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토양 내의 Atrazine의 생물학적 분해 촉진을 위한 활성토의 이용

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Use of Activated Soil to Bioaugment Degradation of Atrazine in Soils

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ABSTRACT

Effectiveness of activated soil containing directly enriched atrazine-degrading soil microorganisms as an inoculant to bioaugment degradation of atrazine in soils was investigated. A Wooster silt loam (Typic Fragiudalf) was spiked with atrazine at a rate of 4 mg/kg soil three successive times to create activated soil. Atrazine degradation was significantly enhanced ($p < 0.05$) after the first treatment. After the second treatment, there was an increase in the number, based on MPN, of microorganisms utilizing atrazine as a C- and N-source by 3 logs and 1 log of magnitude, respectively. Inoculation of typical agricultural soils collected from Ohio with activated soil at a rate as low as 0.5% reduced the extractable atrazine remaining in soils to the level below 2% of that initially recovered (initially added at a rate of 4 mg/kg soil) after 4 days. Inoculation at a higher rate was required to achieve the same result in soils with non-typical properties (pH of 4.5 or organic matter of 43% w/w). Activated soil was stable, in terms of atrazine degradation activity, at least up to 6 months when it was kept at low temperature (< 10^oC) and moistened (water content above 15%). The results of this study indicate that microorganisms capable of degrading atrazine are relatively easily enriched in soil to create activated soil. Use of activated soil can be a practical option for bioremediation of contaminated soils.

Key words : Atrazine, Bioaugmentation, Bioremediation, Activated soil, Pesticide

요 약 문

토양내 atrazine 분해촉진을 위한 생물적 증진제로서의 토양내에서 직접증식된 Atrazine 분해 미생물을 함유하는 활성토의 효용성을 조사하였다. Atrazine 분해미생물을 증식시키기 위하여 Wooster silt loam을 4 mg/kg 농도의 atrazine으로 3회 연속처리하며 atrazine 분해 미생물 수와 atrazine 분해속도를 관찰한 결과, 1회 처리 후 atrazine 분해속도가 현저하게 증가하였고, 2회 처리 후 atrazine을 탄소원과 질소원으로 이용하는 미생물의 수가 각각 10^3, 10^1 배 증가하였다. 증식된 미생물을 함유하는 이 활성토를 atrazine에 오염(초기농도4 mg/kg)된 Ohio의 전형적인 농지 토양에 0.5%비율로 접종하였을 때, 토양내atrazine 농도가 4일만에 초기농도의 2% 이하로 감소하였다. 비전형적인 토양(pH 4.5 또는 유기물함량 43%)에서 같은 효과를 얻기 위해서는 더 높은 비율의 접종이 필요하였다. 활성토는 저온(10^oC 이하) 습윤(수분함량 15%)한 상태에서 최소한 6개월간 안정하였다. 본 연구결과는 atrazine 분해미생물이 토양내에서 비교적 쉽게 증식되며, 이를 함유하는 활성토가 토양내에서의 atrazine 분해 촉진을 위한 접종제로 유용하게 이용될 수 있음을 보여준다.

주제어 : Atrazine, Bioaugmentation, 생물학적 복원, 활성토, 농약

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1. Introduction

The herbicide, atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), is used in many parts of the world to control broadleaf and grassy weeds. Half-life of atrazine in natural soils ranges from a few days to a few months. Atrazine is frequently detected at trace levels in surface and groundwater samples (Goodrich et al., 1991; Thurman et al., 1992) and has been found in water samples at levels exceeding 3 μg/L of the health advisory level set by the U.S. EPA (Gannon, 1992; Koelliker et al., 1986). Therefore, there have been considerable interests in developing management practices that minimize the potential for atrazine pollution of surface water and groundwater resources.

Both aerobic and anaerobic microbial degradation (Chung et al., 1996), as well as sorption, are the key processes governing the persistence of atrazine in the environment. A variety of bacteria (Behki and Khan, 1986; Behki et al., 1993; Bouquard et al., 1997) and fungi (Donnelly et al., 1993; Mougin et al., 1997) capable of degrading atrazine have been isolated.

