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Lipid peroxidation, oxidative stress and respiratory metabolism alteration in the freshwater ciliate *Paramecium tetraurelia* exposed to cypermethrin, a pyrethroid insecticide

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Abstract

Oxidative damage by increased production of reactive oxygen species have been involved in the toxicity of several pesticides. Thus, the aim of this study was to investigate the effect of cypermethrin, a widely used type II pyrethroid, on the oxidative stress biomarkers and the respiratory metabolism of *Paramecium tetraurelia*. Different concentrations of the insecticide (0.05, 0.5, 1 and 2 µg/l) were incubated with *Paramecium* cells. The 96h (IC50) was determined. Variations in lipid content and oxidative stress biomarkers such as: Malondialdehyde (MDA), Glutathione (GSH), Glutathione peroxidase (GPx) and Lactate dehydrogenase (LDH) were carried. Moreover, respiratory metabolism was followed up. The estimated 96h (IC50) value for *Paramecium tetraurelia* exposed to cypermethrin in our study was 1.26 µg/l. Significant decrease was observed in total lipids content. Cypermethrin exposure has led to a lipid peroxidation supported by a significant increase in (MDA) level which might be associated with decreased level of (GSH). (GPx) and (LDH) activities, antioxidant enzymes, were significantly induced. The response was concentration dependent especially for the highest concentration. A strong disturbance in respiratory metabolism was observed. In summary, under the current experimental conditions, lipid peroxidation, oxidative stress and alteration in respiratory metabolism are involved in the toxicity of cypermethrin to the ciliate *Paramecium tetraurelia*. Likewise, due to its susceptibility, *Paramecium* could be used as an ideal model for studying toxicity of environmental contaminants.

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Introduction

In recent decades many new broad-spectrum pesticides have been developed, such as synthetic pyrethroids that have emerged as a major agricultural pesticide in both developing and developed countries owing to their superior insecticidal activity and broad insecticidal range. Unfortunately, the repetitive and indiscriminate use of these insecticides resulted in unintended exposure to animals and humans, which increased the risk of intoxication in non-target organisms (Xiangguo *et al.,* 2011). Pyrethroids have been subdivided into two classes according to their structural, and toxicological differences, type I pyrethroids do not contain a cyano group, while type II pyrethroids contain the alpha-cyano group. Both non-cyano-substituted and cyano substituted pyrethroids shows insecticidal action and low toxicity to Mammals. Their modes of action are apparently worked by keeping open the sodium channels in neuronal membranes but also affect chloride and calcium channels (Steven , 2011).

Cypermethrin is a commonly used insecticide in urban and agricultural environment. As a result of its popularity, this chemical is one of the most common contaminants in the freshwater aquatic system (Carriquiriborde *et al.,* 2007).

Aquatic chemical exposure may be responsible for the induction of oxidative stress in aquatic organisms if the chemical interfere with reactive oxygen species (ROS) production (Jin *et al.,* 2010a). During quite rapid metabolism of synthetic pyrethroids, reactive oxygen species (ROS) are generated. Studies describing the oxidative stress mechanisms in pyrethroids induced toxicity in aquatic organisms are increasing. Many reports have demonstrated the induction of oxidative stress by pyrethroids such as Cyfluthrin, deltamethrin and cypermethrin in different aquatic species: *Cyprinus carpio*, *Danio rerio*, *Penaeus monodon* (Huynh Thi *et al.,* 2012; Sepici-Dinçel *et al.,* 2009; Wenquing *et al.* 2014).

This stress can be monitored by the level of lipid peroxydation (LPO) through the measurement of MDA level and the measurement of antioxidant defenses such as Glutathione (GSH), Glutathione peroxydases (GPx), Catalase (CAT) and respiratory metabolism: Biomarkers are “early-warning” signals reflecting the adverse biological responses toward environmental contaminants that are commonly employed in environmental quality and/or risk assessment (Van de Oost *et al.,* 2003).

Paramecium is useful experimental model that have been widely used to evaluate the health of aquatic ecosystems, due to its easy culture and maintenance in laboratory and for studying possible damages to pollutants in toxicological studies. Further, the unicellular ciliates are very 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 (Madoni *et al.,* 2006).

Cypermethrin has been used indiscriminately in large amounts, and has also been largely involved in progressive pollution for aquatic biota. Therefore, the present study was carried out to investigate the cytotoxicity of this contaminant at different concentrations on lipid peroxydation, antioxidant biomarkers and respiratory metabolism in the freshwater ciliate *Paramecium tetraurelia*.

