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Biochemical characterisation and bioremediation study of dimethoate and chlorpyrifos tolerant bacterial strains isolated from an agricultural soil.

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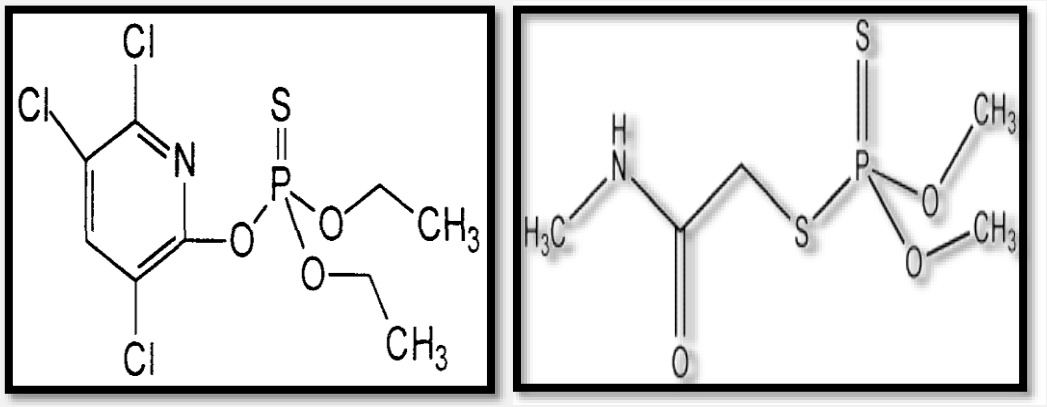
Abstract - Biodegradation processes are increasingly attracting scientists' interest as novel technological methods to detoxify natural matrices contaminated with various pollutants.
Herein, we aimed to isolate, screen and characterize bacteria which tolerate the two pesticides compounds (chlorpyrifos and dimethoate). In order, to distinguish biotic and abiotic pathways responsible of chlorpyrifos and dimethoate break-down, batch of soil have been respectively conserved at their natural form or sterilized. Dissipation of chemical compounds in non-sterile soils followed first-order exponential decay kinetics and the half- life ($t_{1/2}$) of chlorpyrifos and dimethoate ranged from 2.5 to 3.5 and 2.2 to 3.3 respectively. The difference between sterile and non-sterile soils indicates that abiotic dissipation was predominant in the dissipation of chlorpyrifos in Tunisian soil. However, in the case of dimethoate, a microbial activity seems to play a key role in the dissipation of this compound in Tunisian soil.
Bacteria strains that could persist in the presence of the both organophophorous compounds were isolated and identified by biochemical test. The obtained results from pesticides enriched cultures showed the presence of identical strains in both treatments: *Enterobacter cloacae,* and *Panteoa sp.* However, specific strains were isolated only for each active compounds, *Enterobacter cloacae*, *Panteoa sp*, *Pseudomonas putida* and *Enterobacter cloacae*, *Pantoea sp*, *Chromobacterium violaceum*, *Serratia plymuthica*, *Pseudomonas aeruginosa* following treatment with dimethoate and chlorpyriphos, respectively.

Keywords: organophophorous, degradation process, API-Test, soil bacteria

1. Introduction

Organophosphorus pesticides (OP's) represent a large group of highly effective pesticides. The widespread use of OP's has led to severe environmental pollution because these compounds are often transported from their target sites. For instance, OP's may enter aquatic environments *via* soil leaching and/or surface runoff and air drift (Liang et al.2011). . Among this insecticide family, and due to their broad-spectrum activity, chlorpyrifos and dimethoate have been widely used to control insects, particularly in Tunisian orchards. Chlorpyrifos has relatively low water solubility (2 mg.L^{-1} at 25°C), whereas dimethoate is slightly more soluble in water (23-25 mg.L^{-1} at 20°C) (Li et al. 2008). The chemical structures of chlorpyrifos and dimethoate are shown in Fig1.



a. Chlorpyrifos b. dimethoate.

