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Biodegradation of Atrazine in Fertile Nile Delta Clay Soil under an Intensive Multicrop Rotation Farming System

Talaat El Sebaï[1*] • Dalia Barakat[2] • Wafaa M. Abd El-Rahim[1] • Hassan Moawad[1]**

[1] Agricultural Microbiology Department, Agricultural Research Division, National Research Centre, El-Behouth street, Dokki, 12311, Cairo, Egypt
[2] Economical Entomology Department, Agriculture Faculty, Cairo University, Cairo, Egypt
Corresponding authors: * talaatelsebai@yahoo.fr ** wafaa10m@hotmail.com

ABSTRACT

Biodegradation of atrazine in Nile Delta agricultural soils previously treated with atrazine under a maize cropping rotation system was investigated under controlled laboratory conditions. Two techniques were used to follow atrazine biodegradation kinetics: i) incubated soil samples and ii) soil suspension (slurry technique). An enrichment technique and atrazine agar indicator plates were used to isolate atrazine-degrading bacteria. Both tested soils showed high and similar ability to degrade atrazine, in which atrazine dissipated completely without detection of any metabolites in soil suspensions at 7 and 3 days after the first and second enrichment, respectively. Atrazine degradation rate constants (*k*) were significantly increased by repeated enrichment: they increased from 0.4 and 0.5 d^{-1} after the first enrichment to 1.3 and 1.2 d^{-1} after the second enrichment for Soil I and II, respectively. Also, atrazine biodegradation kinetics was strongly affected by atrazine application number where atrazine degradation rate constants (*k*) significantly increased by increasing pre-treatment number. However, the atrazine half-life ($T_{1/2}$) was decreased (but not significantly) by previous treatment times where it was 2.45, 2.32, 2.11, and 1.17 days for T0, T1, T2 and T3 soil samples, respectively. These data suggest the presence of an adapted microbial population in these soils able to metabolise atrazine. This hypothesis was confirmed by isolation of seven bacterial strains characterized by their capacity to degrade a high atrazine concentration in the presence of a carbon source.

Keywords: agricultural soil, biotransformation, enhanced, herbicide, previous treatment

INTRODUCTION

This study focuses on the characterisation of atrazine biodegradation kinetics in soil samples collected from fertile Nile Delta agricultural soil where a rotation system was repeatedly applied; that means the maize crop was planted in rotation with other crops and atrazine was routinely applied when the soil was cultivated by maize. In contrast, the majority of previous studies which demonstrated the accelerated degradation of atrazine were carried out on soil samples collected from agricultural soils under a maize monoculture system and treated annually by atrazine.

