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# Induction of oxidative stress in a freshwater ciliated microorganism *Paramecium sp.*, after treatment with Indoxacarb

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## ABSTRACT

Many years, protozoa are used as cellular models in toxicological studies and as bio-indicators of water pollution. They allow specifying the interaction between a test molecule and target cells. The main objective of our work was to study the effect of an insecticide: Indoxacarb on freshwater ciliated microorganism: *Paramecium sp.* We tested the effect of this xenobiotic at different concentrations (10, 20, 40 and 80µM) on aliquots of 50ml of culture of paramecium done beforehand. The results obtained showed that the growth of paramecium was sensitive at the high concentrations to the product. The toxicity was evaluated by determining the IC 50. The dosage of enzymes of phase II including glutathione S-transferase and antioxidants (catalase and ascorbate - peroxidase) showed fluctuations with time and concentration of the xenobiotic. The measurement of respiratory activity showed inhibition of oxygen consumption reflecting a deleterious effect of this insecticide.



## KEYWORDS

Indoxacarb;
Protist;
Cytotoxicity test;
Oxidative stress;
Respiratory metabolism.

## INTRODUCTION

Since the advent of synthetic pesticides, contamination of the environment has grown to the point where the Earth's surface, as a whole contains molecules of this nature without necessarily having been treated directly[1]. In recent years, it is becoming increasingly aware that pesticides do not act only against the target for which they have been approved but on the whole ecosystem[2]. The effects on biodiversity and including flora and fauna in terrestrial and aquatic are undeniable. Over 90% of synthetic insecticides are organophosphates, carbamates and pyrethroids with localized sites of action in the nervous system[3]. Among these products we have chosen an insecticide: indoxacarb. This is a new broad spectrum insecticide effective against insects[4,5] which shows a low toxicity to mammals and does not show resistance when it is crossed with carbamates, pyrethroids, spinosad, cyclodiene, benzoylureas or organo-phosphates[4]. This is the first insecticide blocking Na + channels of the class of oxadiazine which was marketed. Its selective toxicity against insects is due in part to the fact that this is a pro-insecticide, bio-activated in insects by esterases and amylases[6-8]. It is a modulator of nicotinic receptors of acetylcholine neurons in mammals[9,10], and its activity

is similar to that of pyrazolines.

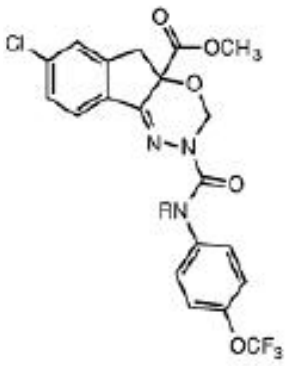
This study was undertaken to better characterize the mechanism of action of the insecticideIndoxacarb at different levels of organization of the cell allowing to specify the interactions between the xenobiotic and cellular targets through monitoring of respiratory metabolism and exactly on the biomarkers that are considered detection tools relevant pollution and thus constitute a new approach to assess the effects of environmental contamination on ecosystems and human health.

## MATERIALS AND METHODS

### Chemicals

### Indoxacarb

(S)–methyl 7-chloro-2,5-dihydro -2 – [[(methoxycarbonyl) [4-(trifluoromethoxy) phenyl) amino] carbonyl] indeno [1,2-e][1,3,4] oxadiazine-4a (3H)- carboxylate.




### Cell culture

The culture of paramecium was performed according to the method of[11]. It is to infuse the hay in a container containing 1 liter of rain water and leave in a warm place (15 to 20 ° c), dark and well ventilated. Few more days later flagellates appeared, these organisms feed at the expense of bacterial veil. The purification of the culture was through multiple subcultures.

### Method of treatment

Xenobiotics were tested in aliquots of 50ml of culture according to[12], four concentrations were chosen: 10, 20, 40 and 80µM (the tests were repeated three times and results were expressed as the average + / - the STDEV).

### Kinetics of growth

The kinetics of growth of paramecium is done by measuring the optical density (OD) at wave length λ = 600 nm as a function of time by spectrophotometry[13]. The toxicity was evaluated by determining the IC 50, that determined the concentration, which, under standard conditions, inhibited 50% of the increase in population[14].