Fig1. The chemical structures of a. chlorpyrifos and b. dimethoate

The environmental behavior of these compounds especially degradation process in the soil represents a fundamental attenuation process governed by both biotic and abiotic factors (Liang et al. 2011) This process could be affected by a variety of interactions among microorganism and a variety of soil constituents. Specially, chlorpyrifos possesses a soil half-life (t1/2) that ranges between 11 and 141 d, depending on the type of soil (Liang et al. 2011). This large variation in half-life has been attributed also to variation in factors such as temperature, moisture content, organic carbon content and pesticide formulation (Li et al. 2007) Chlorpyrifos is rapidly hydrolyzed to its primary metabolite, 3,5,6-trichloro-2-pyridinol (TCP), which is moderately mobile in soil. Previous studies showed that TCP is persistent in the soil, and the $t_{1/2}$ of TCP ranges from 42 to 49 d in surface soils and 64 to 117 d in subsurface soils (Liang et al. 2011)

For dimethoate, the $t_{1/2}$ ranged from 2 to 41 d (Singh & Walker., 2006). Chlorpyrifos, dimethoate and their primary metabolites can accumulate in Tunisian soils if they are continuously applied, which may result in the contamination of groundwater and surface water near the application site. However, data on the environmental risks of these OP's and their metabolites are scarce (Liang et al. 2011). The control of chlorpyrifos, as pollutant, is of a great importance because they are toxic and recalcitrant. The use of bacteria degrading particularly chloryrifos and/or dimethoate for bioremediation of sites contaminated by these molecules has been proved to be the most potential clean-up method (Li et al .2008). In fact, microbial activity has been deemed the most influential and significant cause of organophosphorus pesticide removal. Therefore, biodegradation is considered to be a reliable and cost-effective technique for pesticide reduction and a major factor determining the fate of organophosphorus pesticides in the environment.

There is an increasing need to develop new methods to detect, isolate and characterize the strains playing a part in these degradation processes. These strains could be generalists or specific to a soil which has been treated during several years. This microbial population can exist in rhizosphere soil and have been shown the ability to degrade organic chemicals including pesticides (Yang et al .2005).
In this study, strains of bacteria having the potential to degrade chlorpyrifos and/or dimethoate are isolated from soils having previously received treatment by these active ingredients on Tunisian orchards situated in the southern region of Tunis. Moreover, the identification of these strains will be carried out to compare their ability to degrade these molecules. Through comparative experiments in sterile and non-sterile soils, the potential of strains for the bioremediation of OP's would be also determined. The results of the present study would be used to assess the environment risk of OP's in Tunisian soils and to evaluate, *in situ*, a potential bioremediation process for the reduction of OP's.

2. Materials and methods

2.1. Chemicals and soil

Chlorpyrifos and dimethoate (> 99%) were purchased from Sigma Aldrich. HPLC grade methanol and ethyl acetate were obtained from laboratory of pesticide, The Minister of Agriculture, Tunisia.

Two stock solutions of each pesticide chlorpyrifos and dimethoate (2000 $mg.L^{-1}$) are prepared by dissolving the required amount in ethyl acetate.

Soil samples used in this study are collected from a field site of a typical orchard of the region of Mornag approximately 20 Km away from Tunis (Tunisia). The private farm "Dalia" has been chosen because we find many fruit trees with a good history of pesticide use, according which we have selected chlorpyrifos and dimethoate largely used in this orchard and in the region of Mornag. Soil samples were collected from the surface layer of soil (0–20 cm). They are air-dried at room temperature, mixed thoroughly and sieved (2 mm) to remove stones and debris. Soil samples were stored at 4°C before use. The physico-chemical properties of the soil sample, shown in Table 1, are carried out by a private laboratory Food Quality" located in Tunis (Tunisia).

Table 1 Selected properties of Tunisian studied soil

% clay	32.48
% silt	36.47
% sand	31.05
pH	8.55
Organic matter ($g.kg^{-1}$)	1.7
C/N	10.37
Electrical conductivity	0.25
Total C(calcaire)	33.78
Calcaire actif	11.29
Total N ($g.kg^{-1}$)	0.95
Available P ($mg.kg^{-1}$)	79.21
Available K ($mg.kg^{-1}$)	497.69
Available Ca ($mg.kg^{-1}$)	6521.72
Available Mg ($mg.kg^{-1}$)	669.68
Available Na ($mg.kg^{-1}$)	406.69

2.2. Dissipation studies

Soil samples were collected from tunisian soil from the surface (0-20 cm) which were treated for few years with the same molecules (chlorpyrifos and dimethoate) and the hypothesis is that the soil micloflora is adapted to these compounds.

