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# Performance evaluation of UV/$H_2O_2$ process applied to treat chlorpyrifos ethyl in aqueous solution: Investigation of the genotoxicity using Single Cell Gel Electrophoresis Assay

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## ABSTRACT/RESUME

***Abstract:*** *This study aimed at evaluating the performance of chlorpyrifos ethyl degradation (CP) by a 254-nm ultraviolet irradiation (UV) or in the presence of hydrogen peroxide ($H_2O_2$) or during a UV/$H_2O_2$ process. The degradation rates of chlorpyrifos ethyl, an organophosphate (OP) used in agriculture, were respectively 24 %, 83% and 93%. With the three types of treatments, the degradation of this organophophate showed a pseudo first-order kinetic pattern. The advanced oxidation process with a UV/$H_2O_2$ treatment was found to be a technically feasible approach for the removal of chlorpyrifos ethyl. In addition, the genotoxicity of the studied compound, before and after several treatments, was evaluated* in vivo *in the Mediterranean wild mussels – the* Mytilus galloprovincialis *- using assay comet for the quantification of DNA fragmentation. The percentage of genotoxicity was 42% and 43% after an exposure to 0.66 mg $kg^{-1}$ body weight (bw) and a 0.33 mg $kg^{-1}$ bw of CP, respectively. The genotoxicity decreased at 20% when tested with the CP obtained after a UV/$H_2O_2$ treatment. However, after a four-day biological treatment with Pseudomonas peli, we observed a rate of degradation of 98% and the percentage of genotoxicity was 8% and 15% after an exposure to 0.66 mg $kg^{-1}$ bw and a 0.33 mg $kg^{-1}$ bw of CP, respectively.*

## I. Introduction

For decades, the widespread use of pesticides led to their scattering in the environment with enduring consequences. The organophosphate pesticides (OP) are the most frequently encountered [1]. OP compounds can be highly toxic with a broad range of effects that include cancer, reproductive dysfunctions and neurobehavioral deficits. The chlorpyrifos ethyl (CP) is one of the most widely-used organophosphates or insecticides, used in agriculture. Chlorpyrifos ethyl (CP) is a non-systemic insecticide effective against a wide range of insect pests damaging crops with important economical stakes [2]. The accumulation of CP metabolites in fields and water bodies receiving agricultural runoffs has been studied [3]. It also appears that the subchronic exposure to chlorpyrifos ethyl can cause the alteration in the reproduction of a gastropod – the *Planorbarius corneus* - and in the survival of its offspring [4]. Chlorpyrifos ethyl is degraded to its oxon form, the chlorpyrifos-oxon (CPO), which could lead to a significant contamination when adults and offsprings are exposed, especially the younger ones which are a population particularly at risk when

exposed to CP and CPO [5]. CPO makes a stable covalent adduct on the tyrosine residue of blood proteins for people who ingest CP [6].

Several techniques can be used in order to degrade chlorpyrifos ethyl. Among them is a biological treatment using a pure strain of *Sphingobacterium* sp. JAS3 [7], *Cupriavidus* sp. DT-1 [8] and *Pseudomonas* sp. [9]. Dellai et al., 2013 [10] observed that chlorpyrifos ethyl was completely degraded after a 96-hour shaking incubation of *pseudomonas peli* isolated from the Oued Hamdoun River (Tunisia).

The electrochemical oxidation with a boron-doped diamond can be successfully used to treat wastewater polluted with CP [11,12]. Samet et al. noted that the anodic oxidation with an $Nb/PbO_2$ electrode constituted a viable method for the treatment of effluents contaminated with CP [13].

It appeared that advanced oxidation processes (AOPs) were effective in destroying organic pollutants. Most of them were associated with the production of radical species, mainly hydroxyl radicals ($OH^{\cdot}$), which are strong oxidizing agents (E° = 2.8 vs.ENH at pH 0). These AOPs can include a chemical activation, a photochemical and/or a catalytic activation [14]. A cobalt-60 gamma irradiation was conducted for the treatment of chlorpyrifos ethyl in aqueous solutions. This process proved to be an effective method in removing the CP [15].These different processes were very interesting but very expensive and the unremoved products generated had a potential exposure in food and at home.

