Effects of Atrazine-Mineralizing Microorganisms on Weed Growth in Atrazine-Treated Soils

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The herbicidal effect of atrazine on sensitive plant species was studied in soils inoculated with bacteria capable of mineralizing atrazine. *Nasturtium officinale* and *Solanum nigrum* plants died within 15 days after sowing in soil containing atrazine incorporated at 4 mg/kg. Normal growth of *N. officinale* was obtained when 5 mg/kg atrazine-mineralizing bacteria was mixed into the soil containing atrazine, prior to seeding. Atrazine concentrations in soil declined by 90% within 5 days as a result of the atrazine degradation by the bacteria added to the soil. Normal growth of *S. nigrum* plants was observed in soils receiving only 0.1 mg/kg atrazine-degrading bacteria. The efficacy of atrazine in the presence of atrazine-degrading bacteria was also tested on *N. officinale* and *Agrostis tenuis* in greenhouse trials under simulated field conditions using a commercial atrazine formulation and the herbicide sprayed onto soil in open containers. Here, too, the microbial herbicide breakdown was rapid and the effect of the herbicide on the indicator plants was drastically diminished when the soil was kept under conditions favorable for the atrazine-degrading bacteria.

Keywords: *Weed control; adaptive soils; atrazine; atrazine degradation; atrazine mineralization*

INTRODUCTION

Atrazine [2-chloro-4-(ethylamino)-6-isopropylamino-*s*-triazine] is an effective herbicide for the control of annual broadleaf and some grass weeds, primarily used in maize. It has been extensively used worldwide since its introduction in 1958. Due to its widespread use, its relatively long half-life in agricultural soil (Seiler et al., 1992) and a moderate soil mobility, the herbicide, and its metabolites deethylatrazine and deisopropylatrazine, have been detected in many environmental compartments, especially in surface water as a result of runoff following application and in drainage networks (Buser, 1990; Schottler et al., 1994; Thurman et al., 1994). Up to the 1980s application rates of atrazine of up 20 kg/ha were used for industrial weed control (Humburg et al., 1989). Today, lower rates are recommended, and for many European countries, the atrazine rates applied are below a maximum rate of 1.5 kg of active ingredient (ai)/ha per annum in agricultural crops (Seiler et al., 1992).

Although atrazine has been applied in the field for over 30 years, no enhanced degradation by bacteria in soil leading to adaptive soils has been reported on large scale yet, thus indicating the difficulty of rapid microbial breakdown in the field. Accelerated atrazine degradation in soil has been found in the laboratory only and under certain conditions (Radosevich et al., 1996; Vanderheyden et al., 1997). Numerous research groups tried for many years to enrich microorganisms capable of mineralizing this chemical as a carbon and energy source (Cook, 1987) until Yanze Kontchou and Gschwind (1994) and Radosevich et al. (1995) independently succeeded in isolating atrazine-degrading bacteria. One of these bacteria was identified as a *Pseudomonas* sp. (Yanze Kontchou and Gschwind, 1995). The presence of this culture in soil reduced the degradation half-life of atrazine under unsaturated soil conditions considerably, and the culture kept its atrazine catabolism capacity in sterile moist soil for over 200 days without any supply of carbon (Wenk et al., 1997). ^{14}C-Labeled atrazine added to the soil was mineralized to $^{14}CO_2$. Triazine metabolites were not detected (Wenk et al., 1997).

The application of the bacteria mineralizing atrazine as a carbon source offers potential for remediating contaminated soils and thus preventing soil and water pollution. Microbial methods using aerobic and anaerobic microorganisms for the cleanup of pesticide-contaminated soils have been developed during the past few years (Häggblom and Valo, 1995; Kaake et al., 1992). Most experiences gained from full scale application to bioremediate soils within a reasonable time frame have been obtained by composting soil contaminated with wood preservatives such as chlorinated phenols (Häggblom and Valo, 1995). Plant growth as an indicator for successful soil remediation was integrated in soil remediation studies (Brunsbach and Reineke, 1995). However, the use of such bacterial cultures in the field could result in causally enhanced biodegradation of the herbicide (adaptive soils) and thus to loss of effectiveness as a control agent, as has been observed for other agrochemicals (Lebbink et al., 1989; Felsot and Shelton, 1993; Ou et al., 1993; Robertson and Alexander, 1994). It was the purpose of this study to investigate if similar effects are to be expected if atrazine-mineralizing bacteria are incorporated into soils receiving atrazine for weed control.