2.3. Extraction of chlorpyrifos and dimethoate from soil

Soil samples (5 g dw) were placed into centrifuge tubes, and 25 mL of ethyl-acetate were added respectively to soil treated with chlorpyriphos and dimethoate. The samples (5g) were ultrasonicated for 30 min at 30°C. After that, the soil mixture was centrifuged for 20 min. Each sample was filtered through a Buchner funnel and filtrates were collected in vials of 1.5 mL for further analysis by LC-MSMS.

2.4. Microbial methodology

2.4.1. Bacterial growth media

For bacterial isolation from soils and bacterial characterization performed in the laboratory, the bacterial culture was carried in the Plate Count Agar (PCA), containing 23g gelose per liter of distilled sterile water was used for the isolation of bacteria.

2.4.2. Isolation and culture of strains

In order to isolate bacteria from soil which are involved in degradation of chlorpyrifos and dimethoate, soil samples were collected from agricultural fields where soil had been exposed to the two insecticides during 5 years at least. Soil samples were divided in six pots (50 g of soil in each pot). Chlorpyrifos and dimethoate solutions of $10mL.L^{-1}$ in water (10 fold as recommended for agricultural application) were prepared each day of use from a formulated solution for agriculture use: Robust®

and Biomat® containing respectively chlorpyriphos and dimethoate as commonly used by some farmers of the region of Mornag.
At the laboratory, pots are irrigated on alternate days (the 1st, 3rd, 5th and 7th) to avoid excess irrigation and let the soil to equilibrate for one day and incubated under aerobic conditions at room temperature aiming to stimulate the specific pesticide-degrading microbial community . Excess solution lixiviated from a hole at the bottom of the pots Irrigation consisted of adding to the pot 10mL of commercial chlorpyrifos and dimethoate solutions as 10X concentrated solutions of pesticides to boost the selective pressure on tolerant bacteria. All the experiments were done in triplicate. Another three pots were used as controls irrigated only with distilled water.

The second day of each treatment, soil was sampled for bacterial isolation. One g of soil sample is taken aseptically and diluted in 10 mL of physiological water (NaCl 9‰) From this suspension, the bacteria isolation is done by the technique of diluting counting (three dilutions were prepared 10, 10^{-2}, 10^{-4}) on PCA plates (streaking of 100µL of each and incubation for 16h at 37°C).
We based our primary distinction between the isolates on the size, color and morphology of the colonies on the PCA plates, the homogeneity of colonies is considered as a proof of homogeneity of bacteria (as a primary simplify approach); to confirm the identity of the apparently similarly-shaped bacterial colonies, we analyzed colonies from each plate (after purification with the Z streak microbial technique) by means of API test. The three control pots were useful for comparison of bacteria with treated pots at the beginning in treated pots and in the control pots.
Bacterial strains were isolated and biochemically characterized. Biochemical characterization was performed using API 20E (ref 20 100/20 160) (Biomérieux, Lyon, France).

2.4.3. Preparation of inoculums

Strains cells are pre-cultured in LB liquid (10 g of tryptone, 5 g of yeast extract of and 5g of NaCl per 1liter of distilled water) at 30°C, and the cells were obtained by centrifugation at 5000*g* for 5 min. The pellet is washed twice with sterile water, and cells of strains are inoculated at a concentration of 10^5 CFU $g^{-1}$of dry soil. In fact, inoculum density has a marked effect on degradation of chlorpyrifos and dimethoate. *Enterobacter* sp. is unable to degrade chlorpyrifos below an inoculum density of 10^3 cells g^{-1} (Singh et al.2006).

2.4.4. Soil treatment

In order to prove the effect of bacteria strains in the degradation of chlorpyriphos and dimethoate, we have carried out the degradation process in the soil using bacteria. The bacterial culture suspension (100µL) containing 10^5 CFU per mL^{-1} is inoculated into *Pétri* dishes containing 10g of autoclaved soil and non-autoclaved soil and 2µg.g^{-1} concentration of chlorpyrifos and dimethoate. Uninoculated soil was kept as control. Sterile water was employed to adjust the soil moisture to 30%. The dishes were incubated at 24°C and for different time intervals (0, 3, 5, 7 and 15 days). For each treatment, triplicate soil samples were analyzed by LC-MSMS.

2.5. Chromatographic analysis:

Soil extracts were analyzed using LC-MSMS equipped with a Primacle DBAQ C_{18} column (100 mm X 2,1 mm ;1,9µm). The mobile phase consisted of a mixture of 20% Me OH and 80% H_2O. The flow rate of the mobile phase was 0,2mL min^{-1} and 10µL of standard/ sample solution was injected into the LCMSMS system where the column was kept at40°C in a column oven. MSMS detection was performed using an Applied Biosystems.

