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Comparison among the Efficiency of Different Bioremediation Technologies of Atrazine–Contaminated Soils

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Abstract

In a previous study by the authors, toxicity screening of Atrazine-resistant soil bacteria from different contaminated soils resulted in 23-soil isolate best grown in the presence of herbicide Atrazine. They were identified according to their 16S rDNA sequencing into Enterobacter (*E. cloacae*), Bacillus (*B. cereus* and *B. anthracis*), Pseudomonas (*P. aeruginosa, P. balearica, P. indica* and *P. otitidis*), Ochrobactrum (*O. intermedium*) and Providencia (*P. vermicola*). The 23 resistant isolates were enriched in nutrient broth medium amended with 2 folds the recommended dose (RD) of Atrazine. The enrichment technique resulted in the selection of seven bacterial species belong to 4 genera (Enterobacter, Pseudomonas, Bacillus and Providencia) that were superior in their resistance to Atrazine and exhibited remarkable growth stimulation (70.7-88.7%). These four acclimatized and highly Atrazine-resistant strains have been selected and efficiently used for the degradation of Atrazine-contaminated soil. They were employed in different proposed bioremediation technologies including biostimulation (addition of nutrients to enhance the growth and activity of the indigenous microorganisms), bioaugmentation (seeding the most promising indigenous and exogenous Atrazine degraders to accelerate and help the indigenous bacterial population to achieve high and fast remediation of the contaminated soil) and finally a combination of biostimulation and bioaugmentation technologies to investigate the synergistic or suppressive effects of the two techniques. Results proved that bioaugmentation coupled with biostimulation is the most promising bioremediation technology since it is powerful, economical and environmentally friendly technique for decontamination of Atrazine-contaminated soils.

Keywords: Atrazine; Bacteria; Biodegradation; Growth; Pesticides Toxicity

Introduction

Pesticides and their metabolites are of increasing concern because of their potential impacts on the environment, wildlife, and human health. Contamination caused by pesticides in agriculture is a source of poor environmental water quality in some of the European Union countries [1]. Atrazine, 2-chloro-4-(ethylamine)-6-(isopropyl amine)-s-triazine, is a widely used **herbicide**, non-polar compound and moderately retained by the polar soil colloids. Therefore, it can be washed out from the root zone into ground water resources, especially if applied prior to heavy rainfall or irrigation event [2]. Although prohibited in the **European Union** in 2004, it is still one of the most widely used herbicides in the world **[3].** Atrazine is harmful by inhalation, in contact with the skin and if swallowed [4]. It has been reported to have long-term reproductive and endocrine-disrupting effects as well as being a probable human carcinogen and epidemiological connection to **low sperm levels** in men [2]. Even the concentrations meeting U.S. federal standards may be dangerous, with implications for human **birth defects**, low birth weights and menstrual problems [5] indicating an increase health risks [1]. Compared to the other chlorinated herbicides, Atrazine imposes weaker toxicity on humans. However, severe environmental problems emerged due to herbicides persistence in soils, as well as their runoff to surface and groundwater [6].

Most herbicides decompose rapidly in soils via soil microbial decomposition, hydrolysis, or photolysis. Many modern chemical herbicides for agriculture are specifically formulated to decompose within a short period after application [7]. Atrazine **half-life** in soil ranges from 13 to 261 days [8]. Its fate in soil depends upon many different factors including sorption to soil component, uptake by plants, transport via runoff and leaching, biodegradation, photodegradation, volatilization and chemical degradation [9]. In soil, many microorganisms and plants play the major role in biodegradation and elimination of Atrazine and other pesticides converting them into simpler non-toxic compounds whereas physical and chemical forces have limited effects on pesticides degradation [10,11]. Type of soil determines the mobility and fate of Atrazine through sorption to soil particles. Atrazine is more readily adsorbed on muck or clay soils than on soils of low clay and organic matter content; therefore, adsorption to certain soil constituents significantly limits the downward movement or leaching [12-14].