A number of microbial strains, either as single strain or consortium, have been tested for their ability to bioaugment the remediation of atrazine-contaminated soils and waters. Either specific strains or microbial consortia capable of atrazine degradation resulted in improved atrazine degradation in both laboratory and natural environments (Barriuso and Houot, 1996; Newcombe and Crowley, 1999; Runes et al., 2001; Struthers et al., 1998). In most previous studies on bioremediation of atrazine-contaminated soils and waters using bioaugmentation with atrazine degraders, however, pure cultures of microorganisms have been used as inocula (Grigg et al., 1997; Newcombe and Crowley, 1999) although the majority of species present in an ecosystem are non-culturable (Torsvik et al., 1990).

In this study, the possibility of direct enrichment of atrazine-degrading microbial populations in soil and its effectiveness as inocula to bioaugment the degradation of atrazine-contaminated soil were tested.

2. Materials and Methods

2.1. Chemicals and soils

Standard-grade atrazine was purchased from Chem Service, Inc. (West Chester, PA), and pesticide grade toluene was obtained from Sigma Aldrich (St. Louis, MO).

Soils used were collected from various areas including agricultural and forest areas. Surface (0-20 cm depth) soils with different textures, pH values, organic matter content and land use were obtained. Soils I and II (Wooster silt loam soils) and Soil III (a Fremont sandy loam soil) were collected from an agricultural area in Ohio. Soil IV (a forest soil) was collected from Mohican State Park, Ohio, and had no known history of pesticide application. Soil V (a Muck soil) was obtained from the MUCK branch of OARDC at Celeryville, Ohio, and stored at greenhouse without further treatment.

Soil texture was analyzed by the hydrometer method (Buoyoucos, 1962). Total organic matter was analyzed using the method described by Combs and Nathan (1998), and total nitrogen was determined using a Carbon/Nitrogen Analyzer (Model MAX-CN, Elementar Americas, Inc., Mt. Laurel, NJ). Soil pH

Table 1. Selected properties of soils used for atrazine degradation study

Properties		Soils				
		Soil I	Soil II	Soil III	Soil IV	Soil V
Particle Size Composition (%)	Sand	16.1	12.1	53.7	36.1	Ash Content 33.2 %
	Silt	62.9	72.7	32.9	53.1	
	Clay	21.0	15.2	13.4	10.8	
pH		6.7	6.8	6.6	4.5	5.9
Organic Matter (%)		3.10	3.23	2.74	4.13	43.31
Total Nitrogen (%)		0.17	0.16	0.52	0.15	2.37

was measured by the glass electrode method (McKeague, 1978) in a ratio of 1 : 1 (w/v, soil/water suspension). The properties of the soils used are summarized in Table 1.

2.2. Application, extraction, and determination of atrazine

Atrazine stock solution was prepared by dissolving 20 mg of atrazine in 1 mL of methanol. All experiments in which soils were treated with atrazine at various rates (4, 40 and 400 mg/kg soil) were prepared by applying the atrazine stock solution diluted in sterilized water. Soils were thoroughly mixed with added atrazine using a spatula to achieve uniform distribution of atrazine. The water content of soil reaction mixture was then adjusted to 25% (w/w) with sterilized water.

Atrazine was extracted from soil as follows. Eight mL of distilled water was added to 10 g soil in a 125-mL high-density polyethylene (HDPE) bottle, and was vortexed to disperse soil particles. Ten mL of pesticide-grade toluene was added, and the soil-water-toluene mixture was shaken on a horizontal shaker at 120 strokes per min for 1 hr. The mixture was then centrifuged at 8,000 × g for 10 min, and a 2-mL aliquot of the toluene layer was transferred to a 5-mL glass vial and stored at 4°C until analyzed. Tests indicated that samples could be stored for several months in the glass vials without changes in atrazine concentration.