Materials and methods

Test organisms

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

Test chemical

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

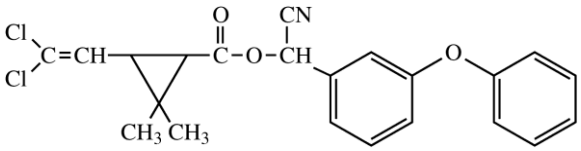

Fig. 1. Chemical structure of Cypermethrin.

Treatment

The habitual culture of *Paramecium tetraurelia* was done in the culture medium described by (Azzouz *et al.*, 2011) at pH 6.5 and 28 ± 2 ° C.

Cells were transplanted each three days for keeping the youthful state of culture (Azzouz *et al.*, 2011; Benbouzid *et al.*, 2012).

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

The treatment was carried at the end of the exponential growth phase (T = 96 h). Assays were done 3 hours after treatment (Azzouz, 2012).

Determination of IC50

The determination of the IC50 (96 h) values was carried out by the kinetic growth method using the linear regression analysis.

Evaluation of total lipids, lipid peroxidation and oxidative stress biomarkers

Lipid estimation

Lipids are determined by the method described by (Goldsworthy *et al.,* 1972) that use the vanillin as reagent and a stock solution of 2.5 mg lipid / ml as standard; the absorbance is measured at a wavelength of 530 nm using spectrophotometer (Jenway 3600).

Lipid peroxidation: (Determination of MDA levels)

According to the method of (Draper and Hadley, 1990), the extent of lipid peroxidation in terms of malondialdehyde (MDA) formation was measured. The samples were ground with TrisHCl (50 mM, pH 7.5), after homogenization, samples were centrifuged. 500 µl of supernatant was added to 2.5 ml of TCA and heated at 100 ° C for 1 h. After cooling, the precipitate was removed by centrifugation. 2 ml of supernatant was added at 1 ml of TBA. After a second heating and cooling, 1.5 ml of Butanol was added. The absorbance of the sample was measured at 532 nm using a blank containing all the reagents except the sample. The result was expressed in µmol/mg proteins

Glutathione (GSH) content

GSH content was determined using the method of (Wechbeker and Cory, 1988). Cells are mixed in 1 ml EDTA (0.02 M). The homogenate was centrifuged. The assay mixture contains 1 ml tris/EDTA buffer (0.02 M, pH 9.6), 0.025 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and the *Paramecium* sample. The absorbance was measured at 412 nm and the amount of GSH was expressed as µmol/mg of proteins.

Glutathione peroxidase (GPx)

The GPx activity was determined spectrophotometrically at 412 nm (Flohe and Gunzel, 1984). 0.4 ml of GSH (0.1 mM) and 0.2 ml of TBS buffer (Tris 50 mM, NaCl 150 mM, pH 7.4) were added to 0.2 ml of homogenate and incubated at 25 ° C for 5 min. To initiate the reaction, 0.2 ml H_2O_2 (1.3 mM) was added. After cooling, the mixture was centrifuged. The final reaction contained 2.2 ml TBS solution, 0.32 ml DTNB (1 mM) and the sample. The result was expressed in µmol GSH/min/mg proteins.

Lactate dehydrogenase (LDH) activity

The LDH activity was measured according to the method of (Hill and Levi, 1954). The final reaction contain 650 µl of substrate buffer (0.2 M, pH 10), 50 µl Co-enzyme NAD and 50 µl of the sample. The absorbance was measured spectrophotometrically at 340 nm for 5 min. The result was expressed as µmol/ min/ mg of proteins.

Polarographic study

Respiratory activity of *Paramecium tetraurelia* is measured using an oxygen electrode type

(HANSATECH), for measuring the production or consumption of oxygen by cells. Its sensitivity permits the detection of concentrations of O_2 under µM (Djebar et Djebar, 2000).

Statistical analysis

The obtained results are represented by the average ± Standard Error. Statistical analysis of data is performed by the Student t test (Dagnelie, 1999).

Results

Determination of IC50

The IC50 was calculated from the linear equation of y = 16.32 × - 17.20 (Fig. 2). The estimated 96h IC50 value for *Paramecium tetraurelia* exposed to cypermethrin was 1.26 µg/l.

