Article

Endocrine Disruption, Cytotoxicity and Genotoxicity of an Organophosphorus Insecticide

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Abstract: In the present study, a battery of biological tests undertaken in vitro and in vivo was used to evaluate the toxic potential of an organophosphorus insecticide, namely Fenitrothion. The cytotoxic effect of pesticide was evaluated with the MTT assay against two human cancer cell lines: Hep-2 and MDA-MB-231. Genotoxicity was also studied using the bacterial VITOTOX® assay. The estrogenic effect was tested using the recombinant yeasts (YES) assay. Likewise, bioluminescence assays using *V. fischeri* and *D. magna* immobilization were performed. The results showed that Fenitrothion exhibits a variable cytotoxic effect depending on the dose as well as the studied cell lines, and no genotoxicity was observed in the tested sample. However, an estrogenic effect was recorded when investigating Fenitrothion using the recombinant yeasts (YES) assay. Analogously, acute toxicity was observed for both organisms and at all tested concentrations of Fenitrothion. Overall, these results underline the crucial importance of in vitro and in vivo bioassays in monitoring toxicity of pesticides.

Keywords: fenitrothion; bioassays; cytotoxicity; genotoxicity; estrogenicity; ecotoxicity

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1. Introduction

Organophosphorus substances (OPs) are the most widely used insecticides worldwide. The OPs also constitute a major group of chemical warfare agents which continue to pose a global human health threat [1].

Fenitrothion [O,Odimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate] is an OPs that has several uses in agriculture [2]. This pesticide is mainly used to control the detrimental effect of insects on rice, cereals, fruits, vegetables, stored grains and cotton. It is also currently used for mosquito control [3]. The total world production of Fenitrothion is estimated to be approximately 15,000–20,000 tons annually. Field doses can exceed 500 g/ha because of the average concentrations of 80 µg/L in the treated water body [4].

Fenitrothion, like all pesticides, acts chemically on effectors that are often involved in vital functions or in reproduction. It disrupts the nerve or the hormonal signaling, the cellular respiration, the cell division or the protein synthesis, thus allowing effective control of the pest organism [5]. It is true that the pesticide is always toxic to the target for which it has been developed but it might not be very specific to a pest organism because of the partially common physiological processes and mechanisms of organisms. As a result, a pesticide, which is intended to control a pest organism, has a more or less extensive

toxicological potential on the other organisms that were not targeted at first. The pesticide professional users or the common individuals are therefore possibly at risk of the improper use of the pesticides which inevitably can cause health problems within hours or days.

These health risks or disorders are developed just like the pesticide mechanisms whilst acting against the pest [5]. Several studies show that human exposure to Fenitrothion could promote the development of neurological [6], immunological [7] and reproductive disorders [8]. More recently, a study in Tunisia was conducted on pesticide handlers showed a decrease in serum and globular cholinesterase activity along with Fenitrothion detection among workers [9].

Fenitrothion also poses a risk on other "non-targeted" organisms including aquatic organisms. This pesticide is classified as highly toxic to aquatic organisms. However, we found that the use of biomarkers is a relevant tool used in signalling the biological signature of the impact or the presence of any contaminant in a living structure. In order to study the ecotoxicological risk associated with the use of Fenitrothion, we tried to determine its toxicity on humans and animals as well as its particular ability to concentrate in the ecosystems as one of the major polluting pesticides.

The type of pollution that affects low-volatility, soil-infiltration and that results from water-dense pesticides is remarkably dangerous. In fact, water pesticides can contaminate groundwater and drinking water for long periods of time when accumulated in highly concentrated levels. In this context, several studies have demonstrated the presence of Fenitrothion residues in various parts of the environment (water, sediment, food, etc.) [10,11] and the potential risk of bioaccumulation in trophic chains as well.

It is within this framework that our research focused on the following objectives.

a. To evaluate the impact of Fenitrothion on the metabolism of two cell lines: Hep-2 and MDA-MB-231.
b. To assess the genotoxicity and estrogenicity of this pesticide using the VITOTOX® test and the YES assay.
c. To evaluate its estrogenic potential using two organisms: the *Vibrio fischeri* and the *Daphnia magna*, belonging to different trophic levels.