To extract atrazine from microbial cultures used for Most Probable Number (MPN) determination, 0.8 mL aliquot of the culture was transferred to Eppendorf tube, and the equal volume of pesticide-grade toluene was added. After mixing on a vortex shaker for 1 min, the mixture was centrifuged at 15,000 rpm (≈ 10,000 × g) in a microcentrifuge for 10 min, and 0.5 mL of the toluene layer was transferred to a 5-mL glass vial and was stored at 4°C until analyzed. As for the atrazine extracted from soil, these samples could be stored for several months without changes in atrazine concentration.

Atrazine concentration in the extracts was determined with a Varian 3700 gas chromatograph equipped with a 3% OV-1 on 100/200 Supelcoport packed column (3 m in length) and a nitrogen-specific thermionic detector. Helium was used as a carrier gas at a flow rate of 28 mL/min. Temperatures were set to 210°C for the injector, 250°C for the column, and 310°C for the detector. The retention time for atrazine was 2.10 min. The average recovery rate of atrazine from soils, when atrazine-treated sterile soils were left at room temperature for 24 hours, was between 82 and 95% at low atrazine treatment rate (4 mg/kg), and above 95% at higher treatment rates (40 mg/kg or higher). The average recovery rate of atrazine under the condition used for MPN was 95%.

2.3. Monitoring the rate of atrazine degradation

Atrazine degradation in this study was monitored by disappearance of parent material during incubation. Disappearance of atrazine was measured based on the amount of extractable atrazine remaining in soils. The amount of atrazine degraded by microorganisms was calculated by subtracting the amount of extractable atrazine remaining in non-sterile soil from that of sterile soil at certain time of incubation. The rate of degradation was primarily expressed by plotting percentage of atrazine remaining at various incubation times compared to the amount of atrazine extracted at time zero immediately after the treatment. The soil reaction mixture was divided into 10 g (as dry weight) subsamples and the subsamples were placed into 125-mL HDPE bottles. Bottles were loosely capped and incubated at 25 ± 2°C. The averaged amount of atrazine initially recovered from triplicate samples at the beginning of the incubation was counted as 100%.

For the test of significant statistical difference in atrazine degradations (as measured by percent recovery of atrazine), Analysis of Variance (ANOVA) was conducted for each incubation time.

2.4. Creation, evaluation, and preservation of enhanced or activated soil

Soils I-V (Table 1) were treated with atrazine at a rate of 4 mg/kg to check the ease of enhancement in a range of soils. Additional treatment of atrazine was applied by two more times only when the extractable

atrazine remaining in soil dropped to the levels less than 20% of the initially recovered amount. Therefore, there was only one treatment of atrazine at a rate of 4 mg/kg for Soil IV and V. These soils treated with atrazine were referred to as "enhanced soil."

Soil activation was achieved by direct enrichment, or acclimation, of atrazine-degrading microorganisms. Soil I was treated three times with atrazine at a rate of 4 mg/kg soil. Atrazine dissipation rate was monitored by the method previously described in section 2.3, and additional atrazine was applied when there was no apparent change for 6 days. During activation total microbes utilizing atrazine as carbon source, and those utilizing atrazine as nitrogen source were counted (see section 2.5). This "activated soil" hereafter, unless otherwise specified, is the soil used as an inoculant for the subsequent experiments.

The activated soil was moisturized to obtain a water content of 25% (w/w) and placed in a plastic container with lid in a cold room (10°C) for storage.

2.5. Enumeration of soil microorganisms

Total microorganisms capable of utilizing atrazine as a carbon or nitrogen source in soil were counted based on MPN method (Alexander, 1982) with a slight modification. TGYe (0.5% tryptone, 0.1% glucose, and 0.25% yeast extract, w/v) was used as a medium for culturing total bacteria. The basal minimal salts nitrogen medium(BMN, described in Behki and Khan, 1986) was supplemented with atrazine (4 mg atrazine/L) and used for microorganisms utilizing atrazine as carbon source. The modified atrazine medium, described by Mandelbaum et al. (1993), containing 4 mg atrazine/L was used for bacteria that can utilize atrazine as a sole nitrogen source. Five replicates of MPN tubes were incubated at 25 ± 2°C for 7 days. Positive tubes for total bacteria were identified by visual turbidity. Those for atrazine utilizing bacteria were determined based on the amount of atrazine remaining in tubes and extracted by the method described in the previous section, after 7 days of incubation. Tubes containing atrazine less than 20% of the initial concentration were considered positive.

