Advances in Environmental Biology, 6(8): 2249-2258, 2012
ISSN 1995-0756

This is a refereed journal and all articles are professionally screened and reviewed **ORIGINAL ARTICLE**

Evaluation of the toxicity of hydrazine carboxylate (Bifenazate) and oxadiazine year (Indoxacarb) Observed in a unicellular eukaryote: *Paramecium sp.*

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Sbartai Ibtissem.; Berebbah Houria.; Sbartai Hana.; Djebar Mohamed Réda.: Evaluation of the Toxicity of Hydrazine Carboxylate (Bifenazate) and an Oxadiazine (Indoxacarb) Grara Nedjoud Observed in a Unicellular Eukaryote: *Paramecium Sp.*

ABSTRACT

In recent years there is becoming increasingly aware that pesticides not act only against the target for which they have been approved but on the whole ecosystem. For this reason we chose Paramecium, eukaryote unicellular, as an alternative model which we tested the effect of tow pesticide. They thus indicate interactions between a test molecule and target cells, to follow the fate of xenobiotics and to identify molecular entities and biochemical pathways disturbed, altered or induced by these elements in order to explain either their toxicity or tolerance by the cell. For concentrations (10, 20, 40 and 80µM) were tested on aliquots of 50ml of culture of paramecium done beforehand. The results obtained show that the growth of paramecium is sensitive to both xenobiotics and for the highest concentration with a more pronounced toxicity for indoxacarb. Toxicity was evaluated by determining the IC 50 and by calculating the percentage of response that evaluates the response of protist towards the pollutant and thus confirms the evolution of the growth curve. The measurement of respiratory activity shows inhibition of oxygen consumption reflecting a deleterious effect of the two pesticides. Finally the risk assessment allowed us to determine through bioassay concentrations with and without effects to aquatic organisms studied (NOEC and LOEC) and the risk index and confirms the effect of the more toxic Indoxacarb compared to bifenazate.

Key words: Pesticide, Growth cell, Respiratory metabolism, Assessment risk.

Introduction

Rich in plant and animal species and micro-organisms, an ecosystem is naturally able to transform and eliminate, in part or in whole, biodegradable substances it receives and thus ensure the maintenance of balance and quality of its waters. But if the abundance of these substances exceeds a critical threshold, their capacities for self-purification are not enough: the pollutant can not be removed fast enough; it accumulates, gradually breaking the dynamic equilibrium of the natural aquatic environment, and can even become toxic. In most industrialized countries, the chemicals used in agriculture are the leading cause of water pollution. Their use has increased from 10 to 1950. Half of which is dumped in the fields does not even end up in plants and fresh water, they act not only against the target for which they are approved, but on the whole ecosystem [1]. The effects on biodiversity, including flora and fauna both terrestrial and aquatic, are undeniable. Since the advent of synthetic pesticides, contamination of the environment has increased to the point where the Earth's surface as a whole contains molecules of this nature without necessarily having undergone treatment direct [2]. 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 two pesticides belonging to two different families. The first is used xenobiotic bifenazate, it is a selective acaricide belonging to the family of Hydrazine carboxylate that controls a variety of parasitic mites of ornamentals. Preliminary studies on the mode of action indicate that high concentrations bifenazate act on receptors postsynaptic GABA nervous system of insects [4]. The second pesticide used is a new broad-spectrum insecticide [5]. This is the first insecticide Na+ channel blocker class of oxadiazines that has been marketed. Selective toxicity against insects is due in part to the fact that it is a pro-insecticide, bio-activated in insects by esterases and amylases [6]. It is a modulator of nicotinic acetylcholine receptors in mammalian neurons [7]. The present study was undertaken firstly to better

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characterize the impact of pesticides on growth and respiratory metabolism and other hand to perform a risk assessment to determine the concentrations without long-term effects for the environment.

Materials and Methods

1. Chemicals:

- Bifenazate:

1 methylethyl 2- (4-methoxy [1,1'-biphenyl]-3- yl) hydrazine carboxylate.