2.6. Data Analysis

Data obtained from the degradation experiments are fit to the exponential decay model ($C= C0\ e^{kt}$) to obtain the degradation rate constant (k). The half-life (t1/2) is obtained from the equation $t_{1/2}$= Ln (2)/k.

3. Results and discussion

3.1. Degradation process of chlorpyriphos and dimethoate

The degradation of the two pesticides in the autoclaved soil and non-autoclaved soil followed first-order kinetics (Fig 2-3). This values was illustrated by the high values of the coefficient of determination (r^2) obtained by fitting the linear first-order kinetics model to the degradation data. A better fit was evident for dimethoate degradation ($r^2 > 0.81$) in all cases compared with chlorpyrifos were r^2 values exceeded 0.85 with the single exception of the degradation of chlorpyrifos in autoclaved soil where the r^2 0.78 and 0.73. Degradation of chlorpyrifos was rapid in all substrates with the values ranging from 2.56 to 3.15 days (table 2). However, the $t_{1/2}$ in autoclaved soil and non-autoclaved soil for chlorpyrifos were similar (2.56 days). Thus, no difference in the $t_{1/2}$ of autoclaved and non-autoclaved soil was observed, indicating that abiotic degradation was predominant in the degradation of chlorpyrifos in experimental soil. Previous study (Liang et al.2011) confirmed these results, the $t_{1/2}$of chlorpyrifos in sterile and non- sterile soil was 10.6 and 8.40 days respectively in native paddy soil.

For dimethoate, the $t_{1/2}$ in autoclaved soil was 3.3 d. In non-autoclaved soil the $t_{1/2}$ was 2.39 d.

Thus, a difference in the $t_{1/2}$ was observed indicating that microbial degradation may play a significant role in the degradation of dimetohate.

In fact, sterilization of soil did not appear to have a significant effect on chlorpyrifos, suggesting that its degradation was mostly abiotic (Kravvariti et al. 2010). This is not surprising considering the high pH of the soil used in the present study (pH=8.55). Previous studies (Racke et al, 2010) have documented the abiotic nature of chlorpyrifos in alkaline soils. Unlike other organophosphorus compounds, chlropyrifos has been reported to be resistant to the phenomenon of enhanced degradation. It was suggested that the accumulation of TCP, which has anti-microbial properties, acts as a buffer in the soil and prevents the proliferation of chlorpyrifos degrading microorganisms (Singh &Walker, 2006). However, for dimethoate, sterilization of soil affect the degradation of this compound indicating that microbial degradation may play a significant role in the degradation of dimetohate.

Table 2: Half-life time of each molecule

Active substance	Type of soil	K(days^{-1})	Half-life time (days)	R^2
Chlorpyrifos	Autoclaved soil	**0,27**	**2,56**	**0,87**
Chlorpyrifos	Non autoclaved soil	**0,27**	**2,56**	**0,85**
Diméthoate	Autoclaved soil	**0,21**	**3,3**	**0,85**
Diméthoate	Non autoclaved soil	**0,69**	**2,29**	**0,81**