2.6. Inoculation of contaminated soil with activated soil

Inoculation of contaminated soils with activated soil and evaluation of its effect on degradation of atrazine were performed as follows. The artificially atrazine-contaminated soils were prepared by applying atrazine to target soils (Soil II, III, IV, and V) at a rate of 4 mg/kg soil. Then the atrazine-contaminated soil was inoculated with activated soil at different rates (0.05, 0.5, and 5%, w/w), and the mixture was divided into 10-g (as dry weight) subsamples and placed into 125-mL HDPE bottles and loosely capped. These bottles were incubated in a temperature-controlled incubator (25 ± 2°C). Triplicate samples were selected at each time of incubation, and the atrazine remaining in soil was extracted and analyzed as described in section 2.2. Atrazine remaining (calculated as a percentage of atrazine initially added and recovered) vs. incubation time was plotted.

For test of significant statistical difference among atrazine degradations (as measured by percent recovery of atrazine) in soils non-inoculated and inoculated with activated soil at different rates, ANOVA was conducted for each individual incubation time for each contaminated soil.

2.7. Stability of activated soil

Stability of activated soil, in terms of atrazine-degrading activity, was investigated by first adjusting water content of activated soil to 25 (w/w), and then storing it at different conditions. Four different storage conditions were imposed as follows: (1) at room temperature without soil water content adjustment; (2) at room temperature and with soil water content maintained between 15 and 25 %; (3) at low temperature (10 or 4°C), and (4) at frozen state (−20°C). Results observed at 10 and 4°C were found to be almost identical, and thus these two conditions were combined and regarded as one condition.

2.8. Statistical analysis

All statistical analyses were done using MINITAB (ver. 13.1, MINITAB Inc., State College, PA). AVOVA

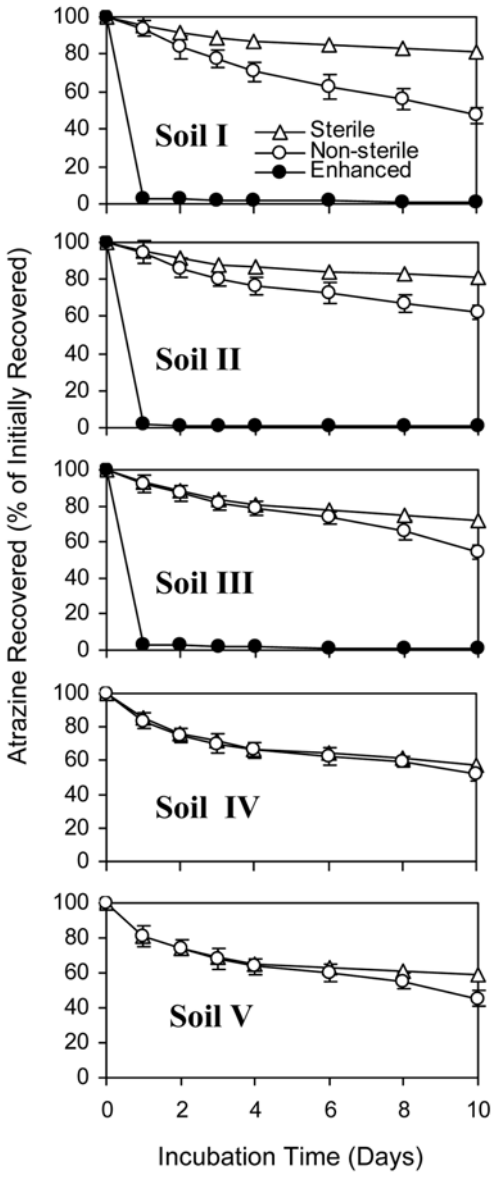

Fig. 1. Degradation and disappearance of atrazine in various soils. Soils treated with atrazine at a rate of 4 mg/kg soil were incubated at 25 ± 2°C. Error bars represent Least Significant Difference (LSD) between treatment means at given incubation time.

was done using the General Linear Model menu of MINITAB, and the least significant difference (LSD) was calculated at 5% level.