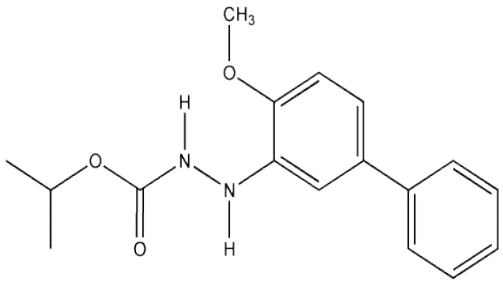

Fig. 1: Formula plane of bifenazate [8]

-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 .

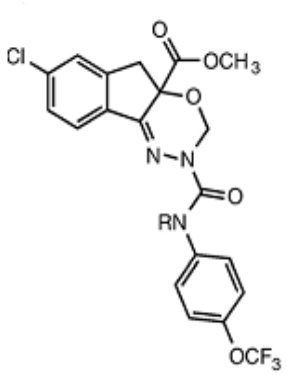

Fig. 2: Formula plane of Indoxacarb [9]

2. Cell culture:

The culture of paramecium was performed according to the method of [10]. 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 appear one flagellates, these organisms feed at the expense of bacterial veil. The purification of the culture is through multiple subcultures.

3. Method of treatment:

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

4. 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 [11].

5. Calculating the percentage response and the IC50:

The toxicity was evaluated by determining the IC 50, which determines the concentration which, under standard conditions, inhibits 50% of the increase in population [12], and the percentage of response which assesses response of protists in respect of the pollutant, according to the equation [13]:

PR = [(NC - BN) / NC] × 100 [15]. The percentage of response positive values indicates a response inhibition of growth, while negative values indicate a stimulation of growth.

6. Polarographic measurement:

The aircraft used is an oxygen electrode, type HANSATECH, which allows the measurement of output or oxygen consumption [14] which is of the order of nano-mole. This device consists of a jacket thermostated containing the cellular suspension, and two electrodes, a cathode (-) of platinum and an anode (+) silver, connected by a bridge containing potassium chloride (KCI). Both jackets are separated by a semi-permeable membrane, allowing diffusion of oxygen dissolved in the culture medium (cell suspension) to electrodes. The device is connected to a PC that can display the different spectra.

7. Risk assessment:

Risk assessment for the two pesticides used in our study was performed using the software $TerraSys_{1.0}$ [15] which was developed to allow the creation of professional analysis of ecotoxicological risks. He has the tools and functionality required to evaluate various situations involving toxic substances in the environment, whether land or water. All stages of the analysis, data entry characterization to final risk assessment, are supported by this software in a consistent and integrated. The ecotoxicological risk assessment by the conventional approach to mathematical modeling has significant sources of uncertainty, particularly related to the actual bioavailability of the contaminants, the limits of the characterizations made, and the availability, validity and representativeness of reference values for different combinations of receptors and contaminants. That is why this software combines mathematical modeling and the use of bioassay to allow the completion of risk assessments as credible and well-founded as possible. In our study, we were able to achieve a bioassay by which we calculated the ECx, NOEC, LOEC and the risk index (RI).

8. Statistical analysis:

The ANOVA two- factor control is used to estimate the differences reported for the various parameters studied [16].

Results:

1. Effect of Bifenazate and Indoxacarb on cell growth paramecia:

Figures 3 and 4 illustrate the effect of bifenazate and indoxacarb on the evolution of the growth of paramecia. All measurements were performed during the exponential phase of growth. Thus, we find that for the controls, the growth appears to decrease gradually until 72 h when the optic density (OD) is 0.135 nm to reach the end of treatment 0.180nm. However, for treatment at all concentrations, growth seems to be affected right from the contact with xenobiotics.

