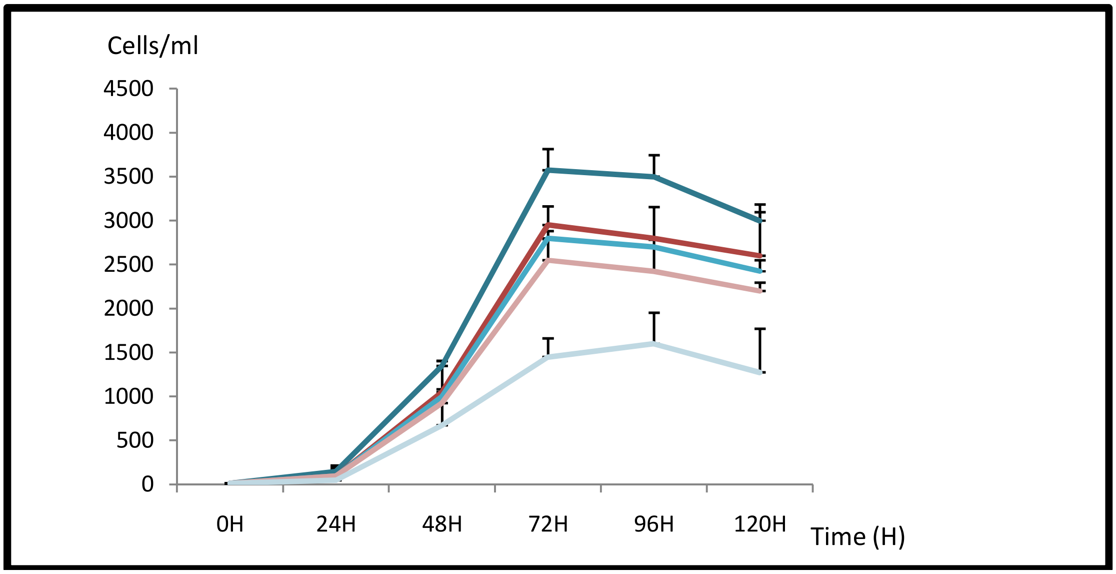

Fig. 2. Effect of cypermethrin on *Paramecium tetraurelia* growth (T 28ºC pH: 6.5)

Thus, the response percentage was positive and show a strong inhibition of microorganisms growth. In fact, it varies from (20%) to (54%) for 0.5µg/l and 2µg/l in which more than half of population is inhibited.

3.3 Protein Estimation

According to the table 2, we note a dose dependent increase of total protein rate in the presence of xenobiotic. The statistical analysis indicates very highly significant differences (*P*<0.001) for the two highest concentrations (1 and 2µg/l).

Table 1. Effect of cypermethrin on *Paramecium tetraurelia* generation number (n) and generation time (g) at 96H

Cypermethrin concentrations (µg/l	Generation number (n)±SE	Generation time (g)±SE
Control	8.07±0.10	11.90±0.15
0.05	7.75±0.04	12.40±0.06
0.5	7.70±0.04	12.47±0.07
1	7.54±0.16	12.74±0.28
2	6.90±0.20	13.85±0.41

Each value is mean of four assays ± Stander Error

Table 2. Effect of cypermethrin on the rate of total proteins in *Paramecium tetraurelia* versus time

Cypermethrin concentrations µg/l	Rate of total proteins µM/mg of tissues±SE
Control	0.283±0.011
0.05	0.294±0.009
0.5	0.339±0.009
1	0.620±0.022 ***p
2	0.772±0.019 ***p

****P =0.001*

3.4 Estimation of Glutathione (GSH) Content

Fig. 5 illustrates the variations of total GSH content in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin.

The result shows that this nonenzymatic antioxidant tends to decrease in dose dependent manner. Thus, the GSH rate is (7.34188758µmol/mg Pro) for the control whereas it is (2.41682134µmol/mg Pro) for paramecia treated with the highest concentration (2µg/l) ie three times less.

The statistical analysis reveals a significant difference (*P*<0.050) for the highest concentration compared to the control.