The best-characterized organisms that degrade Atrazine are of **Pseudomonas** spp. as well as other bacteria [15,16], especially when experienced previous exposure to the pesticide or its analogue [17]. Bioremediation technique is more environmentally safe practice particularly for plant protection and its use should lead to an almost complete disappearance of pesticide pollution [18,19]. For biodegradation of Atrazine, **bioaugmentation** or **biostimulation** are the two options exist [20]. This was confirmed by the study of Chelinho et al., [16] when combining bioaugmentation with *Pseudomonas sp.*

ADP and biostimulation with citrate as an efficient bioremediation tool for Atrazine-contaminated soils. Also plant species with well-developed fibrous rooting systems are most effective in enhancing degradation rates and increasing microbial counts within their rhizosphere [11]. Co-application of glufosinate with nitrogen fertilizers may alter Atrazine cometabolism and extended the herbicide's residual weed control in adapted soils [21,22]. Similarly, the presence of other herbicide such as alachlor altered Atrazine persistence in soil compared to other herbicides [23].

The main objective of the present study was to investigate the remediation capability of the indigenous microbial population in loam-sand soil cultivated with corn maize crop and treated with Atrazine. Levels of degradation using enriched soil natural microorganisms in simulated field conditions system was monitored during cultivation course and the dissipation rate of the Atrazine was estimated.

Materials and Methods

Residual concentrations and bioremediation of the herbicide Atrazine were investigated in sandy-loam soils from two completely different environments (Egypt and Saudi Arabia).

Herbicide: Atrazine

Atrazine (Figure 1) was selected based on its common use for the protection of corn, sorghum crops and sugarcane production from weeds and herbs.

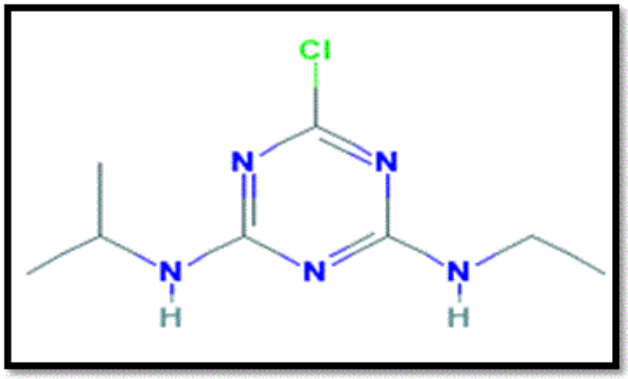

Figure 1: Atrazine Chemical Structure

Soil sampling

Loam Sandy soil samples were collected from two different ecosystems (Hada Al-Shame area, Saudi Arabia ($Soil^H$) and El-Sharqia Governorate, Egypt ($Soil^E$) and used in comparative studies for cultivation of corn to investigate the fate of Atrazine, the applied herbicide. Soils were collected from the top layer of the soil profiles (0-20 cm), passed through a 2 mm sieve, and stored in polyethylene bags until used.

Microorganisms

Twenty three soil indigenous isolates were isolated from different Atrazine- contaminated soils in a previous study by El-Bestawy et al. (2013) [24] (supplementary materials). They were molecularly identified as Enterobacter (*E. cloacae*), Bacillus (*B. cereus* and *B. anthracis*), Pseudomonas (*P. aeruginosa, P. balearica, P. indica* and *P. otitidis*), Ochrobactrum (*O. intermedium*) and Providencia (*P. vermicola*). Among them seven bacterial species belong to 4 genera (Enterobacter, Pseudomonas, Bacillus and Providencia) were found superior in their resistance to Atrazine when enriched in medium amended with 2-fold (2X RD) the recommended dose (RD) of Atrazine set by the Egyptian and Saudi Agriculture Ministries. Therefore, they considered acclimatized, highly Atrazine -resistant and can efficiently be used for the degradation of Atrazine in contaminated soil and/or wastewater. In addition to the indigenous isolates, three exogenous isolates namely *Pseudomonas sp* (PF), *Providencia sp.* (PS) and *Bacillus sp.* (PQ) provided from the collection of the Institute of Graduate Studies and Research (IGSR), Alexandria University were used in the present study. They were isolated from heavily polluted wastewater and environments. Cultures were maintained at 4°C on nutrient agar slants and transferred monthly.