Atrazine (2-chloro-4-(ethylamine)-6-(isopropylamine)-*s*-trazine is an *s*-triazine-ring agricultural herbicide. It has been extensively used all over the world since its introduction in 1958 to control, through photosystem II inhibition, pre- and post-emergence broadleaf and grassy weeds in major crops such as maize (*Zea mays*), sorghum (*Sorghum* spp.) and sugarcane (*Saccharum officinarum*) (Pick *et al.* 1992; Seiler *et al*. 1992; Mandelbaum *et al.* 1993). Up to the 1980s, application rates of atrazine of up to 2 kg of active ingredient (ai)/ha were used for weed control (Humburg *et al.* 1989). To reduce the environmental impact of atrazine, lower rates (<1.5 kg ai/ha per annum in agriculture crops) have been recommended in several countries for the past two decades (Seiler *et al.* 1992). This molecule has a solubility of approximately 30 mg l^{-1} and a half-life in soil of between 15 and 100 days (Protzman *et al*. 1999). Also, it was found to be persistent in groundwater (Agertved *et al*. 1992; Widmer and Spalding 1995). In soil the dissipation of atrazine can be due either to chemical or biological processes. Chemical transformation of atrazine occurs by dechlorination of the atrazine ring, leading to the hydroxylated derivative, hydroxyatrazine. This reaction occurs mostly under acidic conditions and can be catalysed by soil organic matter (Armstrong *et al.* 1967). However, it is widely known that the biotransformation of atrazine is the most effective process for its dissipation in the soil environment, giving rise to deethylatrazine and, to a lesser extent, to deisopropylatrazine. Historically, atrazine was considered to be slowly biodegradable (recalcitrant molecule) in soil due to the halogen and *N*-alkyl substituents, which impede microbial metabolism of the *s*-triazine ring. However, rapid atrazine mineralization has been recently observed in soils that are repeatedly treated with the herbicide (Barriuso and Houot 1996; Ostrofsky *et al*. 1997; Pussemier *et al.* 1997; Yassir *et al.* 1999) and efficient atrazine-degrading bacteria such as *Pseudomonas* sp. (Yanze-Kontchou and Geschwind 1994; Mandelbaum *et al.* 1995); *Pseudaminobacter* (Topp *et al*. 2000b); *Alcaligenes*, *Ralstonia*, *Agrobacterium* (de Souza *et al.* 1998) and *Nocardioides* (Topp *et al.* 2000a) have been isolated from these soils. The development of enhanced atrazine degradation has been positively correlated with atrazine exposure history and soil pH (Barriuso and Houot 1996; Pussemier *et al.* 1997; Vanderheyden *et al.* 1997; Yassir *et al*. 1999; Houot *et al.* 2000; Hang *et al.* 2003; Zablotowicz *et al*. 2007). This indicates that the application of atrazine to some agricultural soils enhances the build up of a microbial population capable of degrading the herbicide. The widespread use, moderate persistence, and mobility of atrazine in soil has led to frequent detection of atrazine at concentration exceeding the US-EPA maximum contaminant level of 3 µg l^{-1} in surface and groundwater (Harman-Fetcho *et al.* 1999; Kolpin *et al.* 2000), in tile drainage (Buhler *et al*. 1993), and in rivers and streams (Solomon *et al*. 1996; Harman-Fetcho *et al*. 1999; Hoffman *et al*. 2000), resulting in concern regarding the impact of atrazine on human and ecological health. Atrazine has been shown endocrine disruptor activity and there is evidence that it interferes with reproduction and development and may cause cancer (Sass and Colangelo 2006). Its metabolites, such as deethylatrazine and deisopropylatrazine may

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also pose health risks (Brouwer *et al.* 1990; Kolpin *et al*. 1998) and thus may pose serious ecological risks. Therefore, there is a considerable interest in the impact of soil management on persistence and atrazine leaching potential.

MATERIALS AND METHODS

Chemicals

Atrazine (96% technical grade) was kindly supplied by the Pesticide Laboratory Centre, Agricultural Research Centre, Ministry of Agriculture, Egypt. The active ingredient (ai) of atrazine was extracted from atrazine (commercial atrazine formulation, 80% ai) for use in this study and its purity was verified by High Pressure Liquid Chromatography (HPLC) analysis. The atrazine stock solution (100-ppm) was prepared in methanol and stored at 4°C. All other chemicals used were of reagent grade.

Soil samples

Soil samples were collected in April 2006 from the top 0–15 cm top layer of two agricultural fields previously treated with atrazine on maize (*Zea mays* L.) crop at Al-Gharbia Governorate, Egypt. The first one (soil I) was cultivated with courgette (*Cucurbita pepo* L.) and the second (soil II) with potatoes (*Solanum tuberosum* L.) at the time of soil sampling. Soils were passed through a 5.0 mm mesh sieve to remove large stones and debris, then stored at 5-6°C in a plastic bag until used. Soil physico-chemical analyses were done by the Soil and Water Department at the National Research Centre. These properties were EC 2.59 dS m^{-1}, pH 7.72, Ca 7.5, Mg 5.0, K 0.9, Na 14.5, CO_3 0.0, HCO_3 0.75, SO_4 20.9, Cl 6.25 meq l^{-1}.