A growing interest in UV activated processes was due to the relatively cheap low-energy UV lamps, the ability to avoid the fouling of UV lamps by using non-contact reactors, and the simultaneous use of UV rays and chemical oxidants like hydrogen peroxide and mercury lamps with a 254-nm emission being most commonly used in order to generate effective ($OH^{\cdot}$). These radicals can oxidize organic compounds (RH) producing organic radicals ($R^{\cdot}$). The possible reactions occurring during a $UV/H_2O_2$ process are as follows (Eq. (1); Eq. (2)):

$$H_2O_2 + h\square \rightarrow 2\ HO^{\cdot} \qquad (1)$$

$$RH + HO^{\cdot} \rightarrow H_2O + R^{\cdot} \qquad (2)$$

More hydroxyl radicals can attack aromatic rings and the removal rate increases. Above 300 mg $L^{-1}$, hydroxyl radicals efficiently react with $H_2O_2$ and produce $HO_2^{\cdot}$ (Eq. (3)):

$$H_2O_2 + HO^{\cdot} \rightarrow HO_2^{\cdot} + H_2O \qquad (3)$$

Since $HO_2^{\cdot}$ radicals are not as reactive as $HO^{\cdot}$ and the enhancement of $HO_2^{\cdot}$ radicals leads to a negligible contribution in the destruction of organic compounds [16]. This technique has several advantages: indeed $H_2O_2$ may be produced thanks to a cheap and efficient environmentally friendly industrial process entailing no sludge formation and achieved at a room temperature [16-18].

After some chemical treatments, more toxic compounds are produced such as chlorpyrifos oxon and diazoxon [19]. The main product resulting from the degradation of chlorpyrifos ethyl is the 3,5,6-trichloro-2-pyridinol (TCP) - which is more mobile than its parent molecule because its water solubility is greater - which causes the widespread contamination of soils and aquatic environments [20]. Then it is necessary to compare both aqueous solutions toxicity before and after treatment in order to complete the treatment evaluation. The use of the comet assay with the *Mytilus galloprovincialis* mussel in order to study the genotoxicity of a chemical compound or industrial wastewater is possible. Biomonitoring is based on the mussel's ability to accumulate chemical contaminants in its tissues to a degree proportional to their bioavailability [21,22].

The purpose of this research was to study the degradation of an organophosphate, the Chlorpyrifos ethyl, by a photochemical $UV/H_2O_2$ degradation process. It enabled the study of the toxicity of a CP solution before and after treatment using a comet assay with *M. galloprovincialis* and was compared to a previous biological treatment [10].

## II. Materials and methods

### II.1. Chemical and reagents

The Chlorpyrifos ethyl ($C_9H_{11}Cl_3NO_3PS$; O,O-diethyl O-3,5,6-trichloro-2- pyridyl; phosphorothioate; molar mass 350.59 g/mole) (99.9 % purity) was purchased from Sigma-Aldrich (Isle d'Abeau Chesnes, France). The molecular structure of the Chlorpyrifos ethyl is given in Figure1. $H_2O_2$ (30% w/w) and ethanol (99.9 % purity) were from VWR (201 rue Carnot 94126 Fontenay sous Bois, France), acetonitrile Hipersolv Chromanorm (99.9 % purity) was from VWR Prolabo (201 rue Carnot 94126 Fontenay sous Bois, France) and H2SO4 (95%) of Normapur quality, from Prolabo (201 rue Carnot 94126 Fontenay sous Bois, France). For the preparation high performance liquid chromatography (HPLC) mobile phase, High purity water was purified using Synergy UV System from Millipore (SAS BP307 78054 St Quentin Yvelynes, France).