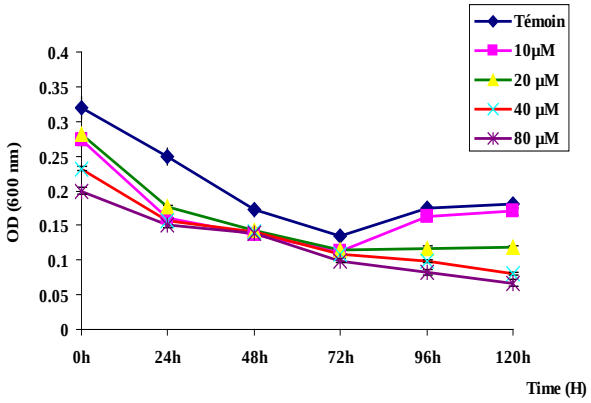

Fig. 3: Bifenazate effect on cell growth of paramecia

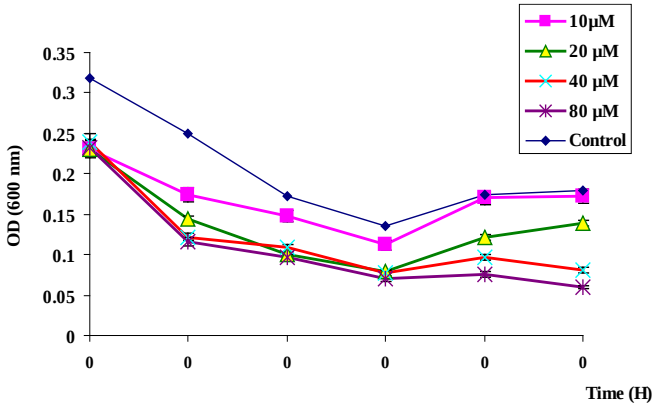

Fig. 4: Indoxacarb effect on cell growth of paramecia

2. Calculating the percentage response and the IC50:

The response rate is a parameter to evaluate the effect of two pesticides with different concentrations confirms the results obtained on the kinetics of growth of the microorganism studied. Figure (5) shows that the percentage of paramecia response is dose dependent and proportional to increasing concentrations of both pesticides. For bifenazate it is: 5.55% to 62.77% of 10µM and 80µM in which more than half of the population is inhibited by this dose. Similarly for indoxacarb where the percentage of paramecia response is dose – dependent, it is respectively: 4.44% to 66.66% and reached 10µM to 80µM.

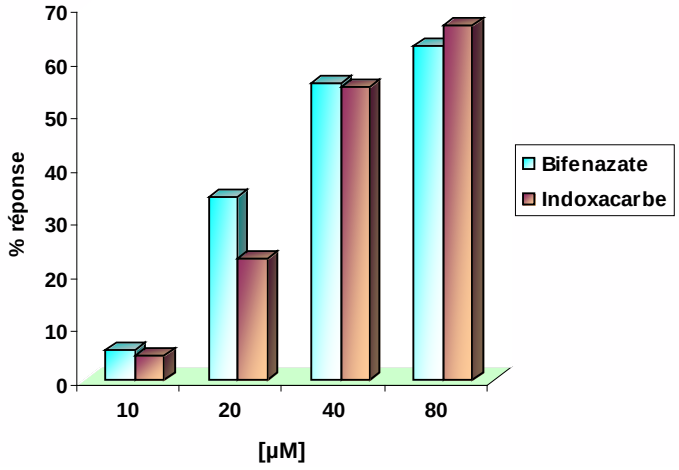

Fig. 5: Evolution of the percentage of paramecia response towards different concentrations of bifenazate and Indoxacarb

To characterize the toxicity, we have calculated the 50% inhibitory concentration (IC 50). Rates obtained were corrected normality transformed into probits and allow for a regression line based on logarithms of the doses used. Since the curve, one can determine all doses remarkable. Note that the inhibitory concentration decreases with exposure time (Table 1). For bifenazate, it is 261.8µM at 72h and 120h to 40.90µM reached. Whereas cells treated with indoxacarb, the IC 50 to 72 is three times lower (83.67µM) than cells treated with Bifenazate, and it is halved to 120 hours.

Table 1: Determination of IC 50

Exposure	IC 50 (µM)	
	Bifenazate	Indoxacarbe
72 h	261.8	83.67
96 h	57.22	52.81
120 h	40.90	44.83

4. Effects of bifenazate and indoxacarb on the respiratory metabolism:

Figures (5) and (6) illustrate the effect of both products on the respiratory metabolism during 5 days (96 h) treatment. We note that the control cells consume oxygen, which varies from 58.57 nmol/ml of O_2 to 39.6 nmol/ml of O_2 on the last day with a peak of 160.71 nmol/ml of O_2 to 24h.

