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SEMIFIELD TESTING OF A BIOREMEDIATION TOOL FOR ATRAZINE-CONTAMINATED SOILS: EVALUATING THE EFFICACY ON SOIL AND AQUATIC COMPARTMENTS

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Abstract—The present study evaluated the bioremediation efficacy of a cleanup tool for atrazine-contaminated soils (*Pseudomonas* sp. ADP plus citrate [*P.* ADP + CIT]) at a semifield scale, combining chemical and ecotoxicological information. Three experiments representing worst-case scenarios of atrazine contamination for soil, surface water (due to runoff), and groundwater (due to leaching) were performed in laboratory simulators ($100 \times 40 \times 20$ cm). For each experiment, three treatments were set up: bioremediated, nonbioremediated, and a control. In the first, the soil was sprayed with 10 times the recommended dose (RD) for corn of Atrazerba and with *P.* ADP + CIT at day 0 and a similar amount of *P.* ADP at day 2. The nonbioremediated treatment consisted of soil spraying with 10 times the RD of Atrazerba (day 0). After 7 d of treatment, samples of soil (and eluates), runoff, and leachate were collected for ecotoxicological tests with plants (*Avena sativa* and *Brassica napus*) and microalgae (*Pseudokirchneriella subcapitata*) species. In the nonbioremediated soils, atrazine was very toxic to both plants, with more pronounced effects on plant growth than on seed emergence. The bioremediation tool annulled atrazine toxicity to *A. sativa* (86 and 100% efficacy, respectively, for seed emergence and plant growth). For *B. napus,* results point to incomplete bioremediation. For the microalgae, eluate and runoff samples from the nonbioremediated soils were extremely toxic; a slight toxicity was registered for leachates. After only 7 d, the ecotoxicological risk for the aquatic compartments seemed to be diminished with the application of *P.* ADP + CIT. In aqueous samples obtained from the bioremediated soils, the microalgal growth was similar to the control for runoff samples and slightly lower than control (by 11%) for eluates. Environ. Toxicol. Chem. © 2012 SETAC

Keywords—Herbicide *Pseudomonas* sp. ADP Simulator Runoff Leaching

INTRODUCTION

Atrazine is one of the most intensively used herbicides worldwide [1]. Due to its moderate to high persistence in the environment, atrazine has been detected above concentration limits defined by several countries [2]. Its toxic effects (especially for aquatic organisms) have also been extensively reported (for a recent review on the environmental fate and effects of atrazine and other *s*-triazine herbicides, see Viegas et al. [2]). As a consequence, atrazine has been banned in Europe [3]. However, its continued use is allowed in Africa [4], Asia [5,6], Latin America [7], the United States [8], and Australia [9].

Atrazine-contamination problems have been reported due to intensive applications [10], accidental spills [11], and deficient storage conditions at industrial units, dealerships, or mix-load sites [12,13]. These represent threats to soil ecosystems and to adjacent or nearby water resources due to edge-of-field runoff or leaching [14,15]. An important cause of nonpoint-source pollution of surface waters is associated with pesticide losses due to runoff [15–17], with peaks of concentration (and toxicity) measured after applications or rainfalls [15,18–20].

To mitigate or prevent the hazard effects of atrazine, research has been undertaken to develop bioremediation methodologies based on the ability of some microorganisms to convert atrazine into less toxic or nontoxic substances [2,14,21]. A bioremediation tool for atrazine-contaminated soils, consisting of soil bioaugmentation with the atrazine-mineralizing bacterium *Pseudomonas* sp. ADP (hereafter designated *P.* ADP) and biostimulation with trisodium citrate (CIT, used as a carbon source) [22], was recently tested at a microcosm scale (15 cm height × 2.5 cm diameter) under different worst-case scenarios of soil contamination. Very promising results were reported, showing a strong and rapid decline of atrazine concentration in soil as well as the effective reduction of soil toxicity to plants, cladocerans, and microalgae within 5 or 10 d [23,24]. Aiming at evaluating the potential of the atrazine cleanup tool [22,24] for routine use under real field scenarios, a scaling up of previous microcosm experiments was considered a crucial step. A further evaluation of the cleanup tool efficacy on both soil and water compartments was carried out at a semifield scale, using a novel cost-effective and standardizable simulator for pesticide applications. Thus, the occurrence of bioremediation and the efficacy of the latter process were investigated. In accordance with previous studies on the potential of this bioremediation tool to reduce atrazine toxicity in soil and aquatic environments [23], the present study also followed an integrated approach. Thus, the assessment of the soil habitat function (ability of soil to serve as a habitat for soil-living organisms) and the soil retention function (ability of soil to adsorb pollutants, avoiding their mobilization via the water pathway) [25], as well as atrazine removal from soil and water (through chemical analysis) at a larger semifield scale, were carried out. The soil retention function and consequently the

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risks of pesticide contamination in groundwaters are correlated with pesticide adsorption to the soil components, especially organic matter, and quantified by the soil adsorption coefficient (K_{oc}) [26].

In addition, a simulator was developed so that the indirect toxic effects on aquatic organisms due to the mobilization of atrazine via the water pathway in soils were evaluated by assessing not only the soil retention function but also toxic effects due to leaching and surface runoff from contaminated soils. Based on the work of Chelinho et al. [23], where results revealed negligible effects on soil invertebrates, no soil animals were used in the present study. Instead, two plant species (a mono- and a dicotyledonous, to investigate possibly different sensitivities to atrazine) and an aquatic microalgal species were selected as representative primary producers of the soil and aquatic compartments, respectively.