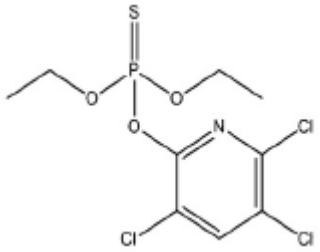

***Figure 1.*** *Chemical structure of chlorpyrifos ethyl*

**II.2. UV radiation source**

The UV source used in this study was the Bioblock Scientific modèle VL 6LM Lamp which produces a highly monochromatic 254 nm UV light.

**II.3. Solution preparation and treatment condition**

A stock solution of a known concentration ($[CP]_0 = C_0$ = 100 mg $L^{-1}$) was prepared by dissolving CP in ethanol. The stock solution was stored at 4°C.

Experiments were conducted with a 100 mg $L^{-1}$ chlorpyrifos solution volume of 50 mL and were magnetically stirred and performed at room temperature. Oxidation ($H_2O_2$ alone) and photo-oxidation ($UV/H_2O_2$) experiments were conducted with $H_2O_2$ solution, which corresponds to an initial hydrogen peroxide concentration ($[H_2O_2]_0$) of 8.5 mol $L^{-1}$. Then the ratio was $[H_2O_2]_0/ [CP]_0$ = 30 mol $mol^{-1}$. Photo degradation (UV alone) and photo-oxidation experiments were conducted with UV lamp at distance of 2 cm. A 1 mL of sample was taken from the reaction mixture for HPLC measurement. All analyses were performed at least in triplicate.

**II.4. Analytical determinations**

The concentration of Chlorpyrifos was monitored by HPLC-UV system with a Waters 515b HPLC pump, equipped with Waters Rheodyne valve with a 20 µL loop, Waters Cartridge Column Novapack $C_{18}$ (4 µm, 150 mm x 3.9). The UV/Visible detector Waters 2489 set at a detection wavelength of 230 nm. The mobile phase consisted of (acetonitril/water 20v/80v) adjusted at pH 3 with $H_2SO_4$ at a flow rate of 1.0 mL $min^{-1}$. The reproductibility of all the measured data were within ± 5%.

**II.5. Biological treatment**

Biological treatment was conducted by *Pseudomonas peli* a non-pathogenic and aerobic bacterium, isolated by Dellai et al., (2013) [10] from Oued Hamdoun River (Tunisia). This natural water was contaminated by organophosphate, in particular by CP. The protocol of biological treatment was well described previously by [10].

**II.6. In vivo toxicity**

Mussel Mytilus galloprovincialis

Toxicity was conducted *in vivo* using Mediterranean Mussel *Mytilus galloprovincialis* (Mollusca, bivalvia) with mean shell length 4 ± 1 cm and weight 30 ± 2 g which were collected from a cultured population located in the city of Bizerte (North of Tunisia). Only male specimens were selected. Mussels were transferred to the laboratory in ice-cold plastic tanks within 2 h after the collection according to the method described by Raftopoulou et al, 2012 [23].

Animal treatment

The mussels were maintained without food supply, for at least 1 week, in order to be acclimated to laboratory conditions. After the acclimation period, 3 groups of mussels (15 mussels/group or subgroup) were placed in static tanks, containing 10 L of natural aerated seawater (recovered from Chebba city (center of Tunisia)).

- Animals of group A were given increasing doses (two subgroup) 0.33 mg $kg^{-1}$ body weight (bw) and 0.66 mg $kg^{-1}$ bw administrated into the posterior adductor muscle of culture medium of *Pseudomonas peli* run without chlorphyrifos ethyl (CP) as negative control group.
- Animals of group B were given increasing doses (two subgroup) 0.33 mg $kg^{-1}$ bw and 0.66 mg $kg^{-1}$ bw administrated into the posterior adductor muscle of chlorphyrifos ethyl treated with *Pseudomonas peli*.
- Animals of group C were given increasing doses (two subgroup) 0.33 mg $kg^{-1}$ bw and 0.66 mg $kg^{-1}$ bw administrated into the posterior adductor muscle of chlorphyrifos ethyl treated with photo-oxidation ($UV/H_2O_2$).