Atrazine degradation kinetics

Soil suspension (slurry) method

To avoid other processes which contribute to atrazine dissipation in particular sorption process, and to increase the atrazine availability to microflora, as well as to facilitate following atrazine residues, atrazine degradation were determined by measuring the changes in aqueous concentrations of atrazine in soil suspensions as follows: 5 g equivalent of dry soil from each soil (used as an inoculate) were suspended in 250 ml Erlenmeyer flasks containing 45 ml of atrazine mineral salts medium (resulting in a 10^{-1} dilution) amended with atrazine (20 mg ai l^{-1}). The atrazine mineral salts medium contained (per liter of deionized water): 1.6 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$; 20 mg atrazine; 10 ml of a sodium citrate stock solution (100 g l^{-1}) 1 ml of a trace element solution; 1ml of a vitamin stock solution and 1 ml of $FeSO_4 \cdot 6H_2O$ stock solution (5 g l^{-1}). The trace element solution contained (per liter of deionized water) 2 g boric acid; 1.8 g $MnSO_4 \cdot H_2O$; 0.2 g $ZnSO_4$; 0.1 g $CuSO_4$; 0.25 g Na_2MoO_4. The vitamin stock solution contained 100 mg l^{-1} of thiamine–HCl and 40 mg l^{-1} of biotin. The $FeSO_4 \cdot 6H_2O$, vitamin stock solutions, and sodium citrate were filter sterilized (pore size, 0.2 µm; Acrodisc, Gelman Sciences) and kept at 6°C and added to the medium after autoclaving. Sterile soil (Cont-P) receiving the same treatment and soil-free control (Cont-N) were used as positive and negative control. Three replicates were prepared for each treatment. The flasks were incubated in the dark at 30 ± 2°C on an orbital shaker at 150 rpm and sampled periodically by removing a 0.5 ml aliquot to microcentrifuge tube. The samples were centrifuged at 12,000 × *g* for 5 min to remove suspended soil and cells prior to HPLC analysis, the supernatants were stored at -20°C until use. Incubation flasks were weighed and corrected for water loss due to evaporation by addition of sterile water just prior to each sampling. The concentrations of atrazine in soil suspensions were estimated by a reverse-phase HPLC system as described below. When close to 90% of atrazine was degraded (about 6 days after the first enrichment) all enrichment cultures were subcultured for a second time on the same fresh medium (by transferring 5 ml of the old medium to the fresh medium) and atrazine concentrations were followed as previously described.

Incubated soil method

Atrazine degradation kinetics in soils was measured in soil samples incubated with the herbicide under laboratory conditions. For this purpose, five groups from each soil were prepared as following: 50 g of soil (on a dry weight basis) were deposited in a closed 500 ml jar then treated with the suspension of commercial atrazine formulation to give a concentration of 0.6 mg ai kg^{-1} air-dried soil, this dose corresponds with the field recommended dose (1.5 kg ai ha^{-1}). The moisture content of the soils was adjusted to 100% of the soil water holding capacity, taking into account the volume of added atrazine suspension. The jars were incubated at room temperature in the dark. The first group (T1S) was sterilised by autoclaving at 120°C for 1 h for three successive days, then atrazine was added; the second group (T0) was not treated with atrazine. The third (T1), fourth (T2) and the fifth (T3) groups were treated once, twice and three times, respectively with atrazine at 15-day intervals between each treatment. Three replicates were prepared for each treatment. After 45 days from the first atrazine treatment, the four groups T1S, T1, T2, and T3 were treated again with atrazine whereas the T0 group was treated with atrazine only once at the beginning of the experiment, as previously mentioned. The atrazine residues were determined at 0, 1, 3, 5, and 7 days after re-treatment with atrazine as follows: 5 g dry soil equivalent were extracted (twice) with 20 ml methanol on a 200 rpm shaker for 4 h at room temperature. Samples were centrifuged for 15 min at 6,000 × *g* and the supernatant was evaporated at laboratory temperature to dryness. The dried residues were dissolved in 2 ml of methanol and analysed by HPLC as described below.

Isolation of atrazine-degrading strains

To isolate atrazine-degrading bacteria, enriched subcultures were streaked on atrazine agar plates as described by Mandlebaum *et al*. (1995). Briefly, suspensions/dilutions of each enrichment were plated on a solidified atrazine mineral salts medium supplemented with 15 g agar l^{-1}. Atrazine was added to the agar medium as a 2 ml methanolic solution to a final concentration of 500 mg l^{-1} that formed a chalky suspension. The plates were incubated at room temperature in the dark and observed daily. Colonies that developed cleared zones in the atrazine medium agar were purified and routinely maintained in this medium. Strains were kept frozen at -80°C with 12.5% glycerol for further work.