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MATERIALS AND METHODS

Soil Characteristics and Cultivation of Microorganisms. The Stein soil used for this study had a pH of 7.8 and contained organic matter (2.6%), clay (25%), silt (33%), and sand (42%). Its maximum water holding capacity (100% WHC), determined according to the method of Wenk et al. (1997), amounted to 326 mL/kg of soil. The soil was steamed, and then it was sieved through a 3 mm grid prior to use and was dried overnight by spreading at 1 cm depth over a shallow plastic tray in an open greenhouse.

The cultivation and harvesting of *Pseudomonas* sp. grown on a Gesaprim emulsion, a commercially available formulation of atrazine, as a single C, N, and energy source was described by Wenk et al. (1997). The bacteria were harvested by centrifugation and washed twice by resuspension in a NaCl solution (0.7 g/L) followed by centrifugation. The bacterial dry weight was determined by drying the wet bacterial biomass at 100 °C overnight. One gram of bacterial dry weight corresponds to about 10^{13} bacteria.

Soil Incorporation and Analysis of Atrazine. In most of the experiments, the herbicide was homogeneously mixed at 4 mg/kg (= 4 ppm) or less into soil to simulate concentrations resulting from a spray application in the field. Assuming an application rate of 1.5 kg/ha and a soil specific density of 1.5 kg/dm^3 of soil, the chemical concentration in the top 5 cm of the soil would be ~2 mg/kg.

Atrazine in 2 g samples was extracted with 5 mL of acetonitrile/water (90% and 10%, respectively) overnight on a rotary shaker (175 rpm). The solvent solution was filtered (0.45 μm, PTFE filter, BGB Analytik AG, Rothenfluh, Switzerland), and atrazine was determined by HPLC (Yanze Kontchou and Gschwind, 1995). The quantification limit of the method was 0.01 mg/L, corresponding to an atrazine concentration of 0.025 mg/kg in soil. The standard deviation of the atrazine concentration in soil of three independent determinations remained below the 5% level (Wenk et al., 1997).

Laboratory Investigation for Residual Activity of Atrazine. The efficacy of different concentrations of atrazine in controlling *Nasturtium officinale* (watercress) and *Solanum nigrum*, both species chosen as being sensitive to atrazine, was determined by applying different rates to the soil. Atrazine was applied in the form of technical grade ai or formulated as Gesaprim FW 500 emulsion. Sowing of seeds of both species was made after incorporation of the herbicide into the soil. These tests were run in duplicate.

Demineralized water and atrazine solution were mixed into the soil with a dough mixer [Hobart (Swiss) AG, Zürich, Switzerland]. About 30 g of the well-mixed soil at a WHC of ~50% was placed into 5 cm Petri dishes with three holes punctured in the base to allow subsequent subsurface watering. The dishes were loosely covered with lids and were placed into 6.5 cm Petri dishes, to which water was added to irrigate the growing plants. An initial irrigation was made with water containing propamocarb as a fungicide (2.5 mL/L Previcur N solution, Maag, Dielsdorf, Switzerland) to prevent the development of fungi during germination. Subsequent irrigation was made with a fertilizer solution containing mainly N, P, and K (2 mL/L; Flory 3, Hauert, Switzerland). Under these conditions, the soil containing atrazine and supporting growth of the plants was constantly saturated with water during the experiment. Each dish was seeded with 0.2 g of seeds and incubated at 20 °C at ~100% relative humidity in a laboratory incubator. Illumination of 12 h/day was given using fluorescent tubes yielding a light intensity of 2000 lx. After germination, the lids of the Petri dishes were removed to allow further growth of the plants.