2. Materials and Methods

2.1. Chemicals

The purity of each compound ranges from 95.2 to 99.6% according to the manufacturer's specifications. The pesticide Fenitrothion (CAS No. 122-14-5), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Dimethyl sulfoxide (DMSO), Penicillin and Streptomycin, Phosphate buffer saline (PBS), Trypsin-EDTA, fetal calf serum (FCS) and the sterile material used for culture (flasks and culture plates) were from Dutscher (Bernolsheim, France). The unspecified chemicals were purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

2.2. Cell Culture

To study the antiproliferative effect of the pesticide, we used two tumor cell lines, namely: the 90 Hep-2 cell line represented by human epidermoid cancer cells [10] considered to originate 91 from a carcinoma of the human larynx [11] and the MDA-MB-231 cell line which is 92 commonly used for the development of new therapeutic approaches in breast cancer [12]. Cells were obtained from the American Type Culture Collection (ATCC, USA) and were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The culture medium (DMEM) was supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin and 10% (for cell multiplication) or 2% (for cell maintenance) heat inactivated (56 °C, 30 min) fetal calf serum (FCS). When the cell culture reached 80% confluence, cells were dispersed with 0.025 M trypsin-EDTA and reseeded in new flasks. The passage number of the cells used in the experiments was between 10 and 25, and the culture medium was replaced every 48 h.

2.3. Choice of Fenitrothion Concentrations

Fenitrothion was used at concentrations between 50 and 400 μM. The concentrations used in this study were selected on the basis of data on pesticides in the form of residues in food, as well as on the basis of maximum residue limits (MRLs) [13] with a simple adjustment. Upon receipt, the pesticide was dissolved in sterile distilled water and stored as stock concentration of 800 μM at −20 °C. Each aliquot was thawed and used only once.

2.4. Evaluation of Cell Viability by MTT Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to check the toxicity of Fenitrothion on Hep-2 and MDA-MB-231 cell lines. This analysis was used to investigate the metabolic activity of the cells since it allows the determination of the mitochondrial function of cells by measuring the activity of mitochondrial succinate dehydrogenase enzyme, which cleaves MTT into a blue colored product (formazan). Briefly, both cell lines were seeded in 96-well plates (at a rate of 5×10^5 cells/mL for Hep-2 cells and 2×10^5 cells/mL for MDA-MB-231 cells) and incubated during 24 h to ensure adequate adhesion of cells. From a Fenitrothion stock solution (800 μM), a series of dilutions was made in the maintenance medium (2% heat-inactivated FCS) to obtain the following concentrations: 400, 200, 100, 50 μM. The growth medium was replaced with different concentrations of Fenitrothion in triplicate, at the rate of 200 μL/well. A control without treatment was carried out by adding 200 μL of the maintenance medium. After 24 h of toxicant exposure, the culture medium was discarded and 100 μL of MTT reagent (0.5 mg/mL) was added to each well. Following incubation for 3 h, each well received 100 μL of DMSO in order to completely dissolve the formazan crystals. Afterward, the absorbance was detected at 570 nm. All tests were performed along two independent experiments.

The results represent the means of the median values obtained and are calculated as a percentage of cell viability relative to the control condition (cells without treatment).

The viability and toxicity percentages were calculated according to the following formulas [14] as follows:

$$\% \text{ viability} = [\text{OD570 nm treated cells}/\text{OD570 nm control cells}] \times 100$$

$$\text{Cytotoxicity } (\%) = 100 - \text{viability } (\%)$$

2.5. Genotoxicity Assessment

The VITOTOX® test was conducted to screen the cytotoxic and the genotoxic potential of Fenitrothion. This test, based on the SOS DNA-repair system, makes use of two genetically engineered bacterial strains: *Salmonella typhimurium* strains TA104 pr1 (Cytox strain) and TA104 recN2-4 (Genox strain). They are used as porter systems for genotoxicity and cytotoxicity [15]. The referred protocol was the Vitotox-10 manual of Gentaur (Kampenhout, BE) as it was previously outlined. In 96-well plates, four concentrations of Fenitrothion (0, 0.25, 0.5 and 1 mM) and positive/negative control substances were prepared. Positive controls were benzo(α)pyrene in the presence of S9 and 4-nitroquinoline-oxide (4-NQO) in the absence of S9. Methanol was used as the negative control. The tests were carried out in the absence and in the presence of a rat metabolic enzymatic fraction (S9) in order to test the genotoxic potential of pure molecules and its metabolites. The genotoxicity and the toxicity measurements were carried out using a luminometer (GloMax®, Promega Benelux bv, Leiden, Nederland) which allows online measurements of the light emitted every 5 min during 4 h. After the measurements were completed, the signal-to-noise (S/N) ratio, i.e., the light output of the exposed bacteria divided by the light output of the unexposed bacteria, was calculated. A compound is considered genotoxic when the max S/N Genox/max S/N Cytox ratio is greater than 1.5 [15].