Note: The selected dose of CP and its degradation derivatives administered to animals did not increase mortality rates, body weight, feed intake and the size and shape of liver and kidney.

After 24 h of incubation in 5 L of natural aerated seawater, mussels were opened, the byssus taken off and the soft body part (gills) removed from the shells in order to assess the genotoxicity.

The protocol of genotoxicity using comet assay was well described by [23,24]. The total score of DNA damage (TDD) was calculated by the following equation (Eq. (4)):

TDD = (Percentage of cells in class 0x0) + (Percentage of cells in class 1x1) + (Percentage of cells in class 2x2) + (Percentage of cells in class 3x3) + (Percentage of cells in class 4x4) (4)

The percentage of tail DNA (%) was calculated using the following equation (Eq. (5)):

Percentage of tail DNA = 100 x ((TDD of treated cells with with CP or treated CP) – (TDD of untreated cells)) / TDD of untreated cells. (5)

### II.7. Statistical analysis

For all our experiments, a one-way ANOVA was used to analyze the differences between groups, followed by a Duncan's test with a threshold of significance of $p<0.01$ and $p<0.001$ to detect specific differences, using a statistical software package (STATISTICA edition 99 Maisons-Alfort-France). We used this post hoc test or multiple comparison tests, to determine the significant differences between a single control group mean and the remaining treatment group means in an analysis of variance setting.

## III. Results and discussion

Kinetic experiments were performed to investigate the effect of different process on the degradation of chlorpyrifos ethyl (Figure 2 and Figure 3). The amount of chlorpyrifos ethyl decreased more quickly with UV/$H_2O_2$ process (Figure 2). This is due to the enhanced production of hydroxyl radicals (Eq. (2). After 90 min of treatment, CP removal decreased.

Chlorpyrifos ethyl is a photolyzable compound at 254 nm. In the presence of $H_2O_2$, the overall kinetics equation includes two components, direct photolysis and degradation by hydroxyl radicals formed (Eq. (6)) [8].

$$-\frac{d[C]}{dt} = \left\{-\frac{d[C]}{dt}\right\}_{photolyse} + \left\{-\frac{d[C]}{dt}\right\}_{^{\bullet}OH} \quad (6)$$

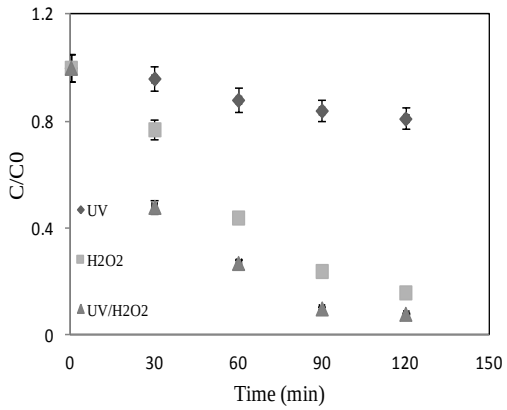


***Figure 2.*** *Time-courses of chlorpyrifos ethyl degradation by UV photolysis, $H_2O_2$ oxidation and UV/$H_2O_2$ photo-oxidation. $C_0$ = 100 mg $L^{-1}$, $[H_2O_2]_0$ = 8.5 mol $L^{-1}$, UV radiation: 254 nm. Data are expressed as mean ±standard deviation*

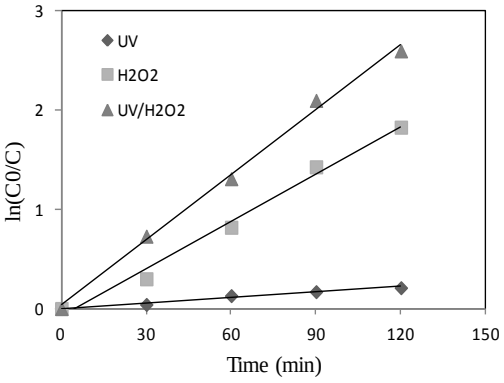


***Figure 3.*** *Plot of ln($C_0$/C) as a function of time for chlorpyrifos ethyldegradation by UV photolysis, $H_2O_2$ oxidation and UV/$H_2O_2$ photo-oxidation, assuming the reaction is pseudo-first order. Data are expressed as mean ±standard deviation*