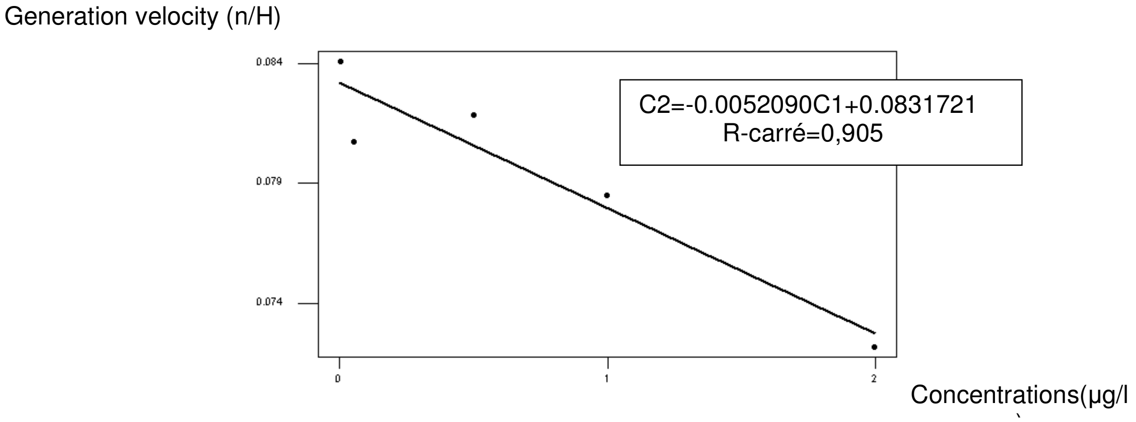

Fig. 3. Effect of cypermethrin on the velocity of generation of *Paramecium tetraurelia* (t=96H)

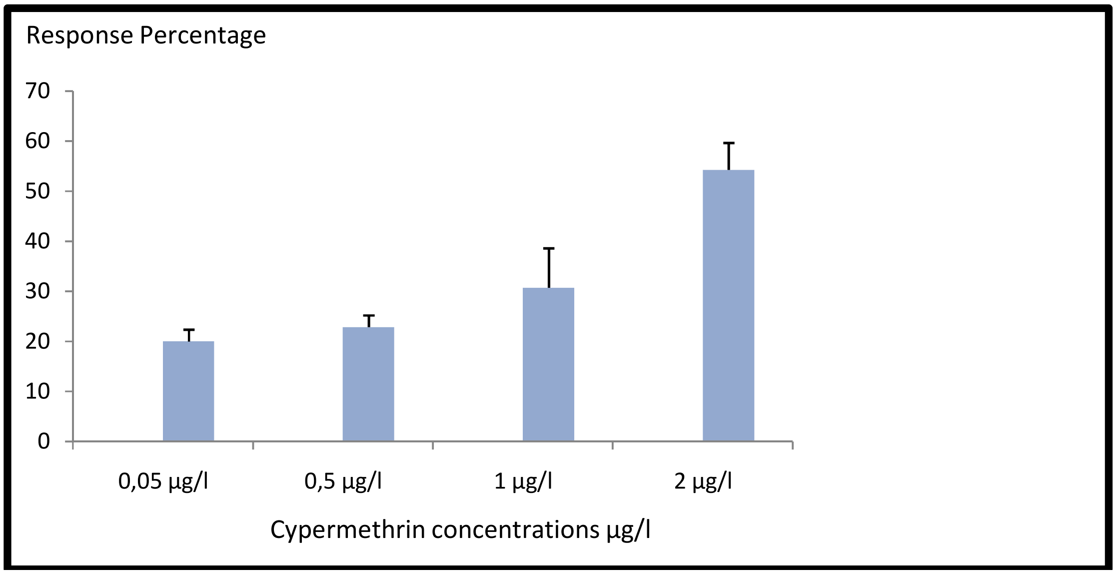

Fig. 4. Effect of cypermethrin on the response percentage at 96H (n= 4)

■ *Response percentage*

3.5 Determination of Glutathione S-Transferase (GST) Activity

The results concerning the variation rate of GST is represented in Fig. 6, it reveals a strong induction of the activity of this enzyme particularly for the highest concentration. Indeed, this induction is in dose-dependent manner: We note an increase of the order of (1.62932472µM/min/mg Pro) for the highest concentration compared to the control which is of the order of (0.59883133µM/min/mg Pro), that is to say, three times higher.