2.6. Estrogenic Activity Assessment

The Yeast Estrogen Screen (YES) assay was used in our study to elucidate the estrogenic activity of fenitrothion. In this assay, recombinant yeast cells "*Saccharomyces cerevisiae*" were transformed with an expression of plasmid containing a gene encoding the human estrogen receptor-alpha (hERα) and a plasmid containing the estrogen-responsive element and carrying the reporter gene lac-Z coding for β-galactosidase [16]. Upon activation of the hER receptor, β-galactosidase was secreted in the medium which catalysed the hydrolysis of the colorless substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) into a yellow product quantifiable by spectrophotometry.

The YES assay was performed according to Le Grand et al. [17] method. Yeast cells *Saccharomyces cerevisiae* were transformed with an inducible plasmid pY60herα containing a gene encoding human estrogen receptor-alpha (hERα) and an expression plasmid YRPE2 encompassing the reporter gene lac-Z coding for the enzyme β-galactosidase.

Transformed yeast cells were grown overnight in a selective medium (6.7 g/L nitrogen base without amino acids, pH 5.8, 20 g/L glucose, plus dropout supplements: DO-ura-leu). Afterward, this preculture was diluted in a rich medium (YPRE, 20 g/L tryptone, 10 g/L yeast extract, 10 g/L raffinose, pH 7.0 and 1% ethanol) so that an $OD_{600nm,l=1cm}$ = 0.1. When $OD_{600nm,l=1cm}$ reached 0.4, hERα expression was induced by 20 g/L galactose.

Yeast cultures (1 mL) from an overnight induction were dispensed into 24-well plates and exposed to different concentrations of Fenitrothion. Assay plates were incubated at 30 °C, 150 rpm. In each test plate, a positive control (17-beta-estradiol (E2) with an initial concentration of 2×10^{-8} M) and negative control (methanol) were included.

After 3.5 h of stimulation, the β-galactosidase activities were monitored. Plates were suspended again in 1 mL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0 and 50 mM β-mercaptoethanol). From this cell suspension, 10 times diluted suspensions were prepared in triplicate. A volume of 250 µL of diluted suspension was transferred in a black 96-well-plate and plates were checked for absorbance at 600 nm in a microtiter plate reader to determine yeast concentration.

The remaining volume was used to monitor β-galactosidase activities. To this end, yeast cells were reconstituted in 1/20 (v/v) volume of acetone:toluene (9:1) for 15 min at 30 °C; then, chromogenic reaction was started with an addition of ONPG (4 mg/mL) and terminated by the addition of 0.3 mol/L Na_2CO_3 when the color of solution became yellow. After centrifugation (11,700 g for 1 min), the OD 420 nm, l = 1 cm values of the supernatant were monitored, and β-galactosidase activity (Activity) was expressed in Miller units according to Miller Equation (1) [18]:

$$\beta\text{−galactosidase activity (Miller units)} = \frac{1000 \times \text{OD420nm}, \ l = 1 \text{ cm}}{t \times v \times \text{OD600nm}, \ l = 1 \text{ cm}} \quad (1)$$

where (t) is the reaction time (min) and (v) is the volume of the cell suspension (0.1 mL).

The data of the dose-response curves were fitted according to the Hill Equation (2) by using Σigma Plot software:

$$Activity = Activity_{[L]\to 0} + \frac{a \times [L]^n}{EC_{50} + [L]^n} \quad (2)$$

For each compound and tested product, the displayed results were expressed in terms of the estrogenic activity. This is calculated with the following Equation (3):

$$\beta\text{−galactosidase relative activity} = \frac{Activity_{[L]} - Activity_{[L]\to 0}}{Activity_{[E2]\max} - Activity_{[L]\to 0}} \quad (3)$$

where $[E2]_{\max}$ corresponds to the required 17β-estradiol concentration for maximal activity.

2.7. Ecotoxicity Investigation

2.7.1. *Daphnia magna* Assay

In order to assess the potential toxicity of Fenitrothion, we selected *Daphnia magna* as one of the species to be exposed. *D. magna* acute toxicity tests were performed according to the ISO 6341 guideline.

First, an artificial freshwater medium was prepared according to Molins-Delgado et al. [19] by adding 10 mL of four different saline solutions containing the needed salts and deionised water up to a volume of 2 L. A series of vessels containing 10 mL of oxygenated medium together with the selected chemical were prepared along with a series of blank control solutions containing only culture medium. Acute toxicity tests were conducted in accordance with [19]. Neonates (<24 h old) in groups of 20 were transferred directly using a disposable Pasteur pipette from the vessels to a Petri dish (10 mL). The animals were exposed to six concentrations of Fenitrothion: 3%, 6%, 12%, 25%, 50%, and 100%. ASTM hard water (ASTM standards used in the production and testing of personal protective equipment) was used as a diluent. Petri dishes were illuminated with a constant light (3000 lux) and kept at 22 °C. The number of immobilized neonates in each vessel was counted using a stereomicroscope. The number of immobilized neonates was correlated to the concentration of Fenitrothion allowing the determination of the 24 and 48-LC_{50} values. Two bioassays proportions were carried out, with two replicates.