Respiratory activity of paramecia to 24h is recorded three times higher than that obtained at 1h. This is probably one of the multiplication of microorganisms. This observation could be explained by the fact that cells treated tend to adapt to the concentrations used, resulting in respiratory activity of paramecia treated much closer to those of control cells.

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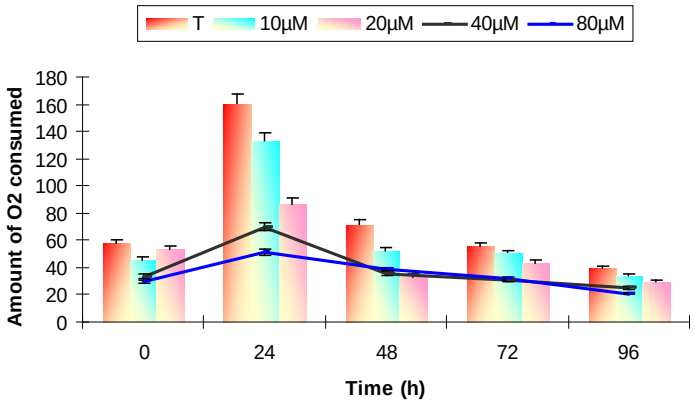

Fig. 6: Effect of bifenazate on respiratory metabolism

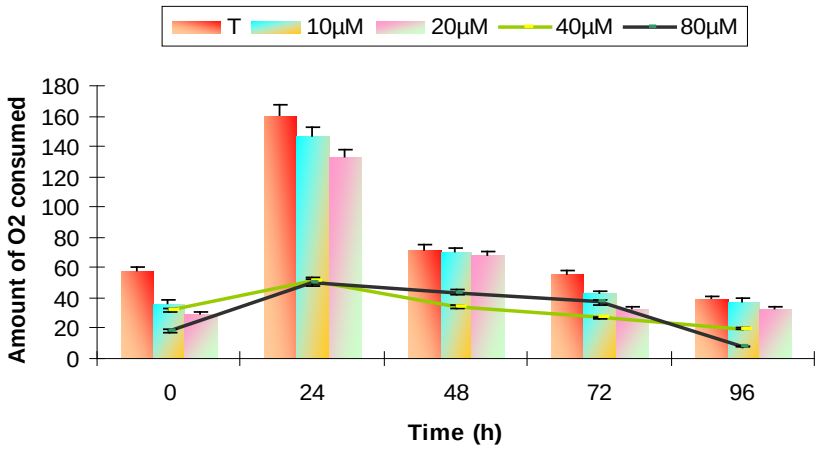

Fig. 7: Effect on the respiratory metabolism Indoxacarb

It is noted that parallel processing by both xenobiotics at low concentrations causes a slight reduction of respiratory activity with time and proportionally compared to controls, it is about 20% at concentrations of 10µM and 20µM to 25%.

For high concentrations, it is found that the variations recorded at 24 hours were double those obtained at 1 hour and does so very highly significant ($p < 0.001$). Beyond 48 hours the respiratory activity is very low and reflects the inhibitory effect of these molecules with a greater effect in reducing the highest concentration 80µM for indoxacarb when paramecia consume only 7.95 nmol compared to bifenazate which consumes about 21 nmol.

4. Risk Assessment:

The software used in our study can be included in the risk assessment results of biotests made from contaminated samples. These results can be used to calculate risk indices qualitative or quantitative (depending on the type of response obtained by toxicological bioassays). It provides a new way of calculating risk index from the results of bioassay. In our work, we undertook a description of a bioassay in which we have summarized the essence of our experiment and our objectives followed by a seizure results in an input interface to allow the use of the results of biotests in order to estimate the risk.

Data obtained after the ecotoxicological analysis of a sample can be multiple. A contaminated sample and whose toxic effects are measured by the bioassay produces a type relationship dose / response as shown in figure (8) for bifenazate and Figure (9) for indoxacarb.