Enhanced Biodegradation Studies in the Laboratory. The same experimental setup as described in the previous section was used for the study of enhanced biodegradation of atrazine. Instead of demineralized water, aliquots of atrazine-mineralizing bacteria (given as dry weight) suspended in an aqueous NaCl solution (0.7 g/L) were added to the soil. Both atrazine and bacterial solutions were filled in individual syringes and were added dropwise and incorporated simultaneously into the soil. Controls receiving no bacteria were run in parallel. After a 15 day incubation period, the plants were cut just above the soil surface to determine the shoot weight.

Atrazine soil concentrations were analyzed in soil taken from the same soil mixtures (~50% WHC) as used for the plant tests. One hundred gram soil samples were placed into closed 250 mL Schott Duran bottles and incubated at the same temperature as the plant biotests. However, in contrast to the biotest soils, which were constantly saturated with irrigation solution, no additional water was added to these samples used for atrazine analysis. Aliquots of 2 g soil were taken every 2 days and frozen at −5 °C until analysis.

Greenhouse Experiments. Tests were performed in plastic boxes 25 cm long, 16 cm wide, and 11 cm deep. About 800 mL of water was added to air-dry Stein soil to obtain a soil moisture of ~50% WHC. Treatments were made by adding 5 mg/kg biomass of atrazine-degrading bacteria to the soil or blank controls. Atrazine was applied by two different methods. The first method was the same as used for the laboratory test, in which atrazine was incorporated into soil using a dough mixer. Each test consisted of 5 kg of soil, bacterial solution (200 mL), Gesaprim emulsion (200 mL of a stock solution containing 1 mL of product diluted in 5 L of water), and 400 mL of NaCl (0.7 g/L). The solutions were added dropwise to the soil during the 1 h mixing procedure. Controls received NaCl solution instead of a bacterial solution.

The second method used to apply atrazine was by spraying the herbicide solutions in a volume of 50 L/ha and at an application rate of 1 kg of atrazine ai/ha. Air-dried soil was put into the boxes to which 400 mL of water with 0.7 g/L NaCl and 200 mL of bacterial solution (or NaCl solution only for controls) were added dropwise onto the soil surface before and after spraying of the atrazine solution. A final 200 mL of NaCl solution was added dropwise to all boxes to set the final soil moisture of ~50% WHC.

No fungicidal solution was added for these trials. Three boxes containing identical soil were used for each test. Soils receiving neither atrazine nor atrazine-degrading bacteria served as a reference for assessing plant growth in the treated soils.

To vary climatic conditions during incubation, the experiment described above was repeated but the boxes of soil were maintained for the first 5 days outside, the remainder kept in the greenhouse, where they received surface irrigation of 2 mm/day. The soils stored outdoors were not watered. After 5 days, the boxes outdoors were also returned to the greenhouse and were subsequently watered daily. All boxes were seeded 5 days after soil preparation with either *S. nigrum* or *Agrostis tenuis*. Soil atrazine concentrations during the experiment were determined from replicate unseeded treatments. Soil sampling was carried out by using a metal corer of 2 cm internal diameter, sampling to a depth of 10 cm.

Chemicals. Technical grade atrazine, analytical master standard (99.2%), and Gesaprim FW 500, a formulation containing 50% (w/w) atrazine only, were supplied by Entwicklungszentrum Agro, Novartis Crop Protection AG, Münchwilen, Switzerland. Acetonitrile (LiChrosolv) for chromatography was obtained from E. Merck, Darmstadt, Germany. Other chemicals were supplied by Fluka Chemie AG, Buchs, Switzerland.

RESULTS

Initial Laboratory Biotests with *N. officinale* and *S. nigrum*. Under the laboratory conditions used, *N. officinale* germinated 4 days after seeding, and all plants grew well up to day 11. At day 15, the leaves of the plants treated with 4 mg/kg atrazine showed evidence of chlorosis and, eventually, plant growth stopped. At day 17, the same was observed for plants treated with 1 mg/kg soil. Figure 1 shows the plants after an incubation period of 31 days. Atrazine application rates of 1.0 and 0.25 mg/kg inhibited plant growth, leading to full mortality. Atrazine concentrations of 0.06 mg/kg did not lead to plant death (Figure 1).