2.7.2. *Vibrio fischeri* Assay

V. fischeri's acute toxicity test was conducted based on the ISO 11348-3 guideline. Each sample was tested in duplicate. A culture medium was prepared consisting of water with 3% NaCl. Standard solutions of the target compounds were prepared at different concentrations in the culture medium *Aliivibrio fischeri* (ATCC® 7744™). Vials containing the bacteria were stored at 20 °C prior to analysis. The analyzer, controlled by the software Microtox Omni (SDI), was equipped with a 30-well temperature-controlled incubator chamber, which was set to 15 °C. In each well, a cell containing culture medium was introduced. The vial with the bacteria was then reconstituted with 5 mL of a saline solution provided by SDI and transferred into a well inside a cell. A few minutes before starting the test, 20 mL of the solution containing the bacteria was added to each cell. After 15 and 30 min, photometric measures of each cell were taken in the analyzer. The recorded absorbance values were then correlated with the different concentrations of Fenitrothion to estimate the 15 and 30 min EC_{50} values.

2.7.3. Acute Toxicity Evaluation

(a) *Daphnia magna*

The percentage of inhibition (I) was calculated following Equation (4) and related to the different concentrations of Fenitrothion using the Hill regression model of Equation (4) and the curve fitting tools of the Graph Pad Prism software:

$$I = \frac{D_i}{D_0} \times 100 \tag{4}$$

where D_0 is the number of initial neonates and D_i is the number of immobilized ones:

In order to correlate the incidence rates estimated with the concentrations of the chemicals, Equation (5) was applied:

$$I = B + \frac{(T - B)}{\left(1 + 10^{((LogEC_{50} - X) \times H)}\right)} \tag{5}$$

where T is the top value of the curve, B is the bottom parameter of the curve, $LogEC_{50}$ is the logarithm of the median effect concentration, X is the logarithm of the compound concentration, and H is the Hill coefficient of the curve. The EC_{50} values were further calculated for the algae using this equation.

(b) *Vibrio fischeri*

The bioluminescence was determined by the absorbance measurements, and then Equation (6) was applied to estimate the incidence rates:

$$I = \frac{ABS_0 - ABS_t}{ABS_t} \quad (6)$$

where I is the percentage of inhibition, ABS_t is the absorbance at the time of the analysis, and ABS_0 is the initial absorbance. Inhibition rates were then correlated with different Fenitrothion concentrations. Toxicity was estimated by calculating the Toxicity Units (TU) according to Equation (7) as described by Tamura et al. [20].

$$TU = 100/ECx \quad (7)$$

where ECx is the X% effect concentration of Fenitrothion. A value of TU < 1 indicates a nontoxic effect, whereas a value of TU > 1 corresponds to a toxic effect.

3. Results

3.1. Effect of Fenitrothion on Cell Proliferation

We exposed two types of human cell lines, epithelial (Hep-2) and mammary (MDA-MB-231), to different concentrations of our molecule of interest. The entry into apoptosis of cells or their death by necrosis is reflected by a slowdown in the metabolic activity. The MTT test measures this drop in the metabolic activity.

The cytotoxic effect of Fenitrothion on cell lines (Hep-2 and MDA-MB-231) was measured after 24 h of exposure to different concentrations (0, 50, 100, 200, 400 µM).

The results presented in Figure 1 show that the cytotoxic effect of Fenitrothion on the cells increased in a dose dependent manner. However, it is much more remarkable in the Hep-2 cell line than in the MDA-MB-231 line. Indeed, the cytotoxicity percentages for the largest dose are 55.653 ± 3.455% and 26.316 ± 1.058% respectively after 24 h of exposure with an IC_{50} determined only for the Hep2 line, equal to 200.577 µM.