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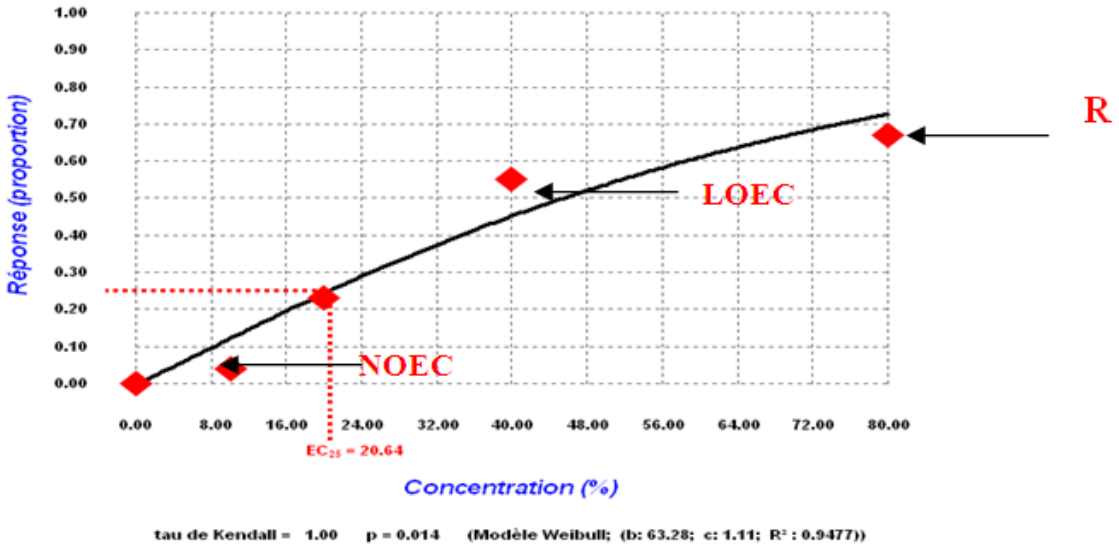

Fig. 8: Relationship dose / response bifenazate calculated from the bioassay

Concentration **(C)** corresponding to a threshold of toxicity, which can be estimated by measuring several variables:

-**ECx:** the effective concentration producing an x percentage effect

-**NOEC**: the highest concentration tested producing no toxic effect statistically significant on target organisms.

- **LOEC**: the lowest concentration tested produced a statistically significant toxic effect on target organisms.

A toxic response (**R)** produced by an undiluted sample. This response is expressed in terms of percentage effect

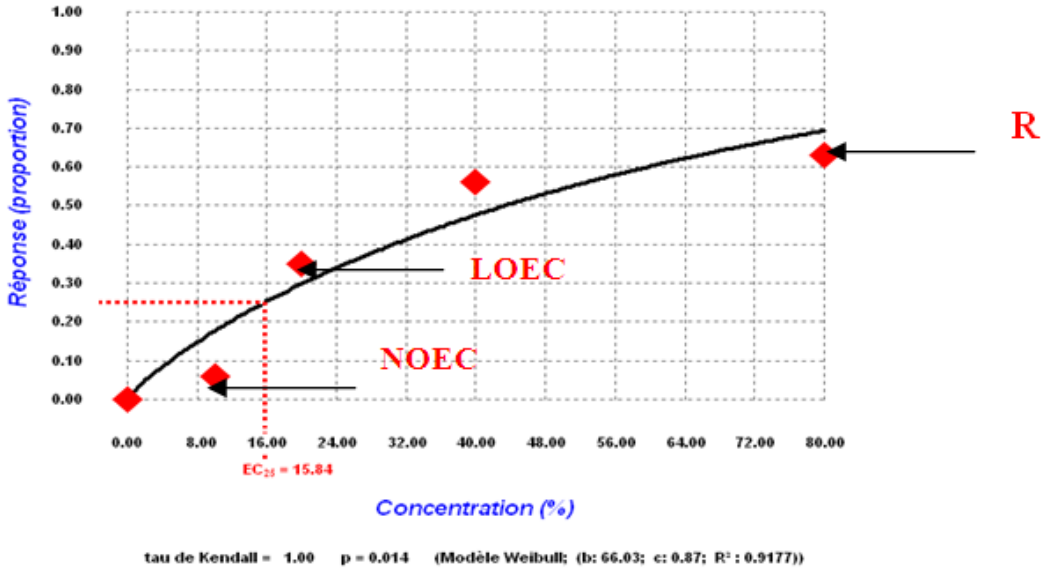

Fig. 9: Relationship dose / response Indoxacarb calculated from the bioassay

The values obtained from the charts by screening for both contaminants are shown in Table (2):