3. Results and Discussion

3.1. Rates of atrazine degradation in various soils

Dissipation of atrazine in natural (non-sterile) soils, presumably disappearance due to biological and/or nonbiological processes (such as adsorption, volatilization, etc.), was relatively slow (Fig. 1). After 10 days of incubation, about 50 to 70 % of the atrazine initially recovered (added initially at 4 mg/kg soil rate) still remained in the soils tested. Atrazine biodegradation seemed to be relatively high in typical agricultural soils (e.g., Soil I, II and III) that may have received previous application of s-triazine herbicide, and atrazine degradation was easily enhanced by repetitive applications of atrazine.

In non-agricultural soil (Soil IV and V), however, both atrazine degradation and enhancement were not observed (Fig. 1). This might be due to the soil pH (a low pH of 4.5 in Soil IV) and organic content (as high as 43% in Soil V).

3.2. Creation of atrazine-activated soil

Atrazine-degrading microorganisms were enriched in soil by applying atrazine (4 mg/kg soil) for three consecutive treatment cycles. The soil used was a Wooster silt loam (Soil I) found to have a higher atrazine degrading activity than the other soils tested. The changes during enrichment process are shown in Fig. 2. The extractable atrazine concentration was reduced much faster during the second atrazine treatment cycle, compared to the first atrazine treatment at a rate of 4 mg/kg soil. The extent of atrazine degradation also increased with each successive treatment cycle; the amount of extractable atrazine remaining in soil was less with each successive treatment.

The number of total bacteria remained almost constant during all three treatments. However, the number of microorganisms utilizing atrazine as a carbon source and a nitrogen source increased by about 3 logs and 1 log of magnitude, respectively, after three treatments. In this study, the population changes during enrichment were not monitored after the third treatment cycle of atrazine. However, further enrichment of atrazine-degrading microorganisms might be possible with additional cycles of atrazine application at the same rate, or by a single treatment at a higher atrazine application rate. The rate of atrazine degradation seemed to be relatively easily enhanced, which is in

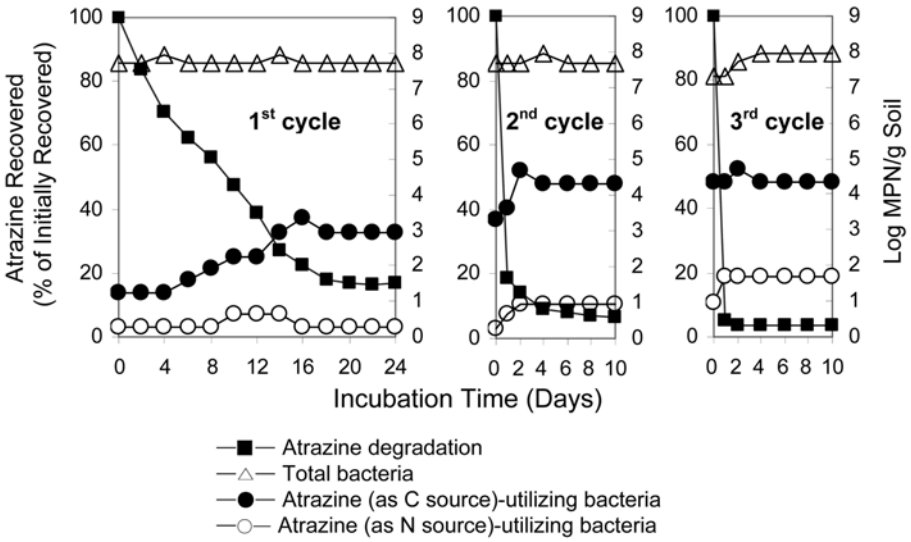

Fig. 2. Atrazine degradation and enrichment of atrazine-degrading microorganisms in soil. Application rate of atrazine at the beginning of each treatment cycle was 4 mg/kg soil.

contrast to the fact that enhanced atrazine biodegradation by repetitive application has rarely been reported.