The statistical study show a significant difference ($P<0.050$) for the highest dose compared to the control.

3.6 Determination of Catalase (CAT) Activity

The effect of cypermethrin on CAT activity is illustrated in Fig. 7. The results show a significant increase for the second concentration (0.5µg/l) and a very highly significant increase ($P<0.001$) of the activity of CAT in cells treated with the highest concentration (2µg/l) compared with the control.

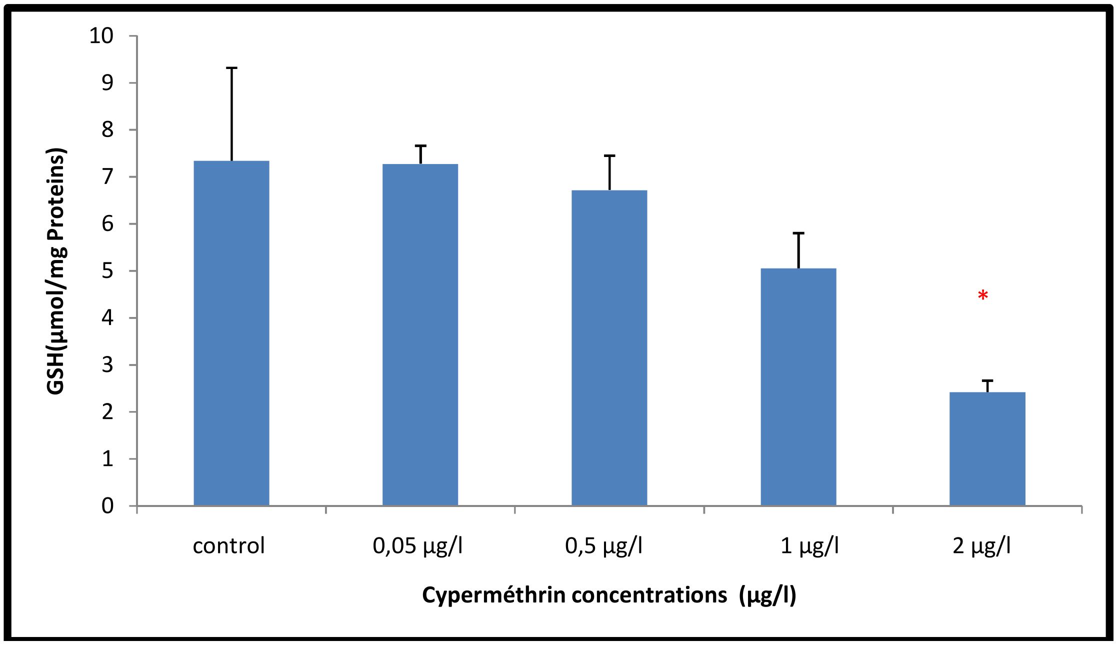

Fig. 5. Variations of GSH content in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin

🟦 *GSH* ** P<0.05*

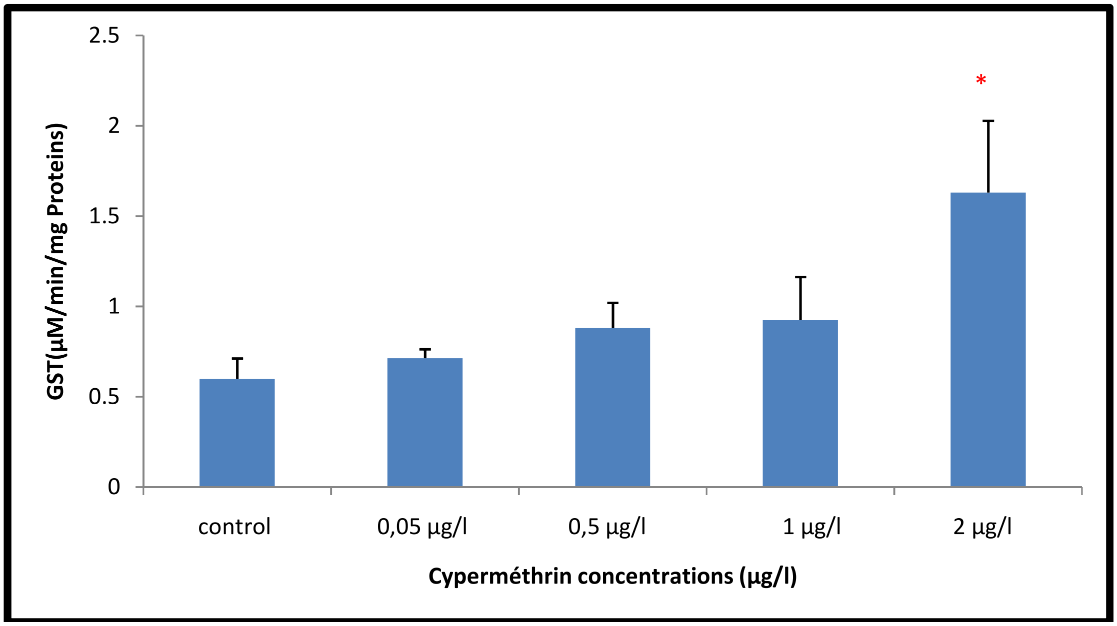

Fig. 6. Variations of GST activity in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin

🟦 *GST* ** P<0.05*

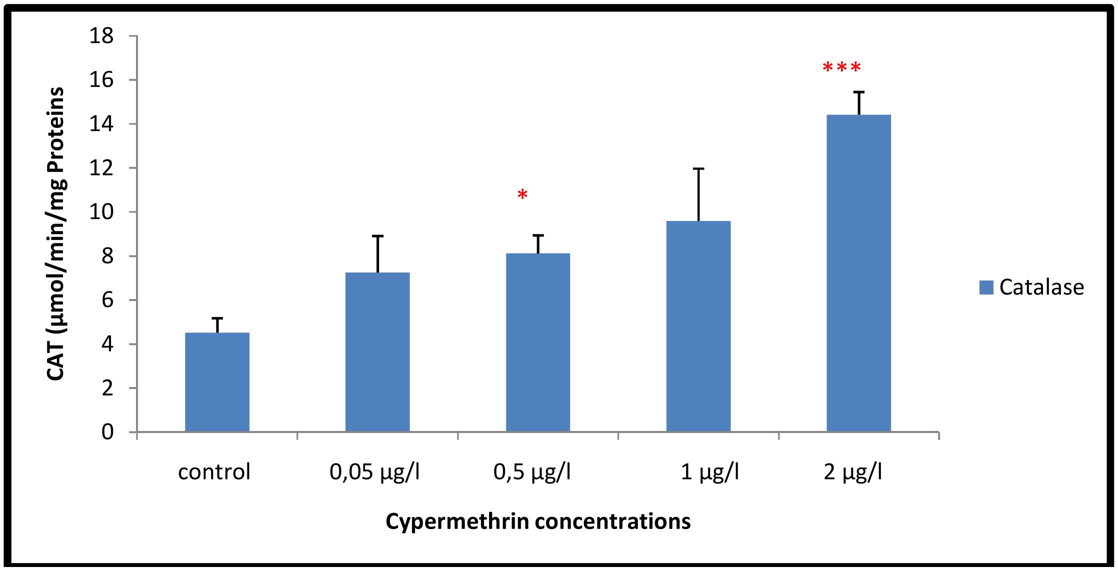

Fig. 7. Variations of CAT activity in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin

🟦 *CAT * P<0.05 *** P<0.001*

4. DISCUSSION

The problem of environmental contamination by the excessive use of pesticides cannot be neglected [50]. Extensive application is usually companied with serious problems and health hazard. It is established that many chemicals, in common use, can produce some toxic effects on biological systems when tested on various type of experimental models through their mode of action or by production of free radicals that damage all cell compounds [51]. In fact, these chemicals act as pro-oxidants [52-58].