Atrazine bioremediation assays

Development of Simulated Natural Soil System: This experiment was performed as a simulation model for natural soil system exposed to repeated applications of Atrazine to determine the possibility to bio-remediate that system under the natural conditions. Two systems (I and II) were developed for the Egyptian and Saudi soils ($soil^E$ and $soil^H$) respectively, cultivated with corn and sprayed with the Atrazine (80% active ingredient) at dose recommended (RD) by the Egyptian and Saudi Agriculture Ministries. Treatability studies took place for 4 weeks in the two bioassays (one for each soil) using 4 cells (for each bioassay). The four cells represent different bioremediation technologies applied to the contaminated soils in the following manner:

Cell (I): Control (sterile soil, i.e. no indigenous or exogenous microbes)

Cell (II): Biostimulation (i.e. soil with only the indigenous microorganisms+the addition of nutrients).

Cell (III): Bioaugmentation (i.e. soil with the indigenous microorganisms augmented by exogenous microorganisms to help the indigenous species in the biodegradation of the herbicide without the addition of nutrients).

Cell (IV): A combination between Biostimulation and Bioaugmentation (i.e. indigenous soil microbes+exogenous microorganisms+addition of nutrients).

Bioremediation of contaminated Egyptian ($Soil^E$) and Saudi ($Soil^H$) in system I and II: Atrazine-free Egyptian ($soil^E$) and Saudi ($soil^H$) was divided and dispensed into 8 cells (25x25x15 cm plastic basin each) where different bioremediation technologies were applied. Cell (I) was considered as control with sterile soil autoclaved at 121°C for 20 minutes to kill and prevent the growth of the indigenous microorganisms. In cell (II) biostimulation technology was applied where soil was enriched with nutrients ($(NH_4)_2SO_4$] and K_2HPO_4 at 250 and 100 mg/kg respectively) to enhance the growth and activity of the indigenous microorganisms [25]. Bioaugmentation technology was applied to the soil in cell (III) where the most promising indigenous and exogenous Atrazine degraders were seeded at definite ratios to accelerate and help the indigenous bacterial population to achieve high and fast remediation of the contaminated soil. A combination of biostimulation and bioaugmentation technologies was applied to the

soil of cell (IV) to investigate the synergistic or suppressive effects of the two techniques [26]. Each cell was filled with (5 kg) of soil where pre-emerge recommended dose of Atrazine was applied (750 g/200 to 600 L water/Fadden).

Bacterial seeding in cells III and IV was carried out by preparing bacterial mixed culture (125 ml nutrient broth) diluted in 250 ml saline solution (0.85%) and poured evenly all around the cell to ensure that they reached the whole cell. Since all the investigated bacteria are aerobic, oxygen supply was very important for microbial growth so that tilling technique was introduced to the 8 cells 5 times per week by spade to provide maximum oxygen. Sterilized distilled water was added to the 8 cells once a week to sustain wetness and avoid dryness or flooding both of which have deleterious effect on bioremediation process and microbial growth. Water poured evenly all around the cells and mixed well with the soil to ensure even distribution and avoid accumulation of water in the bottom of the cell. The total volume of solution added (inoculum, Atrazine, water) was calculated to bring the soil water content to 60% of its moisture holding capacity. Then soils in the 8 cells were thoroughly mixed with Atrazine to form homogenous medium, and incubated in the dark at room temperature. Four replicates of 50 g soil sample each were collected from each cell at 3 days interval till the end of the experiment duration (4 weeks) where Atrazine residues were extracted and determined using GC. Removal efficiency of Atrazine by the selected technologies was calculated and compared.

Atrazine residues analysis

Extraction of atrazine from soil samples: Extraction of Atrazine from soil was carried out using the method described by Polese et al., [27]. Extraction took place by adding 150 ml ethyl acetate (to give the high percentage recovery) to 50 g soil sample in a conical flask with 20 g sodium sulfate anhydrous. The flask was covered and agitated for 3 hours in a mechanical shaker. The extract was carefully decanted and filtrated through a clean pad of cotton. 80 ml from the filtrate was concentrated using a rotary evaporator at 40°C to remove ethyl acetate, the final reconstituted to extract was an adequate volume (1.0 ml) ethyl acetate to injection in GC without clean up.

Extract clean up: The clean-up procedure was done according to the method of Mills et al., [28]. A mixture of an elution solvent system (50% methylene chloride, 1.5% acetonitrile, 48.5% hexane (v/v/v) was used. A chromatographic column containing to gm activated florisil, then the residues from the column were eluted with 200 ml of this mixture. The eluent was evaporated just to dryness as previously described and the residues were ready for chromatographic determination after being re-dissolved in an appropriate volume of ethyl acetate.