The soil prepared by the process described above, which contained an enriched number of atrazine-degraders, is defined as activated soil.

3.3. Atrazine degradation in activated soil

Many chemicals, not toxic at low concentrations and easily biodegraded, are known to become toxic when concentrations exceed their threshold levels. For example, among various factors investigated (e.g., soil type, pH, moisture content, organic matter content, microbial activity, and pesticide concentration), pesticide concentration was found to be the most important factor affecting degradation rate of pesticide mixture of atrazine, captan, carbaryl, 2,4-D, diazinon, fenitrothion, and triflurarin when treatments of 100 and 1,000 mg pesticide/kg soil were compared (Schoen and Winterlin, 1987). Degradation rates in soils treated with pesticide at a rate of 1,000 mg/kg soil were much lower than in soil treated at a rate of 100 mg/kg soil.

In this study, the degradation rates were compared when atrazine was applied at different concentrations(4, 40, and 400 mg/kg soil). A short acclimation or lag period (< 1 day) seemed to exist in the activated soil treated with atrazine at a rate of 400 mg/kg soil,

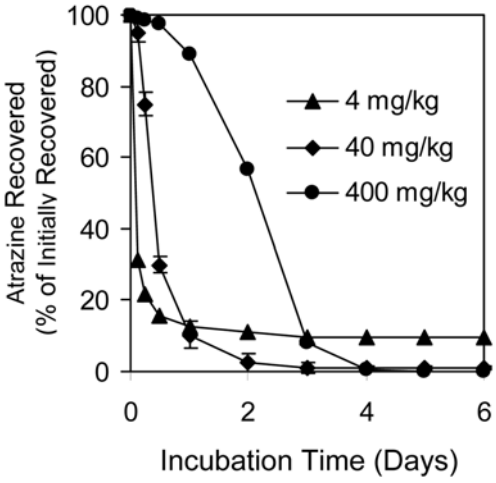

Fig. 3. Degradation of atrazine as a function of atrazine concentration in activated soil. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.

whereas degradation began immediately in the activated soil treated with atrazine at a rate of 4 mg/kg soil (Fig. 3). The overall degradation pattern observed was similar to the results reported by Gan et al. (1996), who found that the amount of atrazine degraded in soil under laboratory conditions also increased proportionally with the increase of concentration of atrazine over the range of 5, 50, 500, and 5000 mg/kg. On the other hand, Grigg et al. (1997) reported incomplete and lower extent of atrazine degradation at a very high initial concentration of atrazine (0.046 mole/

kg 10,000 mg/kg, and 0.23 mole/kg 50,000 mg/kg) by a mixed microbial population. According to the authors, the decreased and the incomplete degradation resulted from decreasing atrazine bioavailability and phosphorous depletion, respectively, rather than the inhibitory effect of high concentration.

Based on the earlier studies (Gan et al. 1996; Grigg et al. 1997) and this report, atrazine degradation by soil microorganisms does not seem to be inhibited even at quite high concentration of atrazine.

3.4. Effect of inoculation of activated soil on atrazine degradation

Inoculation of natural soils with activated soil significantly increased the degradation of atrazine, and the effects of inoculation rates on atrazine degradation in various soils are shown in Fig. 4. Atrazine degradation rates and extents increased with increased inoculation rates ranging from 0.05 to 5% (w/w) on dry weight basis. In all soils inoculated with activated soil at 5% rate, atrazine concentrations were reduced very rapidly from the beginning of incubation. Previously, several studies have shown the accelerated degradation of atrazine in soils augmented with cultured cells (Assaf and Turco, 1994; Yanze Kontchou and Gschwind, 1995) or non-sterile poultry litter (Gupta and Baummer, 1996). Lower degradation in the high organic soil or low pH soil is in agreement with the results given by Yanze Kontchou and Gschwind (1995), who have shown that inoculation of soil with a *Pseudomonas* strain was less efficient at reduced water contents, limited oxygen supply, low pH, and high organic content of soil.