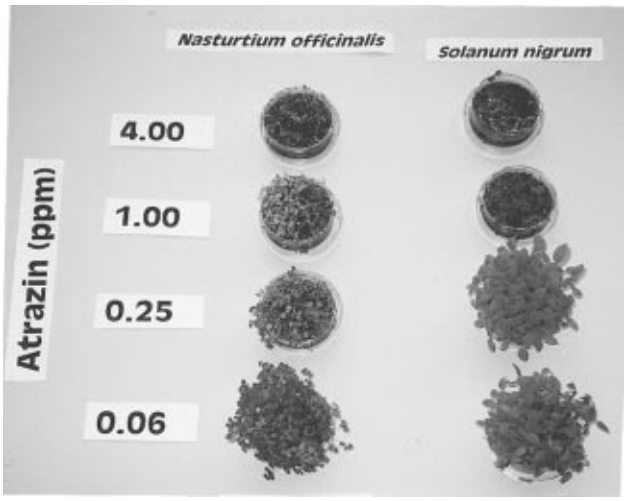

Figure 1. *N. officinale* and *S. nigrum* growing on soils with different atrazine concentrations, after 31 and 29 days of incubation, respectively.

S. nigrum germinated after 8 days. The effect of atrazine on the plants was slower compared to that on *N. officinale*, which developed faster. After 20 days, the shoots of *S. nigrum* treated with 4 and 1 mg/kg atrazine still appeared green but showed much smaller leaves than those treated with 0.25 or 0.06 mg/kg. The plants on different atrazine soil concentrations are shown in Figure 1 after 29 days of incubation. Those grown on the higher atrazine concentrations died, whereas those on soil with ≤0.25 mg/L atrazine did not show any adverse visual effects compared to plants grown on soil without atrazine treatment (data not shown).

Effects of Enhanced Atrazine Biodegradation on Plant Growth. In the subsequent experiments, soil was treated with 4 mg/kg atrazine and, in addition, up to 0.1 g/kg biomass of atrazine-mineralizing bacteria (Figure 2). All soils that had received the atrazine-degrading microorganisms showed good growth of *N. officinale* 16 days after incubation. The treated soil containing 4 mg/kg atrazine but receiving no atrazine-mineralizing biomass was the only one in which the plants died (Figure 2, front row, left).

Because the atrazine-mineralizing bacteria treatment was so effective, the amount of bacterial biomass added was reduced to 0.1−5 mg/kg of soil and *N. officinale* and *S. nigrum* were seeded as test plants. After 15 days, plant growth of *N. officinale* showed a positive correlation with the amount of atrazine-degrading biomass incorporated (Figure 3). The shoot weight of the plants is shown in Figure 4. The highest weight of 1.75 g per Petri dish was obtained in soils receiving 5 mg/kg atrazine-degrading bacteria, and the lowest shoot weight of 0.68 g per Petri dish was obtained in soils with the lowest amount of atrazine-degrading bacteria added (0.1 mg/kg). The addition of >5 mg/kg biomass did not result in any further visual enhancement of plant growth (Figure 2).

To show that growth of the plants was due to degradation of the atrazine in the soil, portions of the unseeded soils were incubated in a closed glass bottle at a soil moisture of ∼50% WHC (instead of 100% WHC of the seeded soils due to the watering procedure). The atrazine concentrations in these soils dropped by 90% within 6 days in soil containing 5 mg/kg biomass (Figure 5). Soils supplied with 0.1 mg/kg atrazine-degrading bacteria showed a 35% decrease of the initial atrazine content. In the planted soils at a soil moisture content (WHC) of 100%, atrazine concentrations are expected to be lower than those measured as the studies of Wenk et al. (1997) showed that the atrazine degradation rates at 50% WHC are slower than those in soils kept under saturated conditions.