### Determination of phase II enzymes and antioxidants

### (a) The rate of glutathione (GSH)

This assay was performed according to the protocol of[15] witch is based on measuring the optical density of the acid 2-nitro-5-mercapturic. This follows the reduction of the acid 5,5 '- dithio -2 - nitrobenzoic acid (DTNB or Ellman's reagent) by groups (-SH) glutathione

### (b) Glutathione S-transférase activity (GST)

Measurement of GST activity is was performed by the method of[16], which is to provide the enzyme substrate [usually chlorodinitrobenzene (CDNB)], which reacts easily with many forms of GST and glutathione. The reaction catalyzed by the combination of these two products leads to the formation of a new molecule that absorbs light at a wavelength of 340 nm.

### (c) Catalase activity (CAT)

The spectrophotometric determination of catalase activity (CAT) was carried out following the method of[17]. The decrease in absorbance was recorded for three minutes (JENNWAY spectrophotometer) at a wavelength of 240nm.

### Polarographic measurement

The aircraft used was an oxygen electrode, type HANSATECH, which allowed the measurement of output or oxygen consumption[18].

### Statistical analysis

The analysis of variance with two controlled factors was used to estimate the differences reported for the different parameters studied.

## RESULTS

### Effect of Indoxacarb on cellular growth

Figure 1 shows a decrease in the growth of cells from the beginning of the second day up to the 4th day.

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Cells treated with 10 µM illustrate a condition almost identical to that of control. For those treated with 20, 40 and 80 µM, a gradual inhibition and an inhibitory effect highly significant ($p<0.001$) of cell growth was observed almost from the second day of treatment.

To characterize the toxicity, we determined the 50%

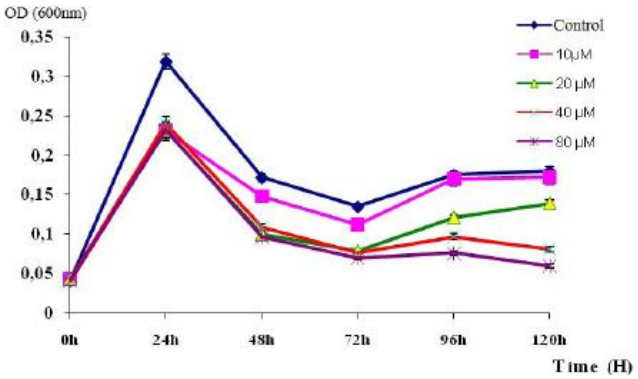


**Figure 1 : Effect of indoxacarb on cellular growth of paramecium**

inhibitory concentration (IC 50). The rates corrected of normality obtained were transformed into probit and allowed to establish a straight-line regression based on the decimal logarithms of the concentrations used. According to the curve, we could determine all the outstanding concentrations (CI 50), and the slope (the slope of the regression line). We noted that the inhibitory concentration decreased with the duration of exposure (TABLE 1).

## Phase II enzymes and antioxidants

**TABLE 1 : Determination of the 50% inhibitory concentration (IC 50) of Indoxacarb**

| Exposition | Indoxacarb | |
|---|---|---|
| | CI 50 (µM) | Slope |
| 72 h | 83.67 | 0.271 |
| 96 h | 52.81 | 0.426 |
| 120 h | 44.83 | 0.243 |

### (a) Glutathione variations (GSH)

The summarized results in Figure 2 represented the variation of GSH according to the different concentrations of the insecticide in a period of time (1h - 96h). After 1 h of exposure, there was a slight induction of GSH $4,310^{-5}$ µM / mg of protein in treated cells at a concentration of 40 µM while it was worth ($2,7^{10-5}$ µM / mg of protein) in control cells.

At 24h of treatment, there was a strong induction

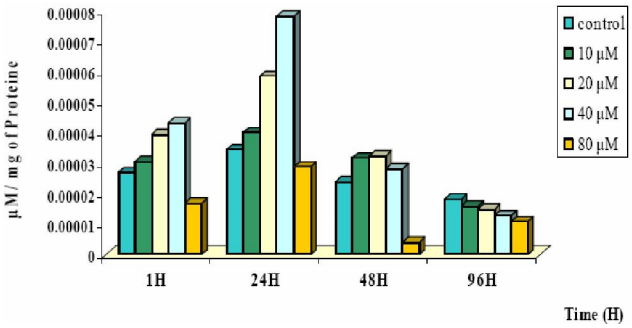


**Figure 2 : Effect of Indoxacarb on GSH induction**

of GSH in most of the treated cells compared to control where the rate peaked at 40µM concentration (about 3 times the control), whereas the highest dose 80µM achieved the lowest level. Finally, the GSH decreased from 48 h with time and concentration in a highly significant manner ($p<0.05$).