Determination of IC50

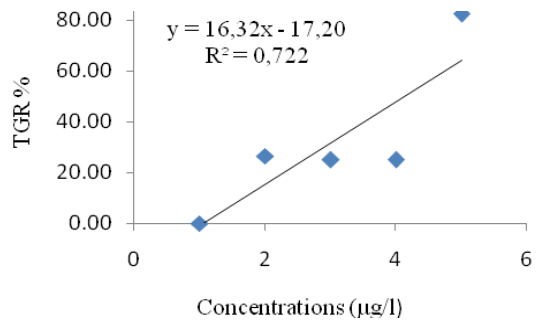

Fig. 2. Effect of cypermethrin treatment on *Paramecium tetraurelia* population (T = 28 °C, pH = 6.5). Determination of IC50 (96h) by the kinetic growth method.

The IC50 was calculated from the linear equation of y = 16.32 × - 17.20 (Fig. 2). The estimated 96h IC50 value for *Paramecium tetraurelia* exposed to cypermethrin was 1.26 µg/l.

Evaluation of total lipids, lipid peroxidation and oxidative stress biomarkers

Lipid estimation

Fig. 3 represents the effect of cypermethrin treatment on the rate of total lipids in *Paramecium tetraurelia*. We observed an important dose-dependent decrease in the total lipids content in the presence of all insecticide concentrations.

The statistical analysis indicated a highly significant difference ($p \leq 0.01$) for the two highest concentrations (1 and 2 µg/l) compared to the control.

Evaluation of total lipids, lipid peroxidation and oxidative stress biomarkers

Lipid estimation

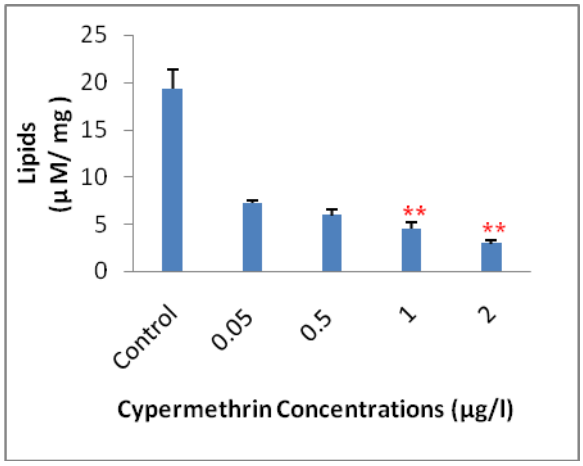

Fig. 3. Variations of Lipids content in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin.

Lipid peroxidation and oxidative stress biomarkers

MDA level in *Paramecium tetraurelia* was found to be increased significantly at different treatment concentrations when compared with the control in a dose-dependent manner as shown in table 1. The maximum increase (249.29%) was observed at the highest concentration (2 µg/l). The statistical analysis revealed a significant difference ($P \leq 0.05$) for the second concentration (0.5 µg/l) and a highly significant difference ($p \leq 0.01$) for the highest concentration (2 µg/l) compared to the control.

The results concerning the variations of total GSH content in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin are represented in table 1. The treatment of cypermethrin caused an important dose-dependent decrease (99.12% - 44.23%) compared to the control. Indeed,

the GSH rate is found to be three times more in the control compared to the highest concentration (2 µg/l). The statistical analysis revealed a significant difference ($p \leq 0.05$) for the highest concentration.

Lipid peroxidation and oxidative stress biomarkers

Table 1. Variations in MDA levels, GSH content, GPx and LDH activities in *Paramecium tetraurelia* exposed to increasing concentrations of cypermethrin for 3h.

Parameters	**Cypermethrin Concentrations (µg/l)**				
	Control	**0.05**	**0.5**	**1**	**2**
MDA	0.393±0.02	0.460±14	0.630±0.07	0.965±0.14	1.058±0.34
(µmol/mg Proteins)	ns	*	ns	**	
GSH	7.341±1.97	7.277±0.38	6.718±0.72	5.053±0.74	3.247±0.23
(µmol/mg Proteins)	ns	ns	ns	*	
GPx	6.875±0.20	8.385±0.18	8.789±0.50	13.707±0.49	18.666±0.37
(µmol GSH/min/mg Proteins)	*	*	**	**	
LDH	10.010±0.77	12.946±1.32	16.373±0.66	18.658±0.66	20.942±0.66
(µmol/min/mg Proteins)	*	**	***	**	

Values are the mean of three essays per treatment with the corresponding standard error .ns, *, **, *** indicate, respectively, statically nonsignificant differences ($p > 0.005$), statically significant differences ($p \leq 0.05$), statically high significant differences ($p \leq 0.01$) and statically very high significant differences ($p \leq 0.001$) relative to the control.