Similar to degradation patterns observed in the non-activated soils (refer to Fig. 1), inoculation effects were greater in soils with neutral pH and low organic matter content (< 10%). A pH value of 4.5 in Soil IV may have inhibited microbial growth or inactivated enzyme function. Lower degradation rate in the Soil V may have been attributed to either lower bioavailability of atrazine because of sorption to organic matter or presence of other substrates that are preferred over atrazine as a carbon source for the microbial population.

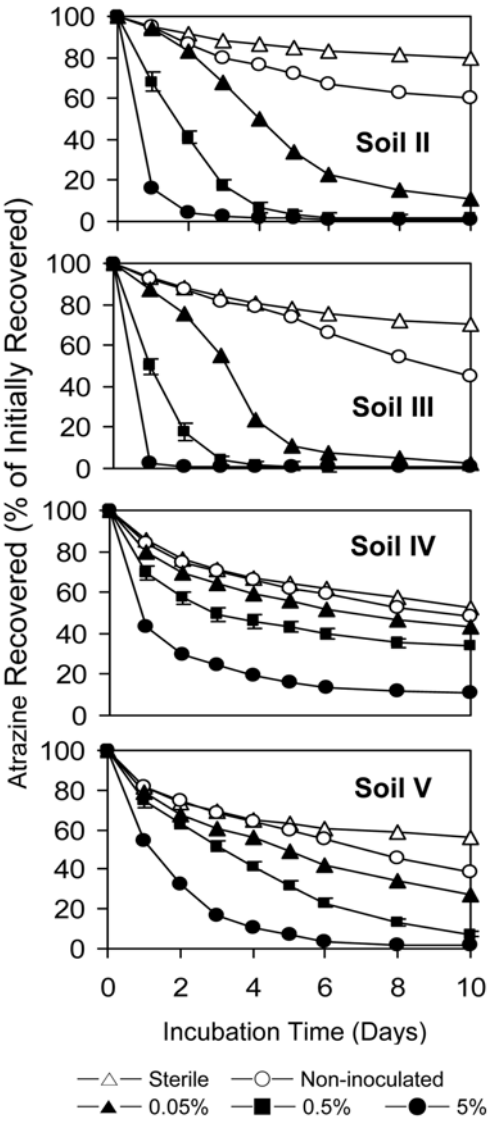

Fig. 4. Effect of inoculation rate of activated-soil on degradation of atrazine in soils treated with atrazine. The rate of atrazine applied to soil was 4 mg/kg soil. Error bars represent Least Significant Difference (LSD) among treatment means, excluding that of sterile soil, at given incubation time.

The relationship between inoculation rate and atrazine concentration on atrazine degradation in Soil II (Fig. 5) revealed that degradation of atrazine started with a short lag when atrazine was applied at a higher rate (40 or 400 mg/kg). Once started, however, degradation was rapid, even in the non-inoculated soils. It seemed that when atrazine was applied at a higher rate (40 or 400 mg/kg soil), it quickly stimulated atrazine degrading activity. This lag in the development of degradation was not evident at application rate of