Table 2: Results of Biotest

	R (%)	NOEC (µM)	EC25 (%)	LOEC(µM)
Bifenazate	80	10	20,64	40
Indoxacarbe	80	10	15,84	20

*

The risk index is used to estimate the magnitude of risk for a given organism exposed to contamination. This value is theoretically the ratio of the concentration of contaminant (s) to which the organism is exposed to the contaminant concentration (s) tolerated by this organization, or corresponding to an effect level considered acceptable.

where:

$$\mathbf{IR = C_{exp} / C_{seuil}}$$

IR: risk index

C_{exp}: concentration of toxicant to which the organism is exposed

C_{seuil}: concentration "threshold" corresponding to the desired level of protection for this organism.

To integrate the results of the bioassay, the risk indices are classified into four categories as follows:
- IR <1: no risk in the sample
- IR ≈ 1: possible risk (value slightly greater than that tolerated)
- IR> 1: presence of risk (value greater than that tolerated by the body)
- IR >> 1: presence of risk (value much higher than that tolerated).

The results are shown in the following table:

Table 3: values of the risk index

	(IR) Bifenazate	(IR) Indoxacarbe
10µM	0,48	0,63
20µM	0,96	1,26
40µM	1,93	2,52
80µM	3,87	5,05

Discussion:

Water quality is essential for the maintaining the health of aquatic organisms and the presence of toxic substances in water is a threat to organisms that living there. The report of the agency for protection of the environment of 1990 suggests that the pollution of rivers and ponds come mainly from agriculture [17]. 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 [18]. Among these organisms, paramecia occupy a prominent place. Paramecia were used in the past for rapid assessment of pesticide toxicity [19,20]. They are protists, ubiquitous in the aquatic environment, characterized by a short life cycle, rapid multiplication [21] and whose normal behavior in nature could be affected in the presence of pollutants, which led us to use them as alternative models for studying the impact of xenobiotics and the assessment of health risks.

Evaluation of cytotoxic effects of a xenobiotic can be performed using different parameters, including cell growth in microorganisms reflects the state of cell metabolism [22]. In our work, a growth inhibition was observed in paramecia treated with bifenazate and indoxacarb and dose-dependent manner. At low concentrations, it appears that xenobiotics have little effect on cell growth, this could be due either to adsorption of the xenobiotic on the cell membrane and the presence of cuticle paramecia, which make them resistant but remains permeable [10], either the influx of the pesticide within cells thus inducing a detoxification mechanism [3]. These results agree with those of [18] which show that monocrotophos (MCP) used in doses of 1 and 10 ppm had no effect on the population density of paramecia after 24 d exposure, but beyond these concentrations a significant decrease is observed. For those treated at 20, 40 and 80 µM, a dose-dependent inhibition of cell growth is observed almost from the first day of treatment. Our results are in the same direction as those reported by [23] who studied the effect of methyl-parathion and Prometryn on the growth of flagellates (*Cryptomonas sp.)* and predators of ciliates (*Urotricha furcata*). Their hand, [24] explain the phenomenon of inhibition of paramecia exposed to acephate, by rupture of the membranes of contractile vacuoles which causes mixing of their contents with the protoplasm and cell volume is increased initially, it follows disintegration of protoplasm and all internal membranes. Thereafter, the outer membrane is also affected.

The percentage of response and IC50 confirm the evolution of the growth curves of cells treated with both xenobiotics studied in our work, and it seems clear that these two pesticides are inhibitors of paramecia with higher toxicity for indoxacarb. However, the ineffectiveness of bifenazate is because it is one of the few acaricides which contains no heavy metals or halogens which could cause different toxic effects when applied at high doses [8] . The Bifenazate is a selective miticide indicated to be neurotoxic. However, this information has not been supported by studies. It is still considered an inhibitor neurons, but we do not know the mode of action [4]. According to [8], bifenazate would be another site that the postsynaptic GABA receptors, most likely encoded by the mitochondrial genome. It is undeniable that one of the first goals that we set

appears to be achieved, not only bifenazate and indoxacarb are toxic xenobiotics to Paramecium, but in addition this cell model is an excellent tool for evaluating potential toxicity in vitro.