Oxidative stress is believed to occur when there is an imbalance in the biological oxidant-to-antioxidant ratio; this can result in oxidative damage to lipid, proteins, carbohydrates and nucleic acids. In most cases, the abnormal generation of ROS, which can result in significant damage to cell structure, is considered an important signal of oxidative damage [59].

Pyrethroids group of pesticides is the most commonly used in agriculture today [2]. However, it have been reported by several investigations conducted in various animal species that this pesticides cause oxidative damage [60-62] through the generation of ROS and can alter the antioxidants or free oxygen radical scavenging enzymes systems in animals, especially, in aquatic organisms [63].

Cypermethrin, is probably the most used pyrethroid. Studies showed that the excessive use can adversely affect most physiological processes [64].

Protists are eukaryotic unicellular organisms and their position in the food web makes them excellent models for predicting the effects of chemicals on aquatic communities. Ciliated protozoa represent a basic component of aquatic environment, where they play critical roles both quantitatively and qualitatively [65].

In this context, the ciliate assay has become a valuable tool for detection of environmental disturbance and for assessment of the trophic state [65-66].

Paramecium is one of the most commonly used ciliated for laboratory research to investigate the direct toxicity of compounds [32,35,67-69].

That is why we chose *Paramecium tetraurelia* as a biological model for elucidating cypermethrin toxicity.

In this study, we were interested in the first time at the effect of cypermethrin on population growth. Our result showed an inhibition in the growth of microorganisms especially for the highest concentrations. Similar results were reported in studies [35,36,70] that investigate the effect of different chemicals on the physiology and morphology of *Paramecium* sp.. [31] Reports that toxics may affect the survival of protozoa in a variety of ways, as the concentration of toxicants in the cell membrane increase and destroy their integrity causing cell death. Toxic affects freshwater ciliates; these effects are perceptible at the population level by reducing the number of cells and on the cellular level by a structural behavioral and physiological damage.

The findings concerning growth kinetics were confirmed, on the one hand, by the decrease in generation number, the decrease in velocity as well the increase in generation time which mean that the proliferation and cellular metabolism were affected [33,71-74]. On the other hand, the positive value of response percentage demonstrated the inhibitory effect of cypermethrin. Indeed, cypermethrin as a lipophilic compound can penetrate into cell, disturbing phospholipid orientation and causing changes in fluidity of membrane [75].

Proteins are one of the major energy reserves present in all organisms, these reserves will be affected by toxicant exposure [20]. In this work, we noted an increase of total protein rate in a dose dependent manner and very highly significant for 1µg/l and 2µg/l. This finding is in agreement with those of [76,77] who showed an increase in the rate of total proteins of paramecia treated with increasing concentrations of Bifenazole and Proclaim.

Our hypothesis is that this increase could be related to the induction of the detoxification process elaborated by this control system which is composed of enzymes, proteins and antioxidant molecules [78].

The antioxidant defense systems are present in all aerobic cells and neutralize the intermediate chemical reactions produced endogenously and/or metabolism of xenobiotics. The antioxidant system activity may undergo an increase or depletion under the effect of a chemical stress [79]. The cells are equipped with both the enzymatic and nonenzymatic antioxidants for combating oxidative stress, which may be either due to increased production of free radical or impaired antioxidant defense or both [80].

Alteration of antioxidant enzymes by cypermethrin has also been reported to be one of the mechanisms of toxicity.

The Glutathione is the major non enzymatic radical scavenger in the animal cells; it is the most abundant thiol, which scavenges residual free radicals resulting from oxidative metabolism and escaping decomposition by the antioxidant enzymes [81]. During the metabolic action of GSH, its sulfhydryl group becomes oxidized resulting with the formation of the corresponding disulfide compound, GSSG (oxidized form) [2,82]. In this work, we noted a significant depletion in a concentration and dose dependent manner. The decrease in total GSH level may be due to the presence of free radicals produced by the insecticide [2,44,77]. In addition, GSH also participates in the detoxification of xenobiotics as a substrate for the enzymes GST and GPX (glutathione peroxidases), so, it plays a crucial key role in cellular defense against pesticides toxicity [83].