HPLC analysis

The concentrations of atrazine in soil slurries and in soil extracts were quantified with a reverse-phase HPLC using a Microsorb-MV C18 column (length 25 cm, internal diameter 4.6 mm, Varian) and a system LC Star (Varian). The isocratic HPLC mobile phase contained methanol/water (75/25, v/v). The flow rate was adjusted to 1 ml min^{-1} and chromatography was conducted at room temperature (30°C) and the atrazine was detected using a UV detector set at 220 nm.

Data analysis

Dissipation of atrazine was fitted to the equation $C=C_0\ e^{-kt}$ where C_0 is the concentration of atrazine at zero time (mg kg^{-1}), k is the first-order rate constant (d^{-1}) and t is time (d). Half-life ($T_{1/2}$) values for atrazine in soil were calculated from the equation $T_{1/2} = ln2/k$.

Statistical analysis

A single factor analysis of variance (ANOVA 1) was realised to determine significant differences in degradation rate constants (k) and half-life ($T_{1/2}$) of atrazine between the two tested soil, enrichment, and atrazine application number at sampling point during degradation kinetics. Data from each treatment were then compared with the control treatment using Fisher's procedure at each point of the kinetics ($n = 3$, $p < 0.05$). The Statview© 4.55 software (Abacus concept) was used to perform statistical analysis.

RESULTS

Atrazine degradation kinetics

In soil suspensions

The results of soil suspension are presented in **Fig. 1A, 1B** in which atrazine residues expressed as percentages of its initial concentration are plotted against time. The two tested soils show a high ability for atrazine degradation; slight but no significantly differences between the two soils were observed. Atrazine was completely dissipated without detection of any metabolites 7 and 10 days after the first enrichment for soil I and soil II, respectively as shown in **Fig. 1A**. On the other hand, the atrazine concentration remained unchanged for sterile soil (Cont-P) and soil-free controls (Cont-N). The increase in atrazine concentration in sterile soil control may be attributed to deadsorption and dissolution of part of the previous atrazine treatment. The second enrichment (**Fig. 1B**) lead to significant enhancement of atrazine degradation, where atrazine was completely dissipated after only 3 days of incubation for the two tested soils. Also the atrazine degradation rate constant (k) was significantly accelerated where it passed from 0.44, 0.52 day^{-1} through the first enrichment to 1.25, 1.23 day^{-1} through the second enrichment for soil I and II, respectively. Little change in atrazine concentration for the two controls (Cont-P and Cont-N) was observed. The enhancement of atrazine degradation rate constant (about 3-fold) after the second enrichment may be explained by an increase in size and activity of atrazine degraders' microflora. These data suggest that the application of atrazine under a maize rotation system may build up the microbial population able to degrade this molecule.

In incubated soil samples

The results from the atrazine degradation kinetics in soil suspensions suggested that the herbicide is attacked by soil microflora in the two tested soils (I and II). However, an