### (b) Glutathione S-transferase activity (GST)

Data obtained after determination of GST specific activity expressed in nmol / min / mg of protein measured in the cells of paramecium were grouped in Figure 3. We noted that the GST increased with time and with increasing concentrations and significantly ($p = 0.05$) and peaked at 48 h at a level double of the control.

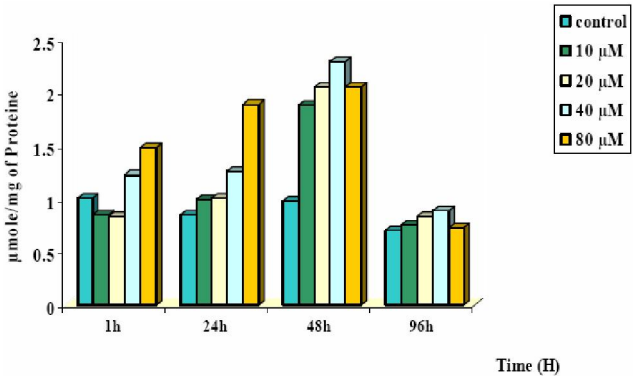


**Figure 3 : Effect of Indoxacarb on GST activity**

### (c) Catalase activity

The monitoring of the catalase activity revealed that it increased from the first h of treatment compared to the control and concentration of (10 and 80 µM). A peak was observed at 48 h for the concentration of 40 µM. However, there was a decrease in catalase at 96 h and very highly significant ($p \approx 0.001$) (Figure 4).

### (d) Ascorbate peroxidase activity (APX)

Figure 5 shows the results of ascorbate peroxidase

activity determination. Note that APX activity increased in a highly significant (p HŠ 0.01) manner with time and concentration and peaked at 48 h right from the lowest concentration where it was approximately three times the control. However, APX activity decreased at the end of treatment and for all concentrations.

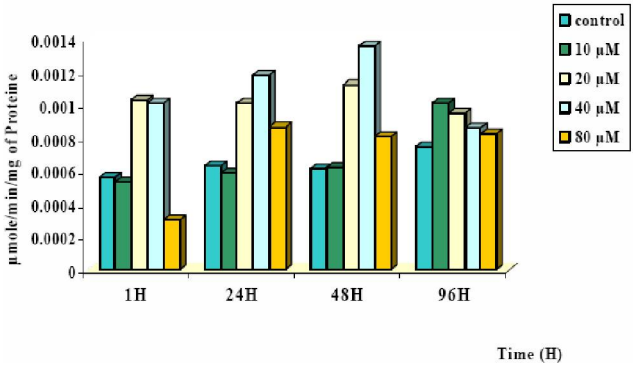


**Figure 4 : Effet of Indoxacarb on catalase activity**

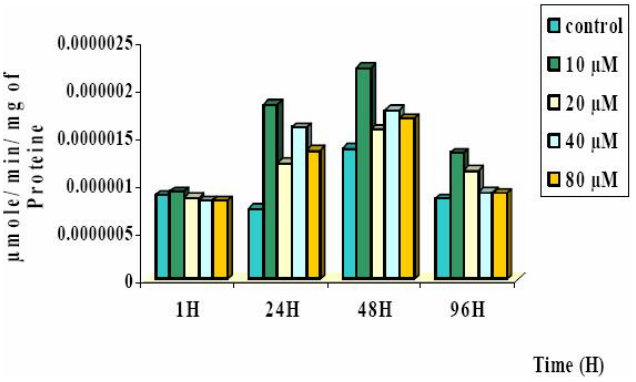


**Figure 5 : Effect of Indoxacarb on ascorbat peroxidase activity**

## Effects of Indoxacarb on the respiratory metabolism

Figure 6 illustrates the effect of Indoxacarb on the respiratory metabolism during 4 days (96 h) of treatment. We noted that the control cells breathed by consuming oxygen, which varied from 58.57 to 39.6 nmol $O_2$ on the last day with a peak of 160.71 nmol / ml of $O_2$ at 24h. The respiratory function registered at 24 h was about three times higher than that obtained at 1h. The parallel processing by Indoxacarb at low concentrations caused a slight reduction of the respiratory function with time and this in proportion compared to controls, it was about 20% at concentrations of 10µM and 25% at 20µM

However, it is important to note that the most significant changes were rated at 24 h.