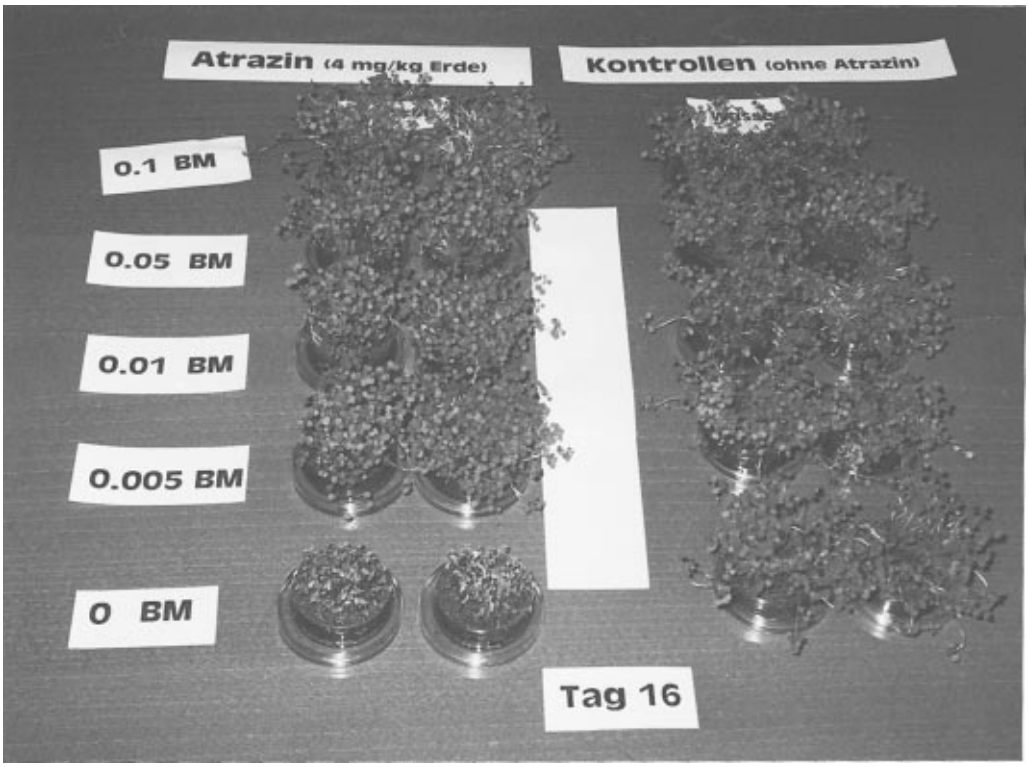

Figure 2. Growth of *N. officinale* on soils treated with 4 mg/kg atrazine (left) and different amounts of atrazine-mineralizing biomass (BM, in g of dry weight/kg of soil) after 16 days of incubation. Control plants grown on soils receiving bacteria but no atrazine are shown in the two rows on the right.

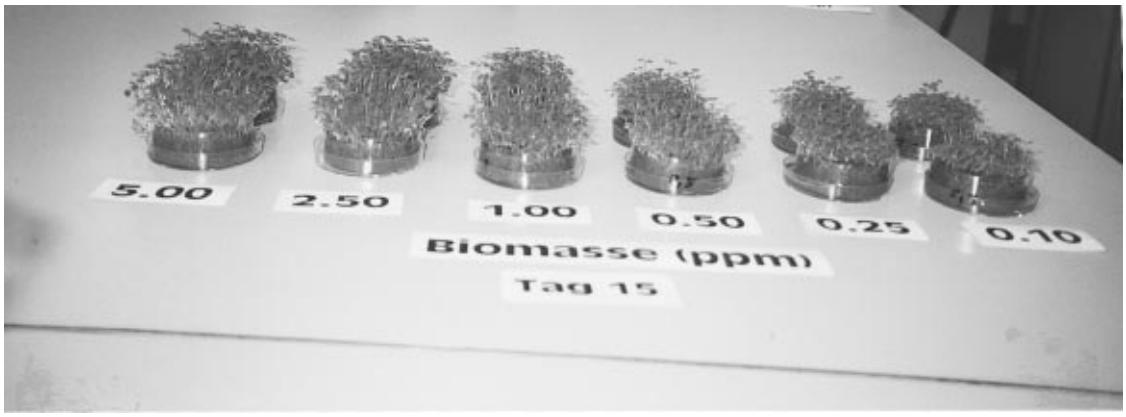

Figure 3. *N. officinale* on soil containing 4 mg/kg atrazine incubated for 15 days with various concentrations (mg of dry weight/kg of soil) of atrazine-degrading microorganisms.