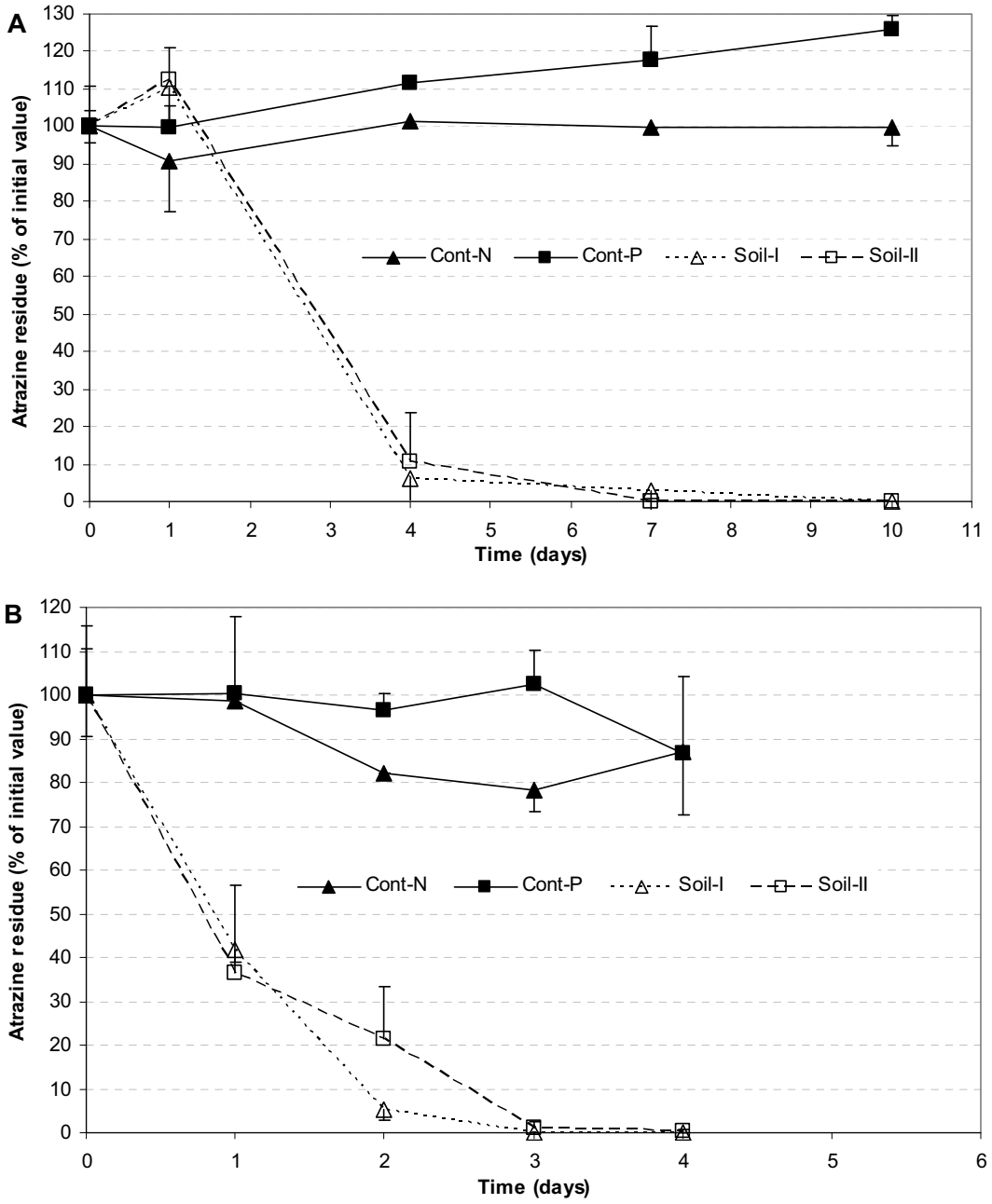

Fig. 1 Degradation of atrazine in soil suspensions prepared from soil (I and II): soil-free control (Cont-N), sterile soil (Cont-P), medium inoculated by soil suspensions prepared from sterile and treated soil, soil I medium treated with soil suspension from soil no. I and soil II medium treated with soil suspension from soil no. II. (**A**) First enrichment; (**B**) second enrichment.

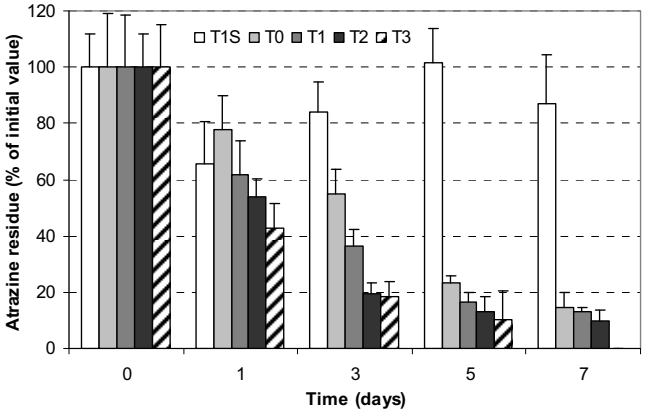

Fig. 2 Degradation of atrazine in soil I in the laboratory using incubated soil samples techniques. T1S soil sterilised and treated, T0, T1, T2, and T3 soil non-treated, treated once, twice, and three times with atrazine in laboratory, respectively.