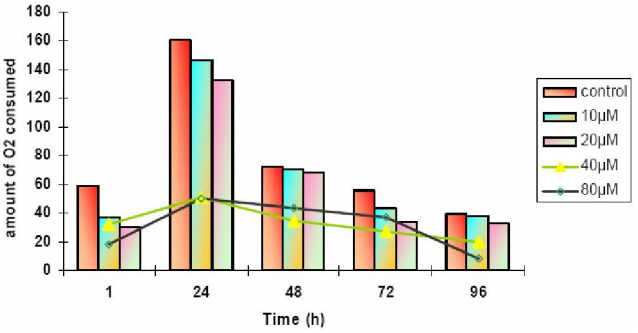


**Figure 6 : Indoxacarb effect on the respiratory metabolism of paramecium**

Regarding the high concentrations, we found that variations in 24 h were twice more important than those obtained at 1h with a much more simplistic to 80µM, the paramecium consumed only 7.95 nmol. After 48, 72, and 96h. The respiratory activity of paramecium was very low and reflected the inhibitory effect of these concentrations and highly significant differences were observed between concentrations and the rate of oxygen consumed ($p < 0.001$).

## DISCUSSION

Many pesticides are used indiscriminately by the farmers to control pests. They are likely to cause water pollution and affect the organisms that inhabit the waters including the paramecium[19]. Paramecia were used in the past for the rapid assessment of the toxicity of pesticides[20-22]. These are protists, ubiquitous in the aquatic and terrestrial environment, characterized by a short life cycle, and a rapid growth[23] and whose behavior in the environment could be affected in the presence of pollutants, which led us to use them as cellular models to study the impact of xenobiotics and the assessment of health risks[13].

The evaluation of cytotoxic effects of a xenobiotic can be performed using different parameters, including cell growth, which reflects the state of metabolism of the cell[13,24]. To reach their molecular targets, the acaricides penetrate inside the body through either the cuticle or the walls of the digestive tract. This penetration occurs at a speed which, for the same toxin, varies from one species to another. If the kinetics of penetration is sufficiently slow, the acaricide can be degraded by the detoxification systems and will have little effect[3]. Thus, our results showed that at low concentrations,

the Indoxacarb has little effect on cell growth. This brings us to confirm the influx of the pesticide inside the cells, despite the presence of the cell membrane that constitutes a barrier against the entry of xenobiotics mass but remains permeable[11] which explains moreover, the toxic effects which were detectable only from 40μM. This is the main similarity between these insects and microorganisms[25]. These results are consistent with those of Ujwala et *al*. (2007)[19] which show a decrease in population density of paramecium exposed to 1 and 100 ppm of monocrotophos (MCP). The same is true for Rouabhi et *al*. (2006)[25]. At high concentrations, a concentration-dependent inhibition of cell growth was observed almost from the first day of treatment. Our results are in the same direction as those reported by Liebig et *al*. (2008)[26] who studied the effect of methyl parathion and prometryn on the growth of flagellates (*Cryptomonas sp.)* and ciliate predators *(Urotricha furcata).* Thus, it seems clear that the xenobiotic is an inhibitor of the growth of paramecium to high concentrations. This toxicity of indoxacarb is partly due to its chemical products containing halogens such as chlorine and fluorine. When microorganisms are subjected to changes in their environment, they are stressed. This stress can be intense, and causes the death of these organisms. It may also be less intense, allowing them to deploy a battery of responses through the activation of detoxification mechanisms to fight, survive and in some cases, to acclimate to this new setting[27]. Biomarkers represent the initial biological response of microorganisms in the face of disruption or contamination of the environment in which they live[28]. Among these biomarkers, the glutathione system is provided by glutathione itself in the presence of several enzymes that are essential elements of this system with the most important is glutathione S-transferase (GST) involved in the reactions conjugation of electrophiles[29]. Glutathione is the most abundant cellular thiol involved in the metabolism in the processes of transport and in protecting cells against the toxic effects of endogenous and exogenous compounds including ORS[30]. By intercepting a hydroxyl radical, glutathione generates superoxide radical that needs to be supported by a superoxide dismutase. In addition to its role as a reducing agent, it also operates a second level in the defense against free radicals through its involvement in the reactions of detoxification catalyzed by glutathione S-transferase[31].