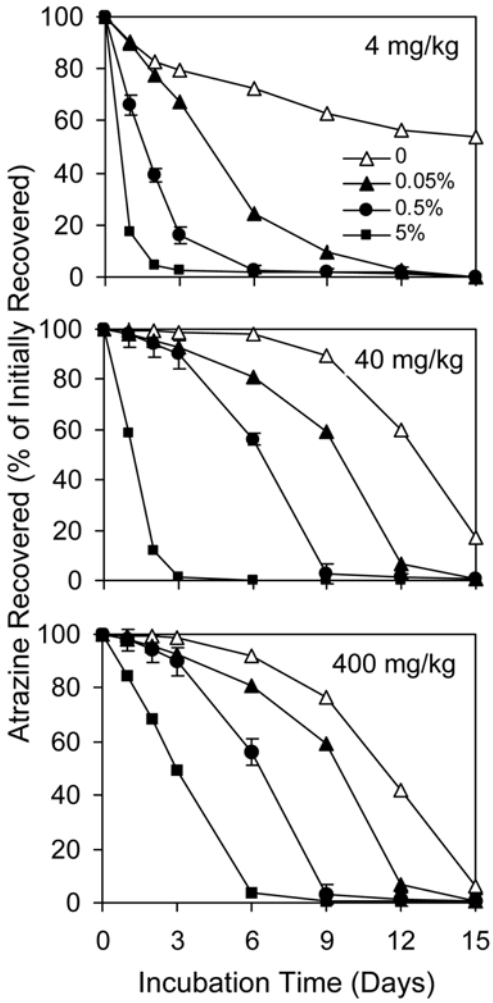

Fig. 5. Effect of inoculation rate on atrazine degradation when atrazine was applied at different concentrations. Artificially atrazine-contaminated Soil II was inoculated with different rates of activated soil. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.

4 mg/kg soil, and stimulation of atrazine degradation was not observed during 15 days of incubation. The extent of degradation in non-inoculated soil treated with atrazine at a rate of 4 mg/kg soil was low and almost completely stopped after only 50% of the initially applied atrazine was degraded. The reason for this might be low concentration of atrazine to support increase in atrazine-degrading microbial population. The results also show that a higher rate of inoculation reduced the lag period even at high atrazine concentrations. The microbial population required for rapid degradation was delayed at the lowest inoculation rate (0.05%) but developed rapidly over just a few days of time to achieve rapid atrazine degradation.

Grigg et al. (1997) suggested that a mixed culture could be used for bioremediation of atrazine at concentrations up to and exceeding those reported for agrochemical mixing-loading facilities; atrazine at agrochemical mixing-loading sites in Illinois occurred at levels up to 1.9 mmole/kg soil (≈ 413 mg/kg soil). On the other hand, this report suggests that activated soil containing directly enriched atrazine degrading microbial consortia is also an effective bioaugmentation option.

3.5. Effect of storage condition on stability of activated soil

Readily usable inoculum is required for immediate availability of inoculant for bioaugmentation and thus successful bioremediation. For practical use of inoculum for bioaugmentation, the inoculum must be stable during long periods of storage. Activated soil, in terms of atrazine degrading activity during storage under various conditions tested, was found to be very stable at least up to 6 months (data not shown).

4. Summary and Conclusion

The rates of atrazine degradation (defined as disappearance of parent atrazine) in natural soils were found to be relatively slow. Depending on soil type and properties, only about 30 to 50% of atrazine added to and initially recovered from non-activated natural soils (4 mg/kg) was degraded after 10 days of incubation at 25 ± 2°C.

It was found that atrazine degrading microorganisms in Soil I (a Wooster silt loam) are readily enriched during repetitive application of atrazine at a rate of 4 mg/kg soil. After three treatments, the number based on MPN, of atrazine-utilizing bacteria as C- or N-source, increased by about 3 logs or 1 log of magnitude, respectively. Atrazine degradation was accordingly accelerated. Increased levels of atrazine degradation were observed in other soils when they were treated with atrazine three times at a rate of 4 mg/kg soil. Exceptions were observed in Soil IV (a

forest soil) and Soil V (a Muck soil), which might not be favorable for microbial growth and/or activity.

Inoculation of atrazine-contaminated soil with activated soil, resulted in significant increase in degradation of atrazine in contaminated soil. This result implies that the activated soil is an effective inoculant to stimulate atrazine degradation in the environments. The activated soil was stable at temperatures below 10°C and moisture content above 15% further suggesting the potential usefulness of these materials to bioaugment the remediation of atrazine-contaminated environments.

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