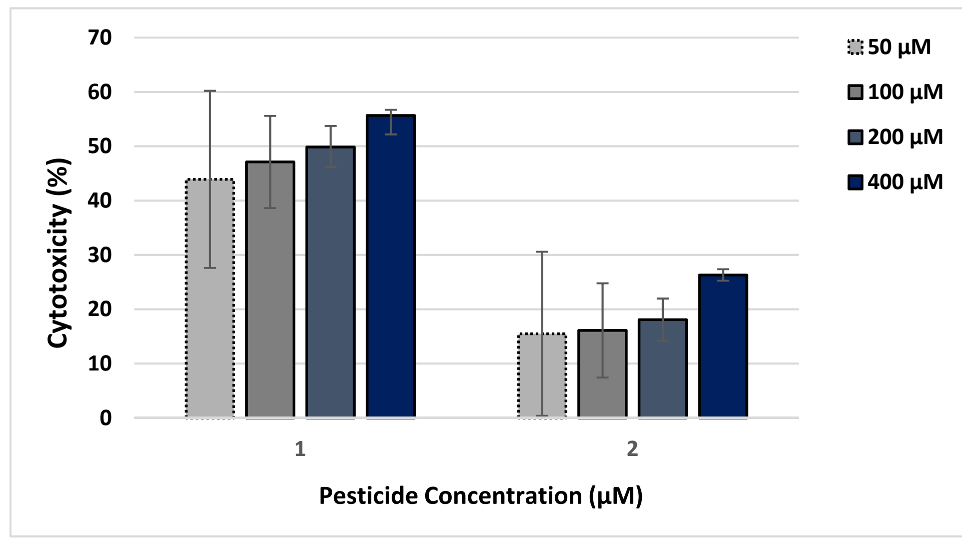


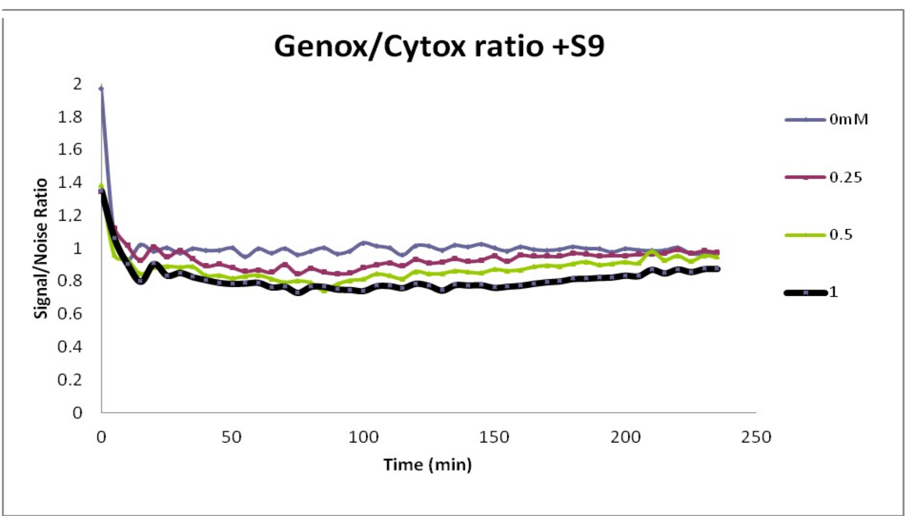
Figure 1. Cytotoxic effect of different concentrations of Fenitrothion on the cell lines Hep-2 and MDA-MB-231.

The cytotoxic effect of Fenitrothion on cell lines (Hep-2 and MDA-MB-231) was measured after 24 h of exposure to different pesticide concentrations (0, 50, 100, 200, and 400 µM) with the MTT test. The results show that the cytotoxicity percentages for the largest dose are 55.653 ± 3.455% and 26.316 ± 1.058%, respectively on Hep-2 and MDA-

MB-231 after 24 h of exposure with an IC_{50} determined only for the Hep2 line equal to 200.577 Mm. All tests were performed for two independent experiments.

3.2. Genotoxicity Assessment

The genotoxicity of Fenitrothion was investigated with the bacterial VITOTOX® assay. It can be seen in Figure 2 that there was no increased light emission in both Cytox and Genox strains for any of the tested doses of Fenitrothion. This was noticed in the absence and the presence of S9 mix (S/N ratios were approximately "1"). This means that no cytotoxic or genotoxic effect was observed in the VITOTOX® assay of the tested concentrations of Fenitrothion.