The GST plays an important physiological role in the protection of cells against toxics and in the initiation of detoxifying against potential agent Alkylation [84,85]. It is enzyme of biotransformation that catalyzes the conjugation of electrophilic substrates to the thiol group of GSH, producing less toxic forms and also lipid peroxides [86,87]. In our study, the increase in GST activity was in dose dependent manner and significant for the highest concentration (2µg/l). The induction of GST activity may be beneficial to handle a stress condition and indicates protection against cypermethrin. [20] Reported an increase in the GST activity by cypermethrin in tadpoles of *D. melanostictus*. [88] Suggested that increase in GST activity is involved in metabolic detoxification of butachlor in *Rhamdia quelen*. Also, [89] reported that this enzyme seems to be implicated in the detoxification of cypermethrin in amphibian larvae.

Catalase is the most important mechanisms against toxic effects of oxygen metabolism. It catalyzed the conversion of hydrogen peroxide into water. This antioxidant enzyme can, therefore, alleviate the toxic effect of ROS [87]. Present study clearly showed a dose-frequency-dependent increase in catalase activity in individuals treated by different concentrations probably due to the intensification of antioxidant activity in Paramecium. CAT is one of the most active enzymes and its level change first following induction of oxidative stress [80]. The present result is consistent with those of [53,54,72] who reported intensification in the Catalase activity in many animal models when treated by pesticides. These results indicate the activation of protective mechanisms necessary for the scavenging of the produced reactive oxygen radicals.

Cytochrome P450 monooxygenases heme-thiolate enzymes catalyzing various reactions, but are best known for their monooxygenase activity, inducing reactive or polar groups into xenobiotics or endogenous compounds [90]. Insect genomes revealed a large expansion of the P450 gene family. Elevated level of P450 activity has frequently been observed in pyrethroid resistant insects populations [91]. Several CYP genes were also linked to pyrethroid resistance [92,93]. These findings validates many P450 as pyrethroid metabolizers [94-97]. The living cell, *Paramecium tetraurelia*, contains a large number of gene families that are involved in processes associated with sensing and responding to environmental cues, such as: P450. Our hypothesis is that CYP450 enzymes are also implicated in the detoxification process of pyrethroids in Paramecium but this requires extensive studies to accurately determine this implication and its mechanisms.

Type II pyrethroids seems be toxic to *Paramecium tetraurelia*, an organism that does not poses a voltage sensitive sodium channel. Likewise, it is established that type II pyrethroids stimulated *Paramecium tetraurelia* back-swimming behavioral, an avoidance behavioral response that is controlled exclusively by Ca++ uptake via voltage sensitive calcium channels associated with the cilia: [98] have characterized the action of pyrethroids on ciliary calcium channel in *Paramecium tetraurelia*. The study was conducted with deltamethrin, the results revealed that the toxic effect of deltamethrin is structurally related, dose dependent and enhanced by depolarization and provide substantial evidence that type II pyrethroids act as potent calcium channel agonists on the ciliary voltage sensitive channel of *Paramecium tetraurelia.* Furthermore, the effect could be due to the Ca++ accumulation in the cell which leads to free radical mediated cell damage [97].

5. CONCLUSION

In summary, under the current experimental conditions, cypermethrin is toxic to the freshwater ciliate *Paramecium tetraurelia*. Exposure to low concentrations of cypermethrin showed significant adverse on growth accompanied with the induction of oxidative damage supported by the decrease in GSH content and the intensification of the antioxidant enzymes such as GST and CAT. It showed be mentioned that other biomarkers of oxidative stress and lipid peroxidation have to be measured, it is the same for the detection and estimation of ROS. A genotoxic study may provide more answers concerning the effects of cypermethrin on *Paramecium tetraurelia.*

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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