Gas liquid chromatography determination: Hewlett Packard GC model 6890 equipped with nitrogen phosphorus Ni63 electron capture detector was used for Atrazine analysis. GC conditions were as follows: PAS-5 capillary column (30 m length×0.32 mm internal diameter (id) ×0.52 µm film thickness), 5% phenyl methyl polysiloxane. N2 at flow rate of 3 ml/min was used as carrier gas. Injector and detector temperature were 230°C and 280°C respectively. The initial column temperature was 180°C for 2 min, raised at 3°C/min, and then held at 200°C for 15 min.

Calibration curve: Stock solution (100 ppm) of Atrazine was prepared in ethyl acetate. Matrix matched calibration standard at the concentration of 2.5, 5, 10 and 40 mg/kg were prepared. Each concentration was injected under the mention chromatographic conditions. The peak area was plotted against each concentration values of r^2 was 0.9994.

Atrazine recovery efficiency study

Atrazine Recovery: Recovery study was carried out to define the efficiency of the determination method. Untreated samples of soil were fortified with known amount of Atrazine active ingredient solutions. Spiked samples were then subjected to Atrazine extraction, clean-up and determination. Average recovery from spiked samples recorded 89%.

Preparation of blank solution: Solvent and the anhydrous sodium sulphate used in the extraction and clean up were subjected also to recovery test to detect any possible traces of the Atrazine in the solvents.

Results

The efficiency of the most acclimatized and resistant indigenous and exogenous bacteria were evaluated for Atrazine degradation and removal from contaminated agricultural soil. Since reduction of the Atrazine was the main target and the key parameter of the present study, its residual levels (RC) and removal efficiencies (REs%) were determined using the selected bioremediation techniques to determine the most efficient. Biodegradation of Atrazine was function of the soil type and bioremediation technique tested. Results (Table 1 and Figures 2-4) revealed the following points:

As a general trend, Atrazine residues (RC) decreased with increasing exposure times regardless soil type or treatment used.

The highest Atrazine residue levels (RC) were detected at the zero time in all treatments. In system I ($soil^E$), Atrazine residue ranged between the highest (30.94 mg/l) in cell I and the lowest (27.43 mg/l) in cell II while in system II ($soil^H$) it was almost the same in the four treatment (30.03-30.85 mg/l).

With time, regular biodegradation took place leading to continuous reduction in Atrazine levels in both systems. On the other hand, significant variations were detected in Atrazine biodegradation among the different treatment in both systems.

In both systems ($soil^E$ and $soil^H$), treatment no IV considered the most effective recording the highest removals of Atrazine followed by treatment III and finally II compared to the control which showed the lowest removals.

In that respect, biodegradation of Atrazine and removal efficiency (RE) in $soil^E$ using treatment IV recorded 99.95% after 15 day. This was followed by treatment III (RE: 99.90%) and finally II (RE: 99.85%) compared to the control which showed the lowest removals (RE: 99.77%) after the same exposure time.

Similarly, in $soil^H$, treatment IV recorded Atrazine RE of 99.96% after 15 day followed by treatment III (RE: 99.94%) and finally II (RE: 99.92%) compared to the control which showed the lowest removals (RE: 99.1%) after the same exposure time.

In both systems, Atrazine was not detected after 18 days except in the control soils and in $soil^H$ using treatment II.

In conclusion, the combination between biostimulation and bioaugmentation remediation technologies considered the most efficient in removing Atrazine contaminants in both systems

compared to the other tested technologies. This combination reduced the residues to its lowest level 0.015 mg/l in soil^{E} (system I) and 0.012 mg/l in soil^{H} (system II) after only 15 day.

Comparing these results indicated that, removal of the Atrazine herbicide from the contaminated soils achieved by the investigated remediation technologies in the following efficiency order: Biostimulation and Bioaugmentation>Bioaugmentation> Biostimulation>Natural (volatilization).