Table 1 First-order rate constants (k; d^{-1}) and half-livese ($T_{1/2}$, d) for Atrazine degradation in: sterile and treated soil (T1S); soil without previously treatment in laboratory (T0); soil treated previously one time (T1); two times (T2), and three times (T3) in the laboratory.

Atrazine treatments	K^*	$T_{½}^{**}$
T1S	0.0171 a	40.53 a
T0	0.2831 b	2.45 b
T1	0.2985 b	2.32 b
T2	0.3278 c	2.11 b
T3	0.5921 d	1.17 b

*K: First-order rate constant, **$T_{1/2}$: half-life (the time for 50% loss of initial concentration), values are means (n = 3). Considering each parameter values followed by different letters are significantly different ($p < 10^{-4}$). Values followed by the same letter do not differ significantly.

understanding of the degradation kinetics of atrazine in soil samples is needed in order to predict the possible behaviour of atrazine under natural field conditions and to study the effect of atrazine pre-treatment on atrazine degradation rate constants (k) and its consequence on atrazine half-life ($T_{1/2}$). For achieving this objective the soil sample incubation technique was used to study the degradation capacity of soil I. The results obtained from the atrazine degradation kinetics are illustrated in **Fig. 2** and **Table 1**. This soil showed high capacity for atrazine degradation, where atrazine was rapidly dissipated even without previous laboratory treatment (T0 samples) where the atrazine half-life was 2.45 days for T0 samples compared with 40.53 days for treated sterilised soil T1S samples. Also, k was significantly affected ($p < 10^{-4}$) by atrazine pre-treatment as shown in **Table 1** where k increased with the number of atrazine applications, which were 0.23, 0.299, 0.338, and 0.592 day^{-1}, for samples T0, T1, T2 and T3, respectively. The atrazine half-life was not significantly affected by the number of atrazine application as shown in **Table 1** and this may reflect the strong adaptation of this soil. These results may be explained by the stability with an increase of the activity of the microbial population responsible for atrazine degradation.

Isolation of atrazine-degrading bacterial strains

Enriched subcultures were streaked on atrazine indicator plates (containing atrazine as the sole nitrogen source at a concentration as high as 500 ppm) as described in the materials and methods and according to Mandelbaum *et al.* (1995). **Fig. 3** shows the clear zones indicating atrazine degradation. Seven out of 12 bacterial strains showed large clearing zones around their growth. Cleared zones around colonies could be due to atrazine being dissolved, transformed and/or mineralised. These results confirm the presence of microbial populations capable of biodegrading atrazine. This also indicates the ability of the strains to

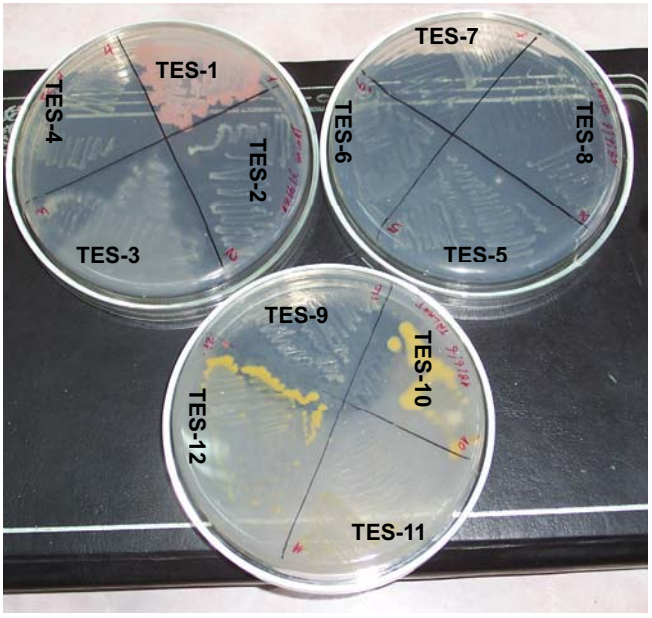

Fig. 3 Atrazine-degrading bacteria showing clearing zones in agar plates supplemented with 500 ppm of atrazine 3 days after incubation. TES-1 to TES12 are the codes of 12 bacterial isolates, seven of which are capable of degrading 500 ppm of atrazine. TES = T. El-Sebai Strain; No control appears in this figure.

metabolize a very high concentration of atrazine in a solid matrix.