In our study, we noticed that when paramecia were subjected to chemical stress, the GSH and GST activity appeared to be sensitive and responded quickly to the presence of the pollutants. This could be explained by the fact that at low concentrations there is a triggering of detoxification systems, which for the most part, consist mainly of enzyme catalase. This allows the cell to tolerate and adapt to xenobiotic thus resulting in an increase in these enzymes (catalase). On the other hand, and according to Ha et *al*. (1998)[32] direct capture of free oxygen radicals (FOR) caused by this insecticide is provided by radical scavengers or compounds by enzyme systems located in the vicinity of the place of initial production. These radicals can be trapped by glutathione, some dipeptides, proteins rich in thiol groups (-SH), amino acids, unsaturated fatty acids nonesterified and phospholipids. At high concentrations, the systems were the very outdated and the enzymes were completely inhibited. This is in perfect agreement with the work of Chaoui et *al*. (1997) and Cho and Park (2000)[33,34]. Enzyme systems involved according to this mechanism have a functioning chain. The enzymes involved are superoxide dismutase, a copper-dependent enzyme that eliminates superoxide radical by converting it into hydrogen peroxide, glutathione peroxidase and catalase, which remove hydrogen peroxide, glutathione reductase, which regenerates glutathione reduced, and glucose-6-phosphatase, which provides the high energy needed to operate the chain of reactions. These systems are likely to wear the massive influx of free radicals[35].

We also showed the increase of catalase activity in paramecia treated with indoxacarb at the start of treatment. This is due to the fact that catalase is considered as an enzyme with a clear answer and rapid contamination by xenobiotics. Indeed, the catalase activity is a transformation of hydrogen peroxide ($H_2O_2$) into water and molecular oxygen ($O_2$). Yet, the production of $H_2O_2$ is induced by the presence of exogenous compounds to the body such as our pesticides[36]. The reactive derivative of oxygen can cause oxidation of macromolecules (DNA, lipids and proteins)[37]. Catalase plays a role in protecting the body against damage from oxidative stress and superoxide dismutase is with the first line of defense[38]. It is considered as one of the

most sensitive biomarker of oxidative stress, especially toward chemical pollutants in the aquatic environment[39]. The decrease in Catalase activity at the end of treatment could be explained by the increased level of ORS resulting from exposure of paramecia to high concentrations of the xenobiotic. Peroxidases are oxido-reductases enzyme that catalyze the oxidation of glycoprotein of many organic and inorganic compounds by hydrogen peroxide ($H_2O_2$)[40]. APX activity, an enzyme, very important in the defense system, induces a response to different treatments in Paramecium. It protects the cell against oxidative damage by $H_2O_2$ toxicity. The increase in APX under oxidative stress caused by this xenobiotic demonstrated its role in the elimination of hydrogen peroxide ($H_2O_2$) formed. It reduces $H_2O_2$ to water using ascorbate as an electron donor resulting from dehydroascorbate. It is recycling ascorbate using GSH as an electron donor and oxidized glutathione (GSSG) is converted to GSH by NADP-H-dependent enzyme glutathione reductase. Indeed, during the phase I metabolism of mono-oxygenases cytochrome P450 catalytic reactions by incorporating their $O_2$ atom in the xenobiotic resulting in greater need for $O_2$[41]. Thus we have shown a disturbance of the respiratory metabolism of microorganisms treated with different concentrations of the xenobiotic studied compared to control cells. Our results are consistent with those of Druez et *al*. (1989)[42] who tested the effect of gossypol on the morphology, mobility and metabolism of *Dunaliella bioculata* (flagellate protists) regarded as a cell model of human sperm. The perturbation of the respiratory activity obtained in our work showed that low concentration of Indoxacarb generated an oxidative stress that led to release of ROS which are known as disruptive of respiratory metabolism[43,44]. The major role of the endogenous production of ROS is the regulation of the activity. These ROS are quickly neutralized by the system of defense / detoxification. The high respiratory activity recorded at 24 hours supported this finding. Also after 24h and after trigger of the defense system of the paramecium, the changes initially recorded at 24 hours were reduced strongly beyond this time. On the other hand, high concentrations of Indoxacarb caused a sharp reduction in respiratory activity of cells closely related to the decrease in the number of paramecium, with a strong release of ROS capable of interfering with the components of the respiratory chain specifically at the site responsible for substrate oxidation from the Krebs cycle causing malfunction which could inhibit it totally. This leads to the apoptosis[45]. Our results are in the same direction as those of Bonsoltane et *al*. (2005)[46] who reported that the stimulation of respiration in the paramecia treated with $NH_4NO_3$. On the other hand the excessive consumption of oxygen could explain the eutrophication of aquatic environments due to pollution by chemicals. The eutrophication of lakes has led to the death by asphyxiation of freshwater fish[47].

After considering all the experimental data obtained throughout the study, it appears that the ciliate protists used in our work is a material of choice for studies in toxicology, and occupies a privileged position in aquatic ecosystems because it is one of the basic elements of food. Hence the need for a deep study of the impact of pollution on our environment is essentially required.

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