RC of Atrazine (mg/l)		Soil^{E}							
		Exposure Time (Days)							
	Cell	0	3	6	9	12	15	18	21
	I	30.94	24.9	19	0.36	0.29	0.07	0.01	ND
	II	27.43	22.71	1.42	0.26	0.07	0.04	ND	ND
	III	29.63	8.01	1.35	0.17	0.06	0.03	ND	ND
	IV	30.08	5.71	1.17	0.15	0.05	0.015	ND	ND
Soil^{H}									
RC of Atrazine (mg/l)		Exposure Time (Days)							
	Cell	0	3	6	9	12	15	18	21
	I	30.16	15.54	17.24	0.48	0.31	0.27	0.037	0.009
	II	30.61	13.56	2.31	0.16	0.069	0.025	0.012	ND
	III	30.85	11.95	2.2	0.08	0.047	0.019	ND	ND
	IV	30.03	5.22	1.69	0.06	0.036	0.012	ND	ND

Table 1: Residual Concentration (RC) of Atrazine in Soil^{E} and Soil^{H} Using the Selected Bioremediation Techniques at Different Exposure Times

Cell (I); control; Cell (II): Biostimulation; Cell (III): Bioaugmentation; Cell (IV): Acombination between Biostimulation & Bioaugmentation;ND: Not detected below the detection limit (0.01 mg/l)

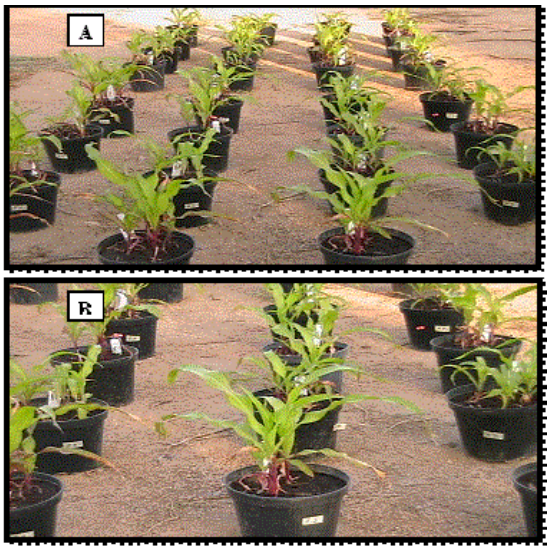

Figure 2: Corn in Soils^{H} and ^{E} 25 Day after Atrazine Application

Discussion

Bioremediation technology (i.e. bioaugmentation and/or biostimulation) considered as an economical and eco-friendly approach and emerged as the most advantageous soil and water clean-up technique for contaminated sites containing heavy metals and/or organic pollutants. Bioaugmentation involves addition of pre-grown microbial cultures to enhance the degradation of contaminants and biostimulation involves injection of nutrients and other supplementary components to the native microbial population to induce propagation at a hastened rate. These are the most common approaches for in situ bioremediation of accidental spills and chronically contaminated sites worldwide. These technologies are controlled by many factors like strain selection, microbial ecology, type of contaminant, environmental constraints, as well as procedures of culture introduction which may significantly enhance biodegradation of the contaminants or may lead to process failure [29]. Effectiveness and limitations of bioaugmentation and biostimulation processes for in situ biotreatment are discussed by many workers [30-32].

Results indicated clearly that combination between biostimulation and bioaugmentation remediation technologies considered the most efficient and best choice in removing Atrazine contaminants in both systems compared to the other tested technologies. This combination reduced the residues to its lowest levels of 0.015 mg/l in soil^{E} (system I) and 0.012 mg/l in soil^{H} (system II) after only 15 day. These results are consistent with those of other workers where microbial biodegradation is considered one of the most viable options for

remediating heavy metal-contaminated groundwater [33] and organophosphate pesticides-contaminated environment [34,35].