DISCUSSION

Biodegradation of pesticides applied to soils is the principle mechanism which prevents the accumulation of these chemicals in the soil and as a consequence prevent their transferred towards other compartments, in particular the surface- and ground-water. One factor that has been shown to increase the rate of microbial degradation of pesticides in the soil is repeated applications of the same pesticides or another pesticide with a similar chemical structure. Enhanced or accelerated atrazine degradation in agricultural soils that follow maize monoculture system and that have a history of atrazine treatment are well documented (Barriuso and Houot 1996; Pussemier *et al.* 1997; Yassir *et al.* 1999; Houot *et al.* 2000; Hang *et al.* 2003; Shaner and Henry 2007; Shaner *et al*. 2007), but few studies on the enhanced atrazine degradation in agricultural soil that follow a maize cropping rotation system have been reported (Zablotowicz *et al.* 2007). The present study indicates enhanced degradation and development of an atrazine-degrading potential under an alternate yearly rotation of atrazine use. We found that the dissipation of atrazine was very rapid in which its half-life was very short (2.45 days) for soil samples (T0) which did not receive atrazine in the laboratory compared to the treated sterilized soil T1S (40.53 days). Moreover, the atrazine half-life decreased by increasing the number of atrazine treatments and became 1.17 days following the third pre-treatment. Also, the results obtained from the soil suspensions showed that the estimated atrazine degradation rate constants (k) increased from 0.4 and 0.5 day^{-1} after the first enrichment to 1.3 and 1.2 day^{-1} after the second enrichment for soil I and II, respectively. These results are in agreement with those reported previously (Zablotowicz *et al.* 2007) and indicated that atrazine mineralization was as rapid in soils under a rotation receiving biannual atrazine applications as in soils under continuous corn receiving annual applications of atrazine. Also the atrazine half-life obtained from our study is consistent with those obtained from previous studies where the half-life of atrazine ranged between 3.5 to 7 days in commercial fields in eastern Colorado, in which, atrazine had been used for 5 years or more

(Shaner and Henry 2007). In Mississippi the half-life of atrazine in fields receiving continuous atrazine for 5 years was 9 days compared with 17 days for an adjacent field that had not received any atrazine (Krutz *et al.* 2007). Our findings indicate that the adaptation of the two tested soils to atrazine treatment may be due to the presence of microbial populations having the potential for atrazine biodegradation. This hypothesis was confirmed by isolating seven atrazine-degrading bacteria on the basis of clear zones observed around the growth of these strains in atrazine agar indicator plates which contain atrazine at high concentration (500 mg l^{-1}) in a medium free from nitrogen. These results are similar to those of Mandelbaum *et al.* (1995) who isolated *Pseudomonos* sp. ADP using advanced subcultures and atrazine indicator plates. This strain showed a clear zone around its growth on the same medium. The same technique was used to isolate numerous species of bacteria capable of metabolizing atrazine from diverse geographical areas, e.g., *Pseudomonas* sp. (Mandelbaum *et al.* 1995; Yanze-Kontchou and Geschwind 1994); *Pseudaminobacter* (Topp *et al*. 2000b); *Alcaligenes*, *Ralstonia*, *Agrobacterium* (de Souza *et al.* 1998) and *Nocardioides* (Topp *et al.* 2000a).

CONCLUSION

The results of this study confirm those of previous studies showing an adaptation of soil microbial populations to atrazine treatment even under a maize cropping rotation farming system. The results obtained from the present study, however, indicate the rapidity of atrazine dissipation without the detection of any metabolites either from soil suspension or from incubated soil samples even without previous laboratory treatments. The presence of microbial populations in both tested soils having the potential to mineralise this molecule was confirmed by isolation of seven bacterial strains from these soils. These isolates are characterised by their strong capacities to degrade a high concentration of this molecule in a solid matrix medium in the presence of a carbon source. Further studies are required to identify and characterise these isolates. This study adds additional information about the biodegradation of atrazine in Egyptian agricultural soil for the first time.

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