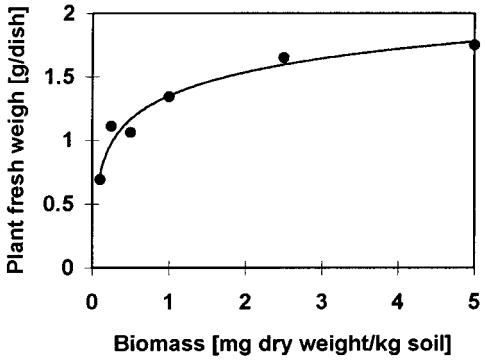

Figure 4. Average shoot weight per dish of *N. officinale* (shown in Figure 3) after the 15 day incubation period on atrazine-treated soil containing different amounts of atrazine-mineralizing bacteria.

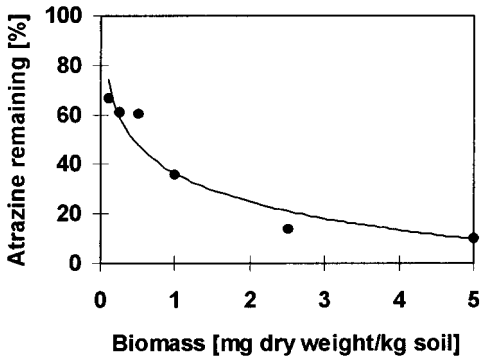

Figure 5. Remaining atrazine levels in soils 6 days after inoculation with different quantities of atrazine-mineralizing biomass. The initial atrazine concentration incorporated into the soil amounted to 4 mg/kg.

S. nigrum germinated 8 days after seeding. Three weeks later, no visual differences in the growth of *S. nigrum* as, e.g., chlorotic leaves were observed in relation to the quantities of bacteria incorporated into the soil (0.1−5 mg/kg, data not shown). Thus, even amounts of atrazine-degrading bacteria as low as 0.1 mg/kg must have resulted in a sufficient extent of atrazine degradation to allow normal plant growth.

In a final laboratory experiment, technical grade atrazine was replaced by the commercial formulation of Gesaprim FW 500. No difference between the atrazine formulations on the response of *N. officinale* and *S. nigrum* was observed. In both cases, the addition of atrazine-degrading bacteria to soil neutralized the herbicidal activity.

Experiments in the Greenhouse. The results obtained for two atrazine application methods, incorporation into soil and spraying, used in the greenhouse trials were similar to those found under laboratory conditions. Normal plant growth of *S. nigrum* or *A. tenuis* was observed in soils receiving atrazine-degrading bacteria, where even spontaneous growth of autochthonous weeds was observed as a result of bacterial herbicide degradation (Figure 6; three boxes each in first and third rows from the left). No plant growth was observed in soils containing atrazine but receiving no atrazine-degrading biomass (Figure 6; three boxes each in second and fourth rows from the left). This result agreed well with the atrazine concentrations found in the soils during the experiment. Atrazine levels in the soil following application by spraying are shown in Figure 7a. The concentration at the start of the experiment was higher than the 4 mg/kg calculated for the average soil concentration because it was not incorporated homogeneously. Five days after bacterial incorporation and at the time seeding took place, most atrazine was already degraded within the soil receiving atrazine-degrading bacteria, whereas the atrazine concentration remained at a higher level for a longer time in soils receiving no atrazine-degrading bacteria. An even faster atrazine degradation was recorded with atrazine mixed into the soil (data not shown). Although the extent of atrazine degradation recorded in the control soils without the addition of bacteria was considerable (Figure 7), the residual atrazine concentrations were apparently high enough to control plant growth (Figure 6, second row from the left). The rapid degradation in the controls might have been a result of microbial contamination since we did not work under sterile conditions in the greenhouse or it may be due to chemical or physical processes or microbial activity of indigenous soil microorganisms.