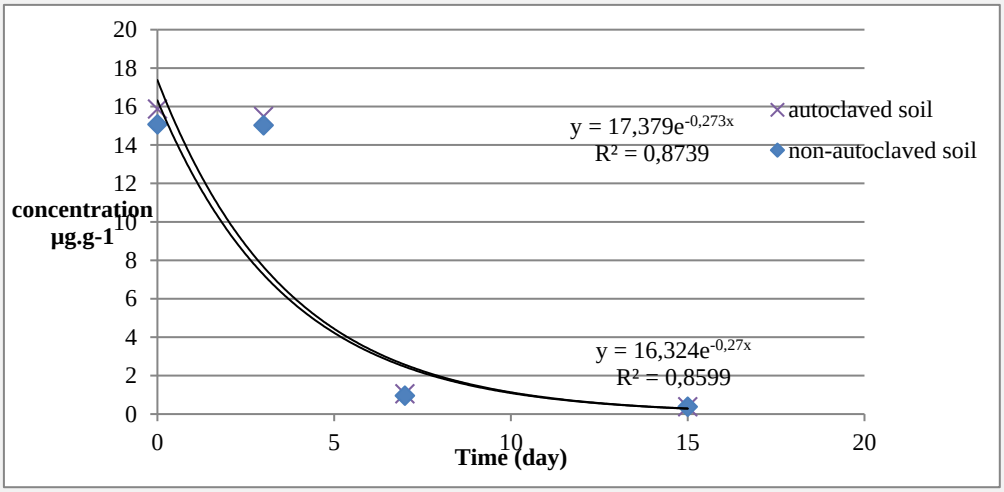


Figure 2. Dissipation of chlorpyrifos in autoclaved and non-autoclaved soil

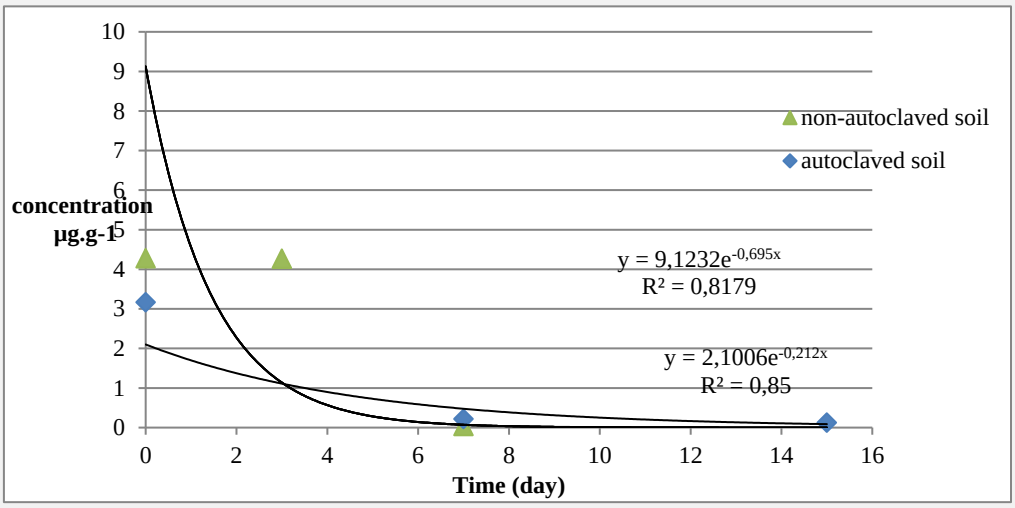


Figure 3. Dissipation of dimethoate in autoclaved and non-autoclaved soil

3.2. Identification of the strains

Results of the first part showed that sterilization of soil decreased the degradation rate, indicating that microorganisms played a significant role in degradation of these compounds. For this reason, we are interested to isolate and identify bacteria from this soil. We based our primary distinction between the isolates on the size, color and morphology of the colonies on the PCA plates; the homogeneity of colonies was considered as a proof of homogeneity of bacteria (as a primary simplify approach) to confirm the identity of the apparently similarly-shaped bacterial colonies. At the beginning in treated pots, as well as in the control pots, there was a high diversity of colonies, in shape, size and color. A decrease in total bacterial number was observed in the treated pots following the first treatment. Biochemical characterization which is a rapid test was performed and coherent results are showed in table 3. Molecular identification will be done later in order to confirm biochemical identification. The degradation of organophosphorus compounds by isolated bacteria has been studied extensively. *Pseudomonas sp* has been reported to have a degrading activity (Goda et al. 2010; Singh &Walker., 2006; Lakshmi et al. 2009; Li et al .2008; Ajaz et al. 2012). Singh &Walker., 2006, showed the ability of *Enterobacter sp* to degrade organophosphorus compounds. *Serratia marscecens* showed 80 % degradation of chlorpyrifos (Lakshmi et al. 2009).

Table 3. Biochemical identification of strains isolated from different soils

Identification of chlorpyrifos-degrading strains	**Identification of dimethoate–degrading strains**
Enterobacter cloacae	***Enterobacter cloacae***
Pantoea spp	***Panteoa sp***
Chromobacterium violaceum	***Pseudomonas putida***
Serratia plymuthica	
Pseudomonas aeruginosa	