Knowing that the microorganisms in the presence of xenobiotics, toxins or peroxide compounds, for example, have the ability to develop a detoxification process and that this process is of a biochemical [25] The most plausible hypothesis advanced is the formation of "free radicals". A first source of radical phenomena is the initial formation of superoxide anion, the most common oxygen free radicals (ROL). Thus we have highlighted our work in a disturbance of respiratory metabolism of microorganisms treated with different concentrations of xenobiotics studied compared to control cells. Upon removal of the xenobiotic, the electrons produced during the detoxification of mono-oxygenases cytochrome P450 [26] will react with oxygen [27]. Oxygen can also react with the electrons escape the respiratory chain [28]. The direct combination of oxygen supplied to the cells with these electrons involves the formation of superoxide anion which is at the origin of the phenomena radical. Disruption of respiratory activity observed in our work shows that low concentrations of bifenazate and Indoxacarb are generating oxidative stress cause the release of ROS which are known as disruptive elements of respiratory metabolism [29,30]. The major role of the endogenous production of ROS is the regulation of the activity. Indeed, radicals can interact directly with molecules containing sulfhydryl groups, and therefore change their conformation. This type of regulation may particularly affect the molecules involved in the transduction mechanisms, such as protein kinase C [31]. Meanwhile, high concentrations of both pesticides cause a significant reduction in respiratory activity of cells closely related to the decrease in the number of paramecia, where a strong release of ROS capable of interfering with the components of the respiratory chain causing and dysfunction of the latter see it stops completely resulting in the early stages of apoptosis [32].

Finally, we completed our study by a risk assessment which aims to determine the levels without long-term effects on the environment of a chemical. This assessment includes two independent components: the component "exposure" is used to predict which compartments of ecosystems potentially affected by the product (land, water, air) and the concentrations of the substance that can be found in these compartments. The component "concentration - effect" is used to determine the danger of the substance to environmental concentrations for the organisms living in enclosures exposed [33]. In our study we limited ourselves to the second aspect that reinforces our results confirming the higher toxicity of Indoxacarb compared to bifenazate and by determining LOEC, NOEC and IR. The risk indices have been calculated on the basis of an effect level (LOEC or ECx), the conclusions drawn about the presence of risk likely reflect a real risk to ecological receptors in the mid to the study. The ecotoxicological risk assessment from the results of Biotests has limitations related to the interpretation Biotest achieved, the limited number of samples available, the types of effects considered and various other factors. A potential significant risk was estimated for the following receivers:

- **Micro-organisms** (a sample of 1 - Confidence level: high). This risk assessment has allowed us to understand the concept of biological response in ecotoxicology and the calculation of threshold values , which justifies the concepts and tools used during this study.

Conclusion:

After reviewing all the experimental data obtained throughout this study, it appears that the protist ciliate used in our work is the material of choice for studies in toxicology, and occupies a privileged position in aquatic ecosystems because it is one of the basic components of food webs where the need for a deep study of the impact of pollutants on our environment. All these results converge to a behavior of this cell model very similar to that of higher organisms, thereby confirming their interest as alternative models for biological bioassays. After this series of results, it appears that bifenazate is less toxic than Indoxacarb because the toxicity of the latter occurs at low doses (20µM) for bifenazate while it appears only from of 40µM and that virtually all the parameters studied in our work. On the other hand and according to the results obtained it appears that bifenazate affects respiratory metabolism which supports the hypothesis of [8] who state that it would act through a different mode action probably related to the mitochondria even if they have not been able to demonstrate in vitro inhibition of complex I / II and III / IV. The last part consists of the risk assessment, we confirmed the toxicity of two pesticides towards these microorganisms and the opportunity we have determined the threshold concentrations (NOEC and LOEC) and index risk..

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