GPx activity was significantly increased in cells exposed to the insecticide in a dose-dependent manner (table 1). This increase is in order of 121.96% and 271.50% for the concentrations 0.05 µg/l and 2 µg/l, respectively. Statistical study showed a significant decrease ($P \leq 0.05$) for the two lowest concentrations (0.05 and 0.5 µg/l) while it is highly significant ($P \leq 0.01$) for the two highest concentrations (1and 2 µg/l).

LDH activity was found to be induced strongly in comparison to control at all exposure concentrations (table 1), with inductions of 129.33%, 163.56%, 186.39% and 209.21% for 0.05, 0.5, 1 and 2 µg/l, respectively. Statistical study revealed a very highly significant difference ($P \leq 0.001$) and highly significant difference ($P \leq 0.01$) for 1 µg/l and 2 µg/l, respectively.

Effect of cypermethrin treatment on the respiratory metabolism of Paramecium tetraurelia

Data concerning the effect of cypermethrin on the respiratory metabolism of *Paramecium* cells are represented in Fig. 4.

Effect of cypermethrin treatment on the respiratory metabolism of Paramecium tetraurelia

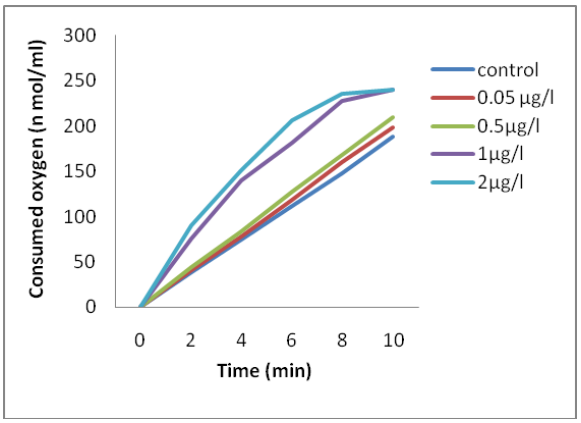

Fig. 4. Effect of cypermethrin treatment on the respiratory metabolism of *Paramecium tetraurelia*.

Results showed that the oxygen consumption in control cells evaluated in a regular and continuous manner versus the time. This consumption increased in a dose-dependent manner at different cypermethrin treatment. The amount of consumed O_2 increased significantly, especially, at the two highest concentrations (1 and 2 µg/l) in comparison to control. Indeed, we noted that the difference in the amount of consumed O_2 at (time = 4 min) was 66 and 77 nmol/ml for 1 and 2 µg/l, respectively, compared to the control. It rose to 69 and 94 nmol/ml at (time = 6 min) for the same concentrations. At (time = 8 min), this difference is between 80 - 88 nmol/ml.

Discussion

Due to the extensive and frequent use of pyrethroids, their residues have often been detected in aquatic ecosystem. Their adverse ecological effects have become an important issue because of their high toxicity to aquatic biota (Yang *et al.*, 2014).

Among the most commonly used biomarkers, those associated with oxidative stress are particularly important, since the mechanisms of toxicity for most pesticides, including pyrethroids, are the production of free radicals, induction of lipid peroxidation (LPO), and disturbance of the total antioxidant capability of the cell (Abele *et al.,* 2011).

In our study, we investigate the oxidative stress and respiratory metabolism disturbance caused by cypermethrin to the protozoan *Paramecium tetraurelia*.

Bioavailability of cypermethrin in the aquatic environment ranged from 0.0046 to 2.8 µg/l (Laabs *et al.,* 2002).The estimated 96h (CI50) value for *Paramecium* exposed to cypermethrin in our study was 1,26 µg/l. This value for *Paramecium tetraurelia* is in a similar range (µg/l) as the value previously reported for *Ceriodaphnia dubia* exposed to α-cypermethrin (about 0.23 µg/l) (Mei-Fang *et al.,* 2011).

The exposure into toxicants is involved in the cascade of events leading to cell toxicity, that arise from the excessive ROS production, which have been reported to attack several biological molecules including lipids, since lipids are one of the major energy reserves (Mohamed *et al.,* 2015). In this work, we noted a decrease of total lipids rate in a dose dependent manner. This finding is in agreement with those of (Sbartai, 2011) who showed a decrease in the rate of lipids of paramecia treated with increasing concentrations of pesticides (Bifenazate and Oxadiazine). The depletion in lipid content observed in this investigation is the result of a progressive lipid oxidation and it can be correlated to their catabolism to the total energy production. It might also be attributed to the impairment in lipid synthetic machinery.