It proves that integration of this equation subject to the initial condition $C=C_0$ at t=0 leads to the following equation (Eq. (7)):

$$Ln\left(\frac{[C0]}{[C]}\right) = (K_{app(photolyse)} + K_{app(^{\bullet}OH)}).t = K_{app(total)}.t \quad (7)$$

In Eq. 7, $K_{app}$ and $K_{app(total)}$ are respectively the rate constant and the global apparent rate constant for CP removal. The above equation corresponds to a pseudo-first-order kinetics reaction model (Fig.3).

The regressions for each of the three processes are distinct from each other. $K_{app(total)}$ of the lines of best fit in Figure 3 were calculated for the different process and presented in Table 1. The goodness of fit of the experimental data to the proposed model attested by high $R^2$ values is also apparent from

Figure 3. The CP degradation by UV/$H_2O_2$ treatment occurs much more rapidly than by using only UV or $H_2O_2$. Therefore, for this substrate, the UV contribution to the whole UV/$H_2O_2$ process can be disregarded.

**Table 1.** Effect of process on the apparents pseudo-first rate constant under the study conditions

| Process | $K_{app(total)}$ ($min^{-1}$) | $R^2$ | $t_{CP.5}$ (min) | $t_{CP.9}$ (min) | $t_{CP.99}$ (min) |
|---|---|---|---|---|---|
| UV | 0.0018 | 0.9766 | 385 | 1279 | 2558 |
| $H_2O_2$ | 0.0151 | 0.9844 | 46 | 152 | 305 |
| UV/$H_2O_2$ | 0.0223 | 0,9956 | 31 | 103 | 207 |

At the time ($t_{CP.5}$) required for CP degradation of 50% (CP.5) (Eq. 6) the kinetic equation is given by Eq. (8) and Eq. (9):

$$\mathrm{Ln}\left(\frac{[C0]}{\left[\frac{C0}{2}\right]}\right) = K_{app(total)} . t_{CP.5} \qquad (8)$$

$$t_{CP.5} = \ln 2/K_{app(total)} \qquad (9)$$

At the same, 90% (CP.9) and 99% (CP.99) was calculated by using the following formulas Eq. (10) and Eq. (11):

$$t_{CP.9} = \ln 10/ K_{app(total)} \qquad (10)$$

$$t_{CP.99} = \ln 100/ K_{app(total)} \qquad (11)$$

The dose require for 50% (CP.5), 90% (CP.9) and 99% (CP.99) removal of chlorpyrifos ethyl is summarized in Table 1. Time for obtain 99 % CP removal are lower with UV/$H_2O_2$ process. There is a significant increase in the value of CP.5, CP.9 and CP. 99 with UV process.

The half-life time of CP is lower for the coupling process (31 $min^{-1}$). Doong and Chang (1997) [25] proposed half-life times of five organophosphate pesticides after treatment by UV/$H_2O_2$ and obtained values between 12.8 and 86.6 min.

In the Figure 4, we compared the removal efficiencies of the target molecule by biological treatment (with *P. peli)*, treatment by photolysis (UV), oxidation ($H_2O_2$) and by photo-oxidation (UV/$H_2O_2$) after 120 minutes of treatment. *P. peli* demonstrated its feasability and efficacy to degrade 98% of CP after 96 h of treatment [10]. The treatment by photolysis at 254 nm is insufficient, representing only 24% removal of CP. The results for the combined treatment by UV/$H_2O_2$ showed better removal of CP by 10% in comparison with the oxidation process alone, this is in accordance with Eq. (4). Zhang et al. (2011) [1] also observed that Chlorpyrifos ethyl could be degraded by ultrasonic irradiation but the toxicity increased.