3.3. Estimation of the chlorpyrifos and dimethoate degrading capacity of bacterial isolates

To reduce the pollution risk of these compounds, microbial degradation technology was evaluated. To this end, about 10^5 CFU g^{-1} of only for *Enterobacter cloacae* and the mixture of bacteria including: *Enterobacter cloacae Enterobacter sakazakii Acinetobacter baumannii Pantoea* spp., *Protoneus penneri Chromatobacterium violaceum Serratia plymuthica Pseudomonas aeruginosa*, were incubated respectively in soil containing chlorpyrifos. For dimethoate, the $_{1/2}$ in autoclaved soil was 3.3d (fig 6). In autoclaved soil inoculated with *E. cloacae* and inoculated with the mixture of bacteria were respectively 2.2 d and 2.56d (fig 6). In non-autoclaved soil inoculated with *E. cloacae* the $t_{1/2}$ was 2.39 d (fig 7). Thus, insignificant difference in the $t_{1/2}$ of autoclaved and non autoclaved soil inoculated with *E. cloacae* was observed. In fact, the addition of *E. cloacae* to autoclaved and non-autoclaved soil

resulted on a rapid degradation of dimethoate where the DT_{50} was about 2 days. The results indicated that soil inoculated with *E. cloacae* had the shorter life-time compared to inoculated soil with the mixture of bacteria as shown by table 4. Obtained results were similar to previous ones shown by (Singh &Walker, 2006) which reported the ability of *Enterobacter* sp. to degrade organophosphorus compounds especially chlorpyrifos by co-metabolic pathway. For dimethoate, the results indicated that soil autoclaved and inoculated with *E. cloacae* had a shorter $t_{1/2}$ compared to autoclaved soil inoculated with the mixture of bacteria (table4), suggesting that this isolate (*E. cloacae*) is more efficient than others to degrade dimethoate. In the present work, the bacterial isolates successfully degraded dimethoate, chlorpyrifos in autoclaved and non-autoclaved soils, suggesting that these bacterial isolates can compete and survive with the local microflora (Singh et al. 2006). The mode of degradation of organophosphorous compounds especially chlorpyrifos by *enterobacter sp* and *Pseudomonas putida* is respectively catabolic and co-metabolic (Singh &Walker, 2006). Although predominance of a particular Gram-negative species has been only reported from a particular region (experimental soil) previous studies have been successful in isolating diverse organisms capable of enhanced OPs degradation from soils sample which also includes a Gram-positive *Bacillus* sp. (Lakshmi et al .2009). The process of biodegradation of compounds is based on detoxifying enzymes. In fact, microbial enzymes that can hydrolyse organophophorus have been identified and characterized from different microbial species. However, despite the apparent diversity of the enzyme systems, most studies of organophosphorus degrading enzymes have focused on organophosphorus hydrolase (OPH) and organophosphorus acid anhydrolase (OPAA) (Singh &Walker, 2006). To conclude, the bacteria strains isolated by incubation with high concentration of chlorpyrifos and dimethoate from Tunisian orchards previously exposed to pesticides per years by biochemical analysis as *Enterobacter sakazakii*, *Enterobacter cloacae* and *Pantoea spp.* We have successfully demonstrated the degradation of OPs by *Enterobacter cloacae.* The Tunisian isolate could play a significant role in pesticides detoxification meanly in the bioremediation of OPs contaminated sites.

Table 4: Half-life time of each molecule

Active substance	Type of soil	K(days^{-1})	Half-life time (days)	R^2
Chlorpyrifos	Autoclaved soil +*E. cloacae*	**0,22**	**3,15**	**0,78**
Chlorpyrifos	Autoclaved soil +bacteria	**0,20**	**3,46**	**0,73**
Chlorpyrifos	Non autoclaved soil +*E. cloacae*	**0,26**	**2,66**	**0,87**
Chlorpyrifos	Non autoclaved soil +bacteria	**0,24**	**2,88**	**0,91**
Diméthoate	Autoclaved soil +*E. cloacae*	**0,31**	**2,2**	**0,87**
Diméthoate	Autoclaved soil +bacteria	**0,27**	**2,56**	**0,88**
Diméthoate	Non autoclaved soil +*E. cloacae*	**0,29**	**2,39**	**0,99**
Diméthoate	Non autoclaved soil +bacteria	**2,14**	**2,32**	**0,8**