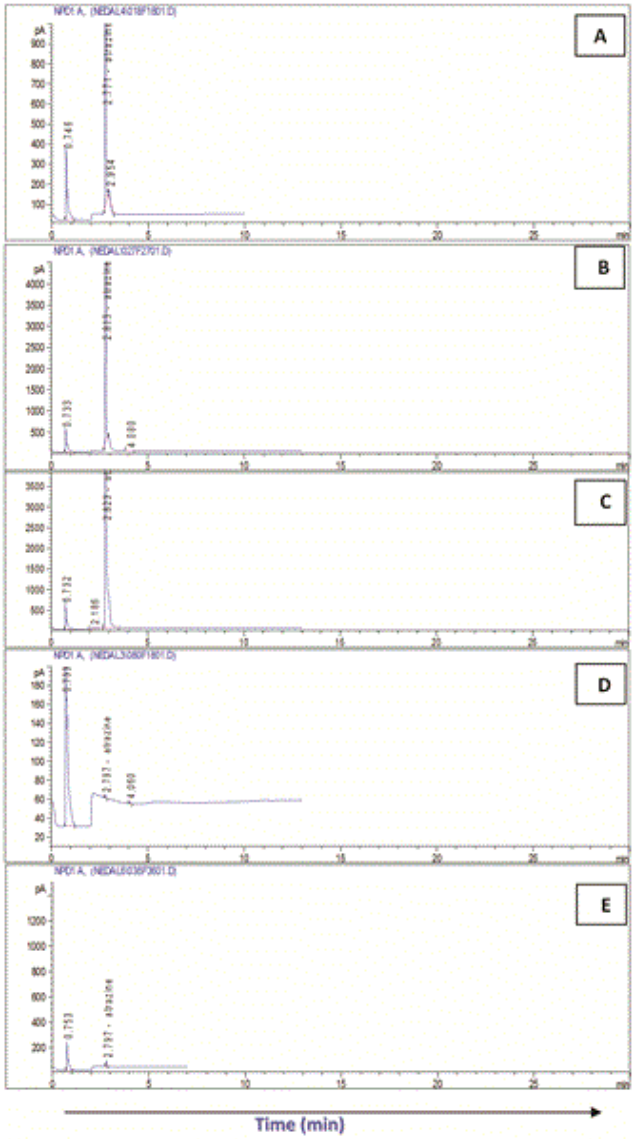

Figure 3: Atrazine Residue after Treatment using Combination of Biostimulation and Bioaugmentation in SoilE at A) Zero B) 3 Days C) 6 Days D) 15 day of Atrazine Application

The initial level of soil contamination controls to a large extent the rate of bioremediation and biodegradation of atrazine. Lima, et al., [30] reported that bioaugmentation with Pseudomonas sp. ADP ($9 \pm 1\times10^{7}$ CFU g^{-1}) resulted in rapid atrazine removal (99% from an initial level of 7.2 ± 1.6 µg g^{-1}) after 8 d independent of citrate. Elevating the initial level to 200×RD of atrazine but with coupling bioaugmentation of Pseudomonas sp. ADP with 2.4 mg g^{-1} citrate amendment (biostimulation) resulted in improved biodegradation efficiency of atrazine (87%) compared to bioaugmentation alone (79%).