A noticeable increase in lipid peroxydation biomarker MDA was observed in *Paramecium* cells treated with different concentrations of cypermethrin. These findings are in good agreement with those of (Xiangguo *et al.,* 2011) who recorded a significant increase in MDA level in embryo-larval stages of zebrafish intoxicated with different doses of cypermethrin. Also, (Oliveira *et al.*, 2012) reported the significant increase of MDA level in the common prawn *Palaemon serratus* intoxicated with deltamethrin, a type II pyrethroid. Our results showed that cypermethrin treatment may result in peroxidation of polyunsaturated fatty acids, leading to the degradation of phospholipids and ultimately result in cellular deterioration (Tappel, 1973). Moreover, Cypermethrin is a lipophilic compound that can penetrate into cells, disturbing phospholipid orientation and causing changes in the fluidity of the membrane.

In our study, the GSH level is found to be decreased in dose dependent manner especially for the highest concentrations (2µg/l). This decrease is may be due to its implication in the capture and sequestration of free radicals produced by insecticide (Nzengue, 2008). Moreover, GSH is used by GST and GPx as

substrate for the detoxification of xenobiotics, so, it plays a major role in the pesticides metabolization by organisms. Studies conducted on paramecia exposed to pesticides demonstrated the same result (Azzouz, 2012). Finally, the toxic effect of xenobiotics may depend on glutathione (GSH) content, in particular, previous studies have illustrated the possible ability of pesticides to induce depletion of GSH content, which may lead to lipid peroxidation and formation of ROS (Itziou *et al.*, 2011).

In our work, GPx activity was significantly increased at all chosen concentrations, suggesting an induction of detoxification. These findings are consistent with those of (Huynh Thi *et al.*, 2012) who reported the induction of GPx activity in the black tiger shrimp *Penaeus monodon* treated with deltamethrin. Also, (Mohamed *et al* ., 2015) stipulated that pyrethroids have the potential to induce antioxidant enzymes such as GPx.

LDH activity was significantly increased in *Paramecium* exposed to low concentrations of cypermethrin demonstrating the metabolic changes induced by the insecticide. This result has also been observed in prawns treated with deltamethrin. Since deltamethrin and cypermethrin belong to the same chemical class (α-cyano), it is likely have a similar molecular mechanism of toxicity. Thus, LDH activity is particularly important when a considerable amount of additional energy is rapidly required which mean that increasing in LDH activity may be due to the ability of paramecia to get additional energy for detoxification and antioxidant protection (Mouneyrac *et al.*, 2011; Diamantino, 2001).

The other interesting aspect of our study was the effect of cypermethrin on the respiratory metabolism of *Paramecium tetraurelia*. The amount of consumed O_2 increased, especially for the two highest concentrations (1 and 2 µg/l) in comparison to control indicating an induction of the respiratory metabolism. Free radicals generated by xenobiotics have the potential to disturb cellular respiratory through the disturbance of mitochondrial respiratory chain. Thus (Azzouz *et al.*, 2012; Benbouzid *et al.*, 2012) showed an inhibitory effect of glyphosate and phosphoramidate on cells. It is plausible to speculate that, in cypermethrin metabolization, cytochrome P450 system is triggered in order to its elimination. Furthermore, the free radicals generated by xenobiotics have the potential to disrupted cellular respiration via the disruption of the mitochondrial respiratory chain and the alteration of ATPases activities, these mechanisms result in a stimulator and/ or inhibitor effect of mitochondrial function by stimulation and / or inhibition of the synthesis or oxidation of ATP. Similarly, the recorded variations may reflect adaptation of organisms, in this case, paramecium, to maintain mitochondrial homeostasis (Paital, 2012)

In conclusion, our results showed that cypermethrin is toxic in concentration dependent manner to the freshwater ciliate *Paramecium tetraurelia*. Since it showed an adverse effect on survival, caused lipid peroxidation, oxidative stress and altered its respiratory metabolism. Furthermore, Comprehension of the mechanisms related to sublethal effects caused by chemicals upon paramecium cells would help to develop sensitive and precise diagnostic tools with a predictive capability in assessing toxic effect, thus contributing to better pond management.

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