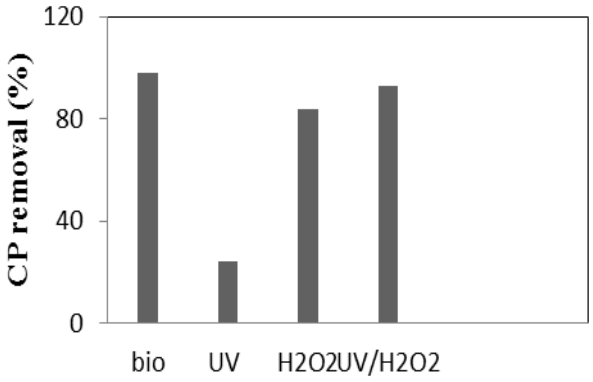


***Figure 4.*** *Degradation of chlorpyrifos ethyl by Pseudomonas peli (bio), by UV photolysis, $H_2O_2$ oxidation and UV/$H_2O_2$ photo-oxidation after 120 min of treatment. Data are expressed as mean ±standard deviation*

However, according to the observation of Dellai et al. (2013) [10] the degradation of some pollutants by *P. peli* doesn't always mean detoxification. For this, in order to examine the treatment level of CP it is necessary to compare the toxicity via genotoxicity of the CP treated biologically (using *P. peli*) , and physico-chemically (using H2O2/UV), two different techniques.

For this purpose, we conducted experiments to evaluate the effect of increasing injection of CP, before and after biodegradation and photo-oxidation treatment, on *M. galloprovincialis*. The *in vivo* genotoxicity of *M. galloprovincialis* was evaluated by monitoring its effects on DNA strand breaks measured with the comet assay also known as single cell gel electrophoresis assay (Mustapha et al., 2013) [23]. Figure 5 showed that the genotoxicity percentage reached about 42% and 43% ($p<0.001$) after exposure to 0.66 mg $kg^{-1}$ bw and 0.33 mg $kg^{-1}$ bw of CP When compared to intact cells (negative control). We have observed a significant dose dependent increase of the total DNA damage in gill cells of bivalves treated with intact CP. Rivandeneira et al. (2013) [4] confirmed our observation and indicated that the subchronic exposure to CP is able to cause alterations in the reproduction of *Planorbarius corneus* and in the

survival of the offspring. In the other hand, the ability to induce DNA damage in gill cells was significantly decreased when tested CP treated with phtooxidation and *P. peli* (Figure 5).

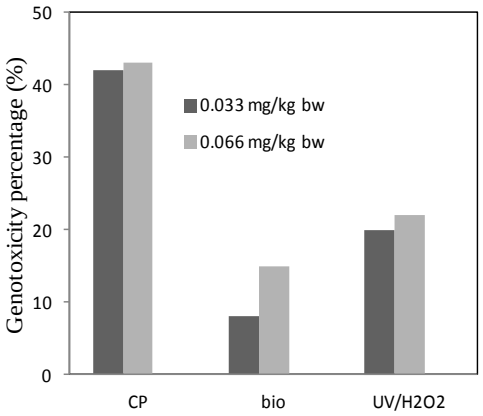


***Figure 5.*** *Comet assay of genomic DNA of gill cells treated with CP solution before any treatment (CP) and after biologique treatment (bio) and UV/$H_2O_2$ photo-oxidation. Data are expressed as mean ±standard deviation*

## IV. Conclusion

The genotoxicity percentage decrease by 50% ($p<0.001$) after photooxidation treatment compared to intact-CP at the high tested dose (0.066 mg $kg^{-1}$ bw). After biodegradation by *P peli* the results were more interesting because the genotoxicity decrease by 81% and 65% ($p<0.001$) after exposure to 0.66 mg $kg^{-1}$ and 0.33 mg $kg^{-1}$ bw of CP respectively. Thus the effect of the exposure dose has an effect on the genotoxicity percentage, especially for biological treatment.

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