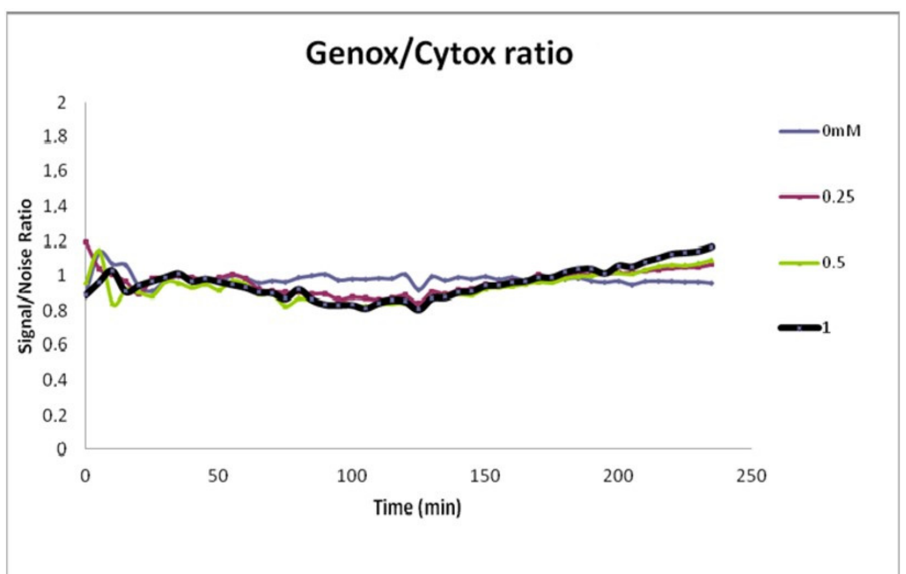
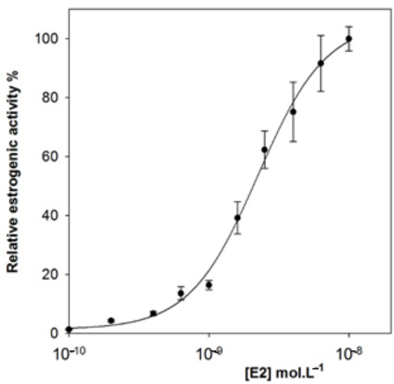


Figure 2. Findings of the VITOTOX® test for both bacterial strains (Genox and Cytox) exposed to different concentrations of Fenitrothion in the presence and absence of S9 mix.

The genotoxicity of Fenitrothion was investigated with the bacterial VITOTOX® assay. It can be seen that there was no increased light emission in both Cytox and Genox strains for any of the tested doses of Fenitrothion, both in the absence or presence of S9 mix (S/N ratios were approximately "1").

3.3. Estrogenic Activity Assessment

The estrogenic activity of pure Fenitrothion was evaluated by measuring the transcriptional activation of hERα; so, co-transformed yeast cells with a hERα-expressing vector and a β-galactosidase reporter vector, were used [16]. Figure 3 displays the dose-response estrogenic activity of the E2 reference molecule, and that of Fenitrothion. All the measured activities were normalized to the E2 maximal activity value. It is worth mentioning that an increase of Fenitrothion molar concentration, from 4.5 to 18 mmol/L, corresponding to a mass concentration of 1.25 and 5 g/L, results in a large and significant increase up to 32% of the observed maximal E2 activity. This relative estrogenic activity of 32% fits with a concentration of estrogens, expressed in E2 equivalents, of 1.35×10^{-9} mol/L. Our results clearly show that the use of this insecticide can induce gene expression through hERα transactivation and can cause adverse effects on human and animal health.



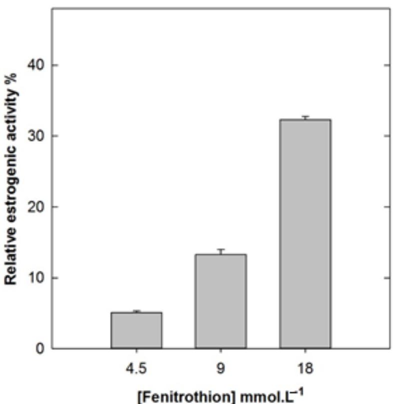


Figure 3. Estrogenic activity of the E2 reference molecule and that of Fenitrothion determined with the yeast estrogen screen (YES) assay.

The estrogenic activity of pure Fenitrothion was evaluated by measuring the transcriptional activation of hERα; so, co-transformed yeast cells with a hERα-expressing vector and a β-galactosidase reporter vector, were used. All the measured activities have been normalized to the E2 maximal activity value. Results show a relative estrogenic activity of 32% fits with a concentration of estrogens, expressed in E2 equivalents, of 1.35×10^{-9} mol/L.

3.4. Ecotoxicological Assessment

When investigating the acute toxicity of Fenitrothion we used two species belonging to two different trophic levels. On the one hand, the freshwater crustacean *Daphnia magna* was chosen as the representative of consumers. On the other hand, the luminescent marine bacterium *Vibrio fischeri* was selected as the representative of decomposers.

3.4.1. Daphnia Magna Assay

In *Daphnia magna*'s immobilization test, the acute toxicity endpoints (EC_{50}) after 48 h exposure of the target, Fenitrothion ranged from 55.3 to 79.8% (Table 1).

Table 1. Median values concentrations (EC_{50}) and toxic units (TU) values of Fenitrothion tested to *Daphnia magna* in 24, 48 h and towards *Vibrio fischeri* after 15 and 30 min exposure time (Test endpoint, bioluminescence).