To determine the effect of climatic conditions on the atrazine-mineralizing bacteria, the experiment described above was repeated but the boxes of soil were kept outdoors and not watered until the seeding took place. At that time, the boxes were also taken into the greenhouse and were watered as described before. During the initial period with the boxes of soil kept outdoors, the soil surface dried out quickly due to the high ambient temperatures (25−30 °C) and the dry weather conditions during the exposure time. The atrazine concentrations in these soils decreased more slowly (Figure 7b) than those in soils kept in the

Figure 6. Plant growth in soil boxes kept in the greenhouse 34 days after application of atrazine by spraying onto (1 kg/ha) or incorporation (4 mg/kg) into the soil. In the foreground, right and left, are two control soils receiving no herbicide treatment. In the background on the very left side are three boxes of soil with atrazine-degrading biomass and atrazine applied by spraying; second row from the left, three boxes without atrazine-mineralizing bacteria and atrazine applied by spraying; third row from the left, three boxes with atrazine-mineralizing bacteria and atrazine incorporated into the soil; very right row, three boxes receiving no atrazine-mineralizing biomass and atrazine incorporated into the soil.

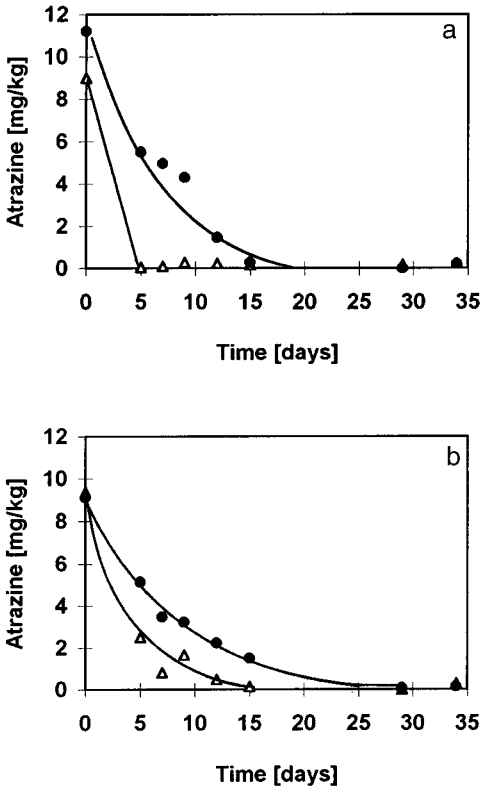

Figure 7. Atrazine concentrations in soils with atrazine applied by spraying (1 kg/ha), (△) with and (●) without atrazine-mineralizing bacteria incorporated into soil: (a) soil kept in the greenhouse for the whole incubation period; (b) soil kept outdoors without watering for 5 days before being taken into the greenhouse, seeded, and watered.

greenhouse and watered daily (Figure 7a). The plant growth observed was poor, independent of the presence or absence of atrazine-mineralizing bacteria, indicating that the atrazine-degrading bacteria remained less active.

DISCUSSION

Microbial enrichments and isolates capable of mineralizing atrazine rapidly and completely have been found and isolated from fields with a long history of atrazine application or from samples within atrazine production sites (Assaf and Turco, 1994; Mandelbaum et al., 1995; Radosevich et al., 1995; Topp et al., 1996; Yanze Kontchou and Gschwind, 1994). A great application potential of these atrazine-degrading bacteria is expected in the field of industrial water treatment and soil remediation. A few examples studying the application of special microorganisms for pesticide removal have been published so far. Hallas et al. (1995) treated glyphosate-contaminated waste water in a bioreactor at a high efficiency using immobilized bacteria capable of degrading this herbicide and applying hydraulic retention times of <30 min. At similar rates, atrazine was mineralized by immobilized bacteria used for this study for decontamination of polluted ground water (Stucki et al., 1995). Feng et al. (1997) described a system using immobilized cultures to clean industrial waste water containing a degradation product of the insecticide chlorpyriphos.