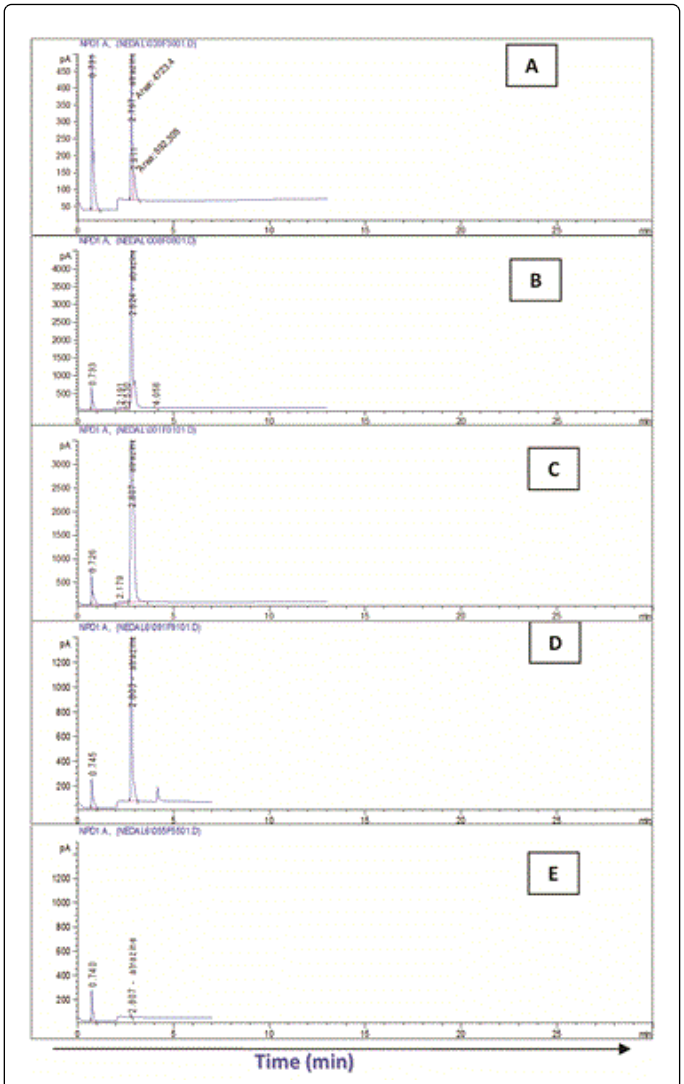

Figure 4: Atrazine Residue After Treatment using Combination of Biostimulation and Bioaugmentation in Soil[H] A) Zero time B) 3 Days C) 6 Days D) 9 Days E) 15 Day of Atrazine Application

These results suggested that bioremediation is valuable tool for efficient removal of atrazine from contaminated field soils to minimize atrazine and its chlorinated derivatives from reaching water compartments. In a similar manner, the combination of bioaugmentation-biostimulation approach coupled with rye cultivation showed the most profound effect on Trinitrotoluene (TNT) degradation, a commonly used explosive for military and industrial applications, which cause serious environmental pollution after 28 day [31].

The high efficiency of biostimulation and/or bioaugmentation was also confirmed for biodegradation of petroleum hydrocarbons. Amendment of indigenous microbial populations with a lignocellulosic substrate and bioaugmentation with two strains of white-rot fungi (i.e., *Trametes versicolor* and *Lentinus tigrinus*) enhanced the native microbiota which promoted the highest biodegradation of creosote, even of those with five aromatic rings after 60 treatment days [32]. Importance of biostimulation in the

bioremediation of soils contaminated with Atrazine is well documented. Degradation of 14C ring labelled atrazine was monitored in laboratory incubations of soils supplemented with 0, 10, 100 and 1000 μg g^{-1} sucrose concentrations. Another experiment was carried out to determine the effect of carbon amendment on total microbial biomass and soil respiration with different concentrations of sucrose and non-labelled atrazine. Atrazine dealkylation was enhanced in treatments with 100 and 1000 μg $g^{-1}$of sucrose added. Hydroxyatrazine (HA) metabolite was formed in the control (no sucrose) and in the presence of 10 μg g^{-1} of sucrose, whereas desethylatrazine (DEA) was only detected in treatment with 1000 μg g $^{-1}$ sucrose. Results indicate that total microbial biomass increased significantly ($P<0.001$) with the addition of 1000 μg g^{-1} sucrose [36].

Bioaugmentation may be done by single or many microorganisms. Bioaugmentation of soil with mixed bacterial consortium enhanced the rate of atrazine degradation in a highly polluted soil [37]. Zhang et al. (2012) [38] reported isolation of four-member mutualistic bacterial consortium (DNC5) capable of metabolizing atrazine from corn-planted soil. *Arthrobacter sp*. DNS10 found to be the primary organism capable of mineralizing atrazine to cyanuric acid. *Bacillus subtilis* DNS4 and *Variovorax sp*. DNS12 are secondary strains that utilized cyanuric acid during atrazine degradation process. The growth and the degradation rate of the consortium DNC5 were faster than that of the single strain DNS. The high degradation ability of the consortium showed good potential for atrazine biodegradation. This information contribute toward a better understanding about metabolic activities of atrazine degrading consortium, which are generally considered to be responsible for atrazine mineralization in the natural environment.

Therefore, the present results supported by other workers clearly indicated that bioaugmentation coupled with biostimulation is a powerful, economical and environmentally friendly bioremediation technique that is very promising for decontamination of Atrazine-contaminated soils.

Conclusion

In the present study results of greenhouse bioassay used in the indoor experiments proved to be a simple and sensitive tool in detecting small amounts of herbicides present in the soil. Results confirmed the effect of soil characteristics as well as herbicide concentration applied on its environmental fate in the tested soils. Combination between biostimulation and bioaugmentation remediation technologies considered the most efficient in removing atrazine contaminants from the tested soil systems compared to the other tested techniques. Results indicated that, removal of the herbicide atrazine from the contaminated soils achieved by the investigated remediation technologies in the following efficiency order: Biostimulation and Bioaugmentation>Bioaugmentation>Biostimulation> Natural (volatilization).

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