Compound	**Bioindicators**					
	Daphnia magna **EC_{50} (mgL^{-1})**			***Vibrio fischeri*** **EC_{50} (mgL^{-1})**		
	24 h	**48 h**	**TU**	**15 min**	**30 min**	**TU**
Fenitrothion	79.8	55.3	1.26–1.80	21.61	10.29	4.62–9.71

In *Daphnia magna*'s immobilization test, the acute toxicity endpoints (EC_{50}) after 48 h exposure for the target Fenitrothion ranged from 55.3 to 79.8% (after 24 h). In the *Vibrio fischeri* bioluminescence test, the EC_{50} value was calculated after 15 min and also after 30 min exposure to Fenitrothion the value was in the range 10.29–21.61%.

In the case of a 48-hour exposure, the obtained value of the mean 48 h for EC_{50} was 55.3%. As has been shown in this work *Daphnia magna* immobilization at increasing Fenitrothion concentrations was monitored. According to these results, it appears that the Fenitrothion mechanism of toxicity to *D. magna* depends on the concentration of the compound. The higher the concentration, the higher the toxicity rate. From the immobilization test, a toxic effect was observed for the Fenitrothion. This toxic effect was displayed within the concentration studied range.

As it was indicated, growth inhibition increased with the increasing of the Fenitrothion concentration. The toxicity of Fenitrothion, as it was expected, increased with the exposure time in *D. magna*; after 48 h the number of immobile *D. magna* had approximately doubled compared to that measured after 24 h.

3.4.2. *Vibrio fischeri* Assay

In the *Vibrio fischeri* bioluminescence test, the EC_{50} value was calculated after 15 min and also after a 30 minute-exposure to Fenitrothion; it was in the range 10.29 to 21.61%, as shown in Table 1. Fenitrothion, therefore, was considered to be toxic to *Vibrio fischeri*. There was little change in toxicity after 15 and 30 min of exposure.

Acute toxicity of Fenitrothion was thus observed. The value of EC_{50} was calculated after exposing the algae for 15 min and for 30 min. The results of ecotoxicity of Fenitrothion on *Vibrio fischeri* differs depending on the exposure time. The estimated ecotoxic effect on *V. fischeri* exceeds 50% after 15 min of exposure time. When comparing the values that could be calculated from EC_{50} for 15 min and 30 min, it is observed that there was a significant inhibition. The observed toxicity at 15 min showed that this type of organism is resistant during the first 5 min.

4. Discussion

Despite their beneficial effects, pesticides have a harmful impact on humans and on the quality of the environment and foods [21,22]. Organophosphate insecticides are among the most widely used pesticides in Tunisia and the rate of poisoning by these pesticides represents 11% of all acute poisonings according to the Toxicology Reference Center in Tunis (center of urgent medical assistance and resuscitation CAMUR) [23]. Fenitrothion is an organophosphate pesticide which is used as a study molecule in our work for several reasons.

This insecticide is not only toxic to the target for which it has been developed but also to other non-target organisms including humans and the aquatic organisms.

Human exposure to this chemical can enhance the development of serious pathologies affecting the nervous system [6], the immune system [7] and the reproductive system [8].

Several in vitro studies have shown the cytotoxic [13] and genotoxic [24] effect of Fenitrothion.

This corroborates the results of our study, which has a major objective, the investigation of the effects of Fenitrothion on the 2 cell lines Hep-2 and MDA-MB-231.

Our results showed that Fenitrothion affects the metabolism of Hep-2 cells as well as the MDA-MB-231 cells, thus resulting in an increased dose-dependent cytotoxicity. This confirms the work of Li and Zhang [25], who demonstrated that the cytotoxic effect of an organophosphate pesticide on the multiplication of FG-9307 cells is dose-dependent. Our results show that the antiproliferative activity of Fenitrothion is found to be more important against Hep-2 cells than against MDA-MB-231 cells. Thus, we can conclude that the cytotoxic effect of pesticides depends on the cell type used [26].

Exposure of different cell lines to Fenitrothion can affect their cell proliferation. This biochemical reaction, among others, can be due to a direct interaction of this organophosphate pesticide with the glucocorticoid receptors, as in the case of MDA-MB-231 cells [27].

The cytotoxicity of Fenitrothion may also result from its ability to interact with the macromolecules and the cellular structures. Moreover, it proved to be able to disrupt several cellular functions. In fact, malformations of the mitochondrial structure (destruction of mitochondria by loss of ridges) were described by Adamski et al. [28].

Biochemical alterations in lipids, in proteins and in the DNA/RNA structures were demonstrated by Ukpebor et al. [24]. Likewise, the ability of Fenitrothion to generate oxidative damage through lipid peroxidation [13], the production of reactive oxygen species [29] and the disruption of the function of anti-oxidant enzymes [13] were also demonstrated in human cancer cells.