Since the atrazine-degrading organisms completely mineralize the molecule at high rates (Wenk et al., 1997; Yanze Kontchou and Gschwind, 1995), their inoculation into atrazine-contaminated soils at dealer sites could speed up remediation and thus could yield an economic advantage toward alternative remediation techniques. The bacterial strain used in this study was applied without any amendments, whereas the application of other atrazine-degrading pure cultures such as *Pseudomonas* ADP need the supply of an additional carbon source such as citrate to mineralize the herbicide efficiently (Mandelbaum et al., 1995). The addition of a carbon source is regarded as critical because the atrazine degraders have to compete with other soil microorganisms for the easy degradable carbon source

and because too high additions of citrate might lead to oxygen depletion in the soil.

The data presented show that the addition of the *Pseudomonas* sp. led to a rapid atrazine degradation in moist soil accompanied by the loss of activity of atrazine on sensitive plant species because the major part of the chemical was degraded during germination of the seeds (Figures 3, 5, and 6). This indicates that the herbicidal activity of atrazine can be lost on bioremediated fields. The loss of atrazine activity was dependent on the amount of atrazine-degrading biomass added (Figures 2 and 3). Atrazine sensitive plants germinating within a short period of time such as *N. officinale* were not killed but still retarded in their development with 0.1 mg/kg atrazine-degrading bacteria present in soil (Figure 4). The atrazine activity was lost completely in moist soils, however, on plants such as *S. nigrum* with a longer germination period than *N. officinale*, during which most of the atrazine was degraded. Amounts as low as 0.1 mg/kg atrazine-degrading bacteria were sufficient for this effect, which is equal to an amount of 0.15 kg of bacterial dry weight/ha, assuming a soil specific density of 1.5 kg/dm^3 and a homogeneous distribution of the bacteria in the top 10 cm of the soil. Thus, an application rate of 1.5 kg of bacterial dry weight/ha incorporated into or sprayed onto the soil might lead to successful bioremediation provided ideal field conditions are available.

The importance of ideal field conditions for rapid atrazine removal is shown in the experiment with soils left outdoors following application just for 5 days, during which time the soil surface dried. As a result of the dry soil conditions, the biological atrazine breakdown was slowed and the herbicide remained active (Figure 7b; Wenk et al., 1997). Because predictions about the bacterial breakdown are still difficult under field conditions, further investigations are needed to define better the conditions to be considered for a successful bioremediation.

A new field of application of the atrazine-degrading microorganisms might be the reduction of the risk of environmental pollution. Residual herbicides such as atrazine produce a lasting effect in the soil to control weeds germinating in the period after application. While this is beneficial from an agronomic viewpoint, from an environmental perspective, however, the slow degradation rate increases the risk of contamination of environmental compartments such as soil, water, and air. Results obtained in the present study indicate that the application of bacteria to degrade the herbicide to reduce the risk of environmental pollution can result in a too rapid degradation and thus in the loss of controlling activity of such chemicals. Accelerated breakdown of several pesticides in soils has been reported, sometimes occurring after several years of application (Felsot and Shelton, 1993; Ou et al., 1993). The reasons for this phenomenon were ascribed to the development of microorganisms adapting spontaneously to the presence of the agrochemical in the soil (Verhagen et al., 1995; Robertson and Alexander, 1994). So far, no report of enhanced biodegradation of atrazine in the field resulting in a loss in efficacy has been published. Nevertheless, in most of the published literature, atrazine is reported as being biodegradable. The rates of degradation were slow and the extent of mineralization reported remains only a low percentage of the amount applied (Solomon et al., 1996; Willems et al., 1996).

To reduce the environmental risk, it might be a challenge to find the balance between application of a chemical for pest control and its intended biological degradation.

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