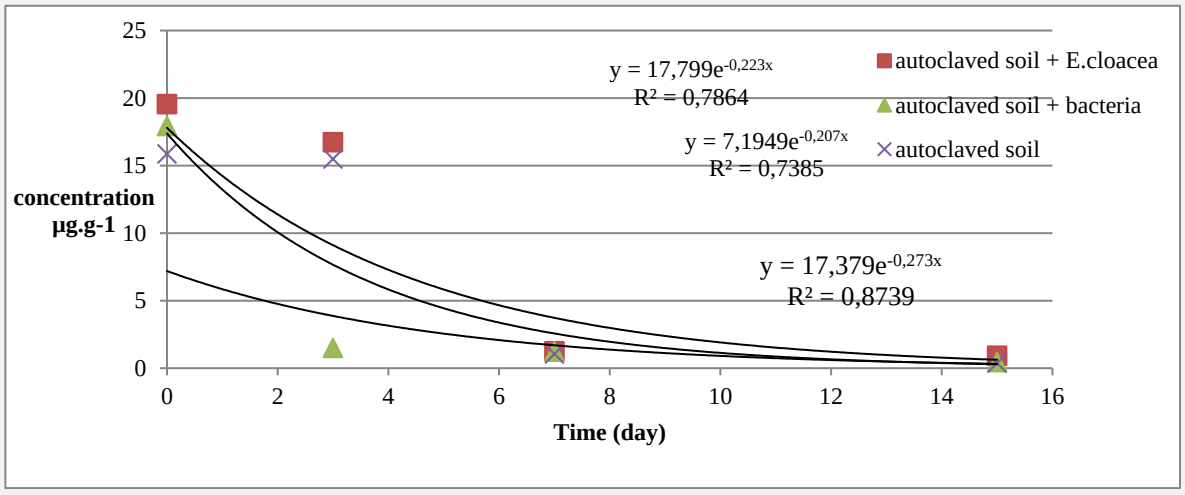


Figure 4. Dissipation of chlorpyrifos by strains in autoclaved soil

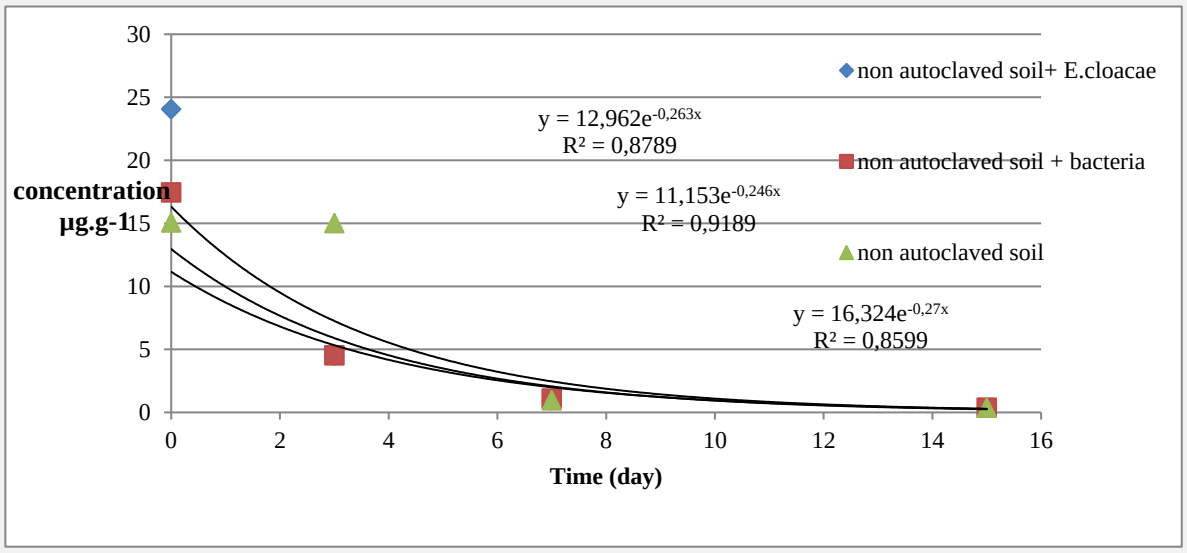


Figure 5. Dissipation of chlorpyrifos by strains in non-autoclaved soil

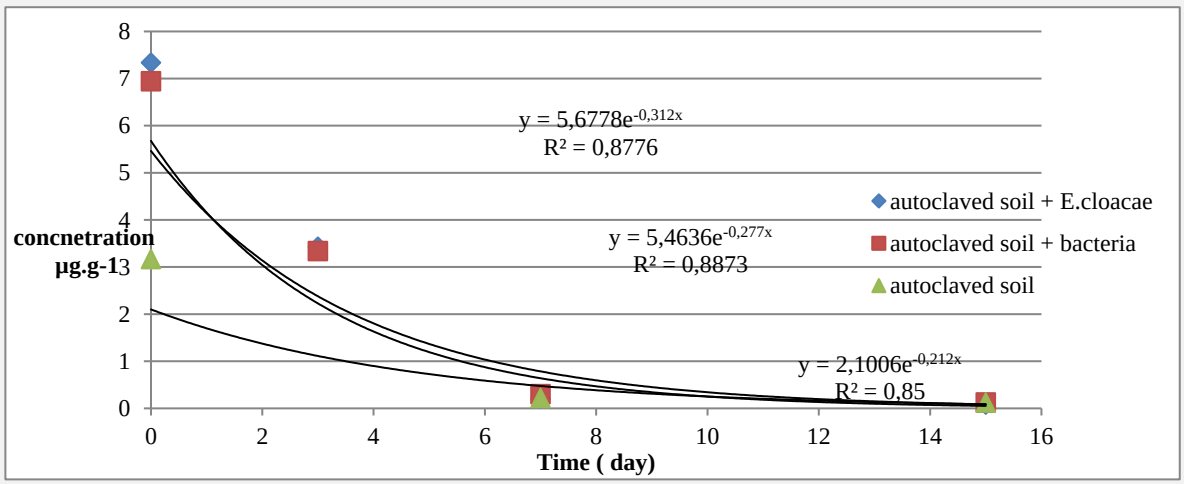


Figure 6. Degradation of dimethoate by strains in autoclaved soil

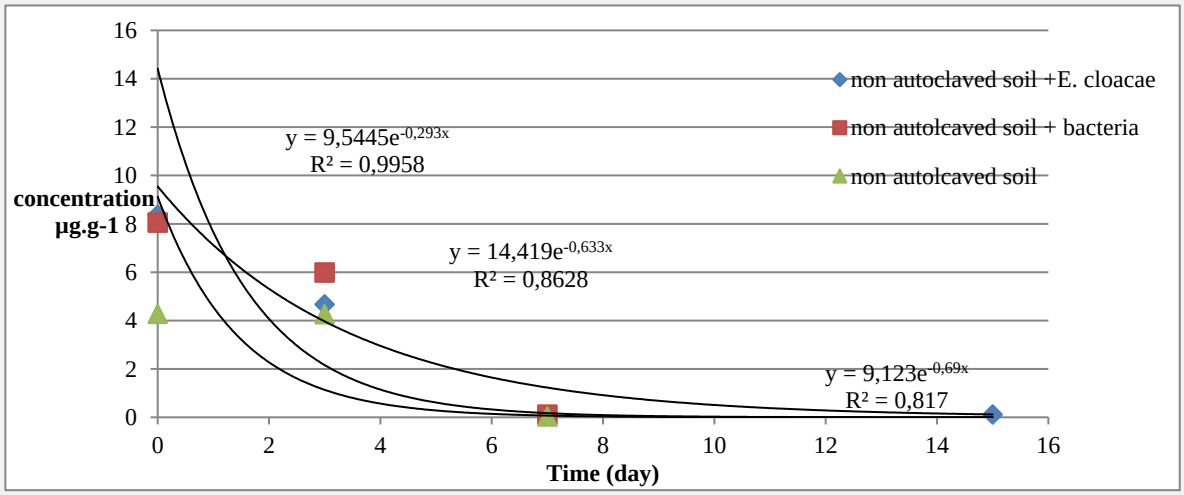


Figure 7. Dissipation of dimethoate by strains in non-autoclaved soil

4. Conclusion

This study confirms the aptitude of the soil microflora of Tunsia to transform chlorpyrifos and dimethoate. In fact, soil pesticide dissipation is effectively performed consecutive to an inoculation, although biodegradation process is not highlighted.

Bacterial strains isolated by incubation with high concentration of chlorpyriphos and dimethoate from Tunisian agriculture soils previously exposed to pesticides per years, were identified by biochemical test respectively as *Enterobacter cloacae , Pantoea spp ,Chromobacterium violaceum Serratia plymuthica Pseudomonas aeruginosa and Enterobacter cloacae Panteoa sp Pseudomonas putida.*

In fact, studies based on selective isolation and characterization of pesticide degrading microorganisms are crucial for understanding the variety of mechanisms related to the accelerated degradation in the ecosystems. For example, chlorpyriphos, which was previously thought to be immune to the enhanced biodegradation, has now been shown to be efficiently biodegraded by bacterial system. This bacterial strains identified could play a significant role in pesticides detoxification meanly in the bioremediation of water contaminated by organophosphorous insecticides.

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