The genotoxicity assessment of Fenitrothion was performed using the bacterial VITOTOX® assay. It is a quick and simple genotoxicity screening assay which was shown to be effective in detecting chemical compounds that can cause genetic damage [15]. In the VITOTOX® test, no evidence of a cytotoxic or genotoxic activity was observed at the tested doses of Fenitrothion. Similar to our findings, Fenitrothion was found to be non-mutagenic and non-clastogenic in yeasts and rats [30].

Several other studies have reported that the cytotoxicity induced by Fenitrothion against breast cancer cells may also result from its binding to the estrogen receptor. It can function as an endocrine disruptor [31]. This falls within the scope of our third objective in this study, namely in the determination of the estrogenic activity of Fenitrothion using the YES assay. This bio-tool is widely used for monitoring pesticides which disrupt the estrogenic function. Findings from this bioassay confirm that Fenitrothion exhibits an estrogenic response in exposed yeasts. Our findings corroborate the previous studies reporting the possible environmental estrogenic and anti-estrogenic activities of Fenitrothion in human breast cancer MCF-7 cells [31].

Once present in the environment, Fenitrothion can have harmful impacts on both our flora and fauna—that is why it is mandatory to evaluate its ecotoxicological effects. The acute toxicity of Fenitrothion was investigated using a battery of two ecotoxicological tests: *Daphnia magna* and *Vibrio fischeri* tests. Both bioassays revealed that Fenitrothion possessed acute toxic effects for both target organisms (*V. fischeri and D. magna*), and these effects were dose-dependent. Several studies have underlined the ecotoxic effect of Fenitrothion for the *D. magna* bioindicator [32] but very few studies have evaluated the toxicity of Fenitrothion in particular concerning the bacterium *V. fischeri* [33] and concerning bacteria in general. Consequently, our study is complementary to the previously performed biochemical studies.

For instance, in this study, the LC_{50} (48 h) value for Fenitrothion was higher than that reported by [34] and [35]. The toxicity of Fenitrothion may be related to the numbers of P, S and Cl in the composition of each agricultural chemical [34,35].

It is worth mentioning that crustaceans, whose physiology presents many analogies to that of insects, are particularly sensitive to insecticides [36].

Many studies have underlined the acute and chronic toxicity of this compound towards fish and crustaceans [37,38] but also towards microalgae [39]. Other authors [40] have reported different values than those obtained in our study using the Microtox™ system. This is probably related to the variations in the cell suspension and the problems of reproducibility (within one laboratory and also between laboratories).

The toxicity assessment using: ecotoxicity, cytotoxicity and genotoxicity tests as well as the application of new molecular approaches, contributes to the evaluation of the potential ecotoxic effect of the pesticide at different levels of biological organization. These results encourage international commissions to move towards replacement and/or bioremediation procedures for this insecticide as is the case with Chlorpyrifos because of its confirmed neurotoxicity [41,42].

Likewise, biological control has paved the way for several molecules, active materials and organisms to be effective and ensure the role of insecticides. We can take the example of essential oils and their potential in the biological fight against the white fly *Bemisia tabaci* Genn [43].

The combination of active substances with different modes of action, and leading to synergy, is a promising and still under-exploited path. The objective sought in perspective is to increase the effectiveness of the treatment while reducing the doses of the active substances used. This is why we have already started a study on a possible synergy of action between Fenitrothion and a vertebrate antimicrobial peptide (results not shown). Indeed, antimicrobial peptides are produced by living organisms and offer strong possibilities in agriculture because new compounds can be developed on the basis of natural structures with improved properties of activity, specificity, biodegradability and toxicity [44].

5. Conclusions

In our work, we investigated the effect of an organophosphate pesticide, Fenitrothion, either in vitro or in vivo on different models. The results revealed an antiproliferative effect of the pesticide which depends both on the dose and on the cell line used. In addition, a significant estrogenic effect was determined during the YES test on yeasts, which may point to the mechanism of action of the pesticide on cells. The VITOTOX® test did not reveal any genotoxicity, but this in no way diminishes the toxicity of this insecticide. Indeed, the toxicity data helped in assessing the risk posed by Fenitrothion to aquatic biota. In fact, the acute toxicity of fenitrothion against two different aquatic species has proven to be very obvious. These results prompt us to look for other less harmful and more effective molecules against pests that can replace this kind of chemically synthesized pesticide. Indeed, in perspectives we are studying the effect of the mixture between Fenitrothion and an antimicrobial peptide on butyryl cholinesterase inhibition activity and the possibility of substitution of the pesticide by a natural molecule.

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