MATERIALS AND METHODS

Laboratory simulators

Laboratory simulators ($100 \times 40 \times 20$ cm; length, width, and height, respectively) were used to test the fate and effects of pesticide applications, mimicking different worst-case scenarios of soil and water contamination (see below) while allowing the collection of soil and aqueous samples (runoff and leachate). They consisted of two adjoining stainless steel trays of the same size, movable relative to each other, allowing the independent regulation of slopes. The bottom of each tray was slightly funnel-shaped and equipped with a tap at the bottom of the funnel to collect leachate samples. One of the trays was also equipped with a U-shaped channel with an opening at the center to drive and collect the runoff samples (Fig. 1).

Experimental design

The bioremediation efficacy of the atrazine cleanup tool was evaluated by performing three experiments with the simulators set up to represent three different worst-case scenarios of atrazine contamination for soil and aquatic organisms. To assess soil toxicity, that is, the soil habitat and retention functions [25], a first experiment (A) was carried out with the two trays of the simulators in the horizontal position (slope of 0%), to maximize the amount of pesticide remaining in the soil (see scheme A of Table 1). A second experiment (B) was carried out with both trays of the simulators at a slope of 42% (see scheme B of Table 1), to assess the maximum risk for aquatic organisms due to surface runoff originating from severe and unpredictable rainfalls. Finally, to assess the highest risk for aquatic organisms due to leaching, a third experiment (C) was carried out with one tray of the simulator at a slope of 42% and the other in the horizontal to mimic locations where leaching to groundwater is maximized by the occurrence of a field with an inclination adjacent to a flat area, which may receive runoff inputs (see scheme C of Table 1).

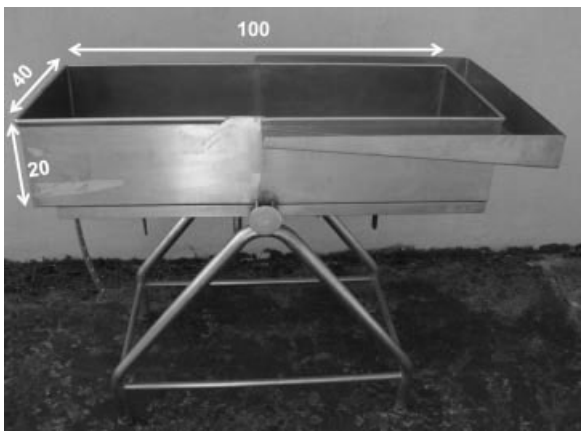

Fig. 1. Laboratory simulator developed to evaluate the bioremediation efficacy of an atrazine cleanup tool at a semifield scale. Dimensions are expressed in centimeters. See *Laboratory simulators* section for further details.

For each experiment, three simulators/treatments were set up. The first treatment was the control (Ct), with no herbicide or bioremediation tool sprayed onto the soil. The second treatment featured soil sprayed with 10 times the recommended dose (RD) of Atrazerba FL (~500 g atrazine/L; Sapec) for weed control in corn plantations (10×2 L Atrazerba/ha, equivalent to ~13.3 mg atrazine/kg of soil dry wt, assuming a soil density of 1.5 g/cm^3 and herbicide incorporation up to 5 cm depth) and with the bioremediation tool (*P.* ADP + CIT, see below). This treatment is hereafter designated as B (bioremediated) treatment. The third treatment was soil sprayed solely with 10 times RD Atrazerba. This treatment is hereafter designated as NB (nonbioremediated) treatment (Table 1). Because the present study intended to simulate a field application of pesticides with further collection of soil and aqueous samples for ecotoxicological evaluation (the assessment was not done directly in the laboratory simulators), the experiments were not replicated. Each simulator was filled with a 5-cm layer of 1-cm (diameter) glass beads (to facilitate leachate percolation) and a 15-cm layer of a sandy loam soil (pH = 6.14, 3.10% organic matter, 62.4% sand, 21.2% silt, 16.4% clay, 0.83 mg/kg total N, 0.0125 cmol/g CEC, and 32.8% water holding capacity) analyzed according to methods in Lima et al. [24]. Soil was representative of a corn-production field with no history of pesticide applications (Coimbra, central Portugal). The soil (top 10 cm) was collected one day before the start of each experiment, and major stones and vegetation were manually removed. During all experiments, the simulators were placed under a 7-m^2 (3.5×2 m) semiopen space, protected from direct sunlight and rain but exposed to outside temperature and humidity.

At the beginning of each experiment (day 0), the soil surface was sprayed with 10 times RD of Atrazerba for both the NB and B treatments (Table 1). This high Atrazerba dose was intended to represent worst-case scenarios of atrazine contamination, such as accidental spills [11], careless disposal [12,27], and intensive use [7] or overuse [10]. After spraying, a rainfall was simulated using artificial rain, prepared by diluting a stock solution in distilled water (1:10 ratio, v:v). The stock solution consisted of a mixture of $(NH_4)_2SO_4$ (925 mg); NaCl (386 mg); $CaCO_3$ (200 mg); $MgSO_4$ (180 mg); KCl (37 mg); KH_2PO_4 (14 mg); $NaNO_3$ (40 mg); HNO_3 3.5 M (2.0 ml); and HCl 1.0 M (1.0 ml) in distilled water at a final volume of 1 L [28]. The top 5 cm of soil was then mixed with a garden rake to facilitate the herbicide incorporation into the soil. For B treatments, a mixture of *P.* ADP + CIT (bioremediation tool) was sprayed onto the soil surface (see *Bioaugmentation agent* for details on the preparation of the inoculum) approximately 2 h after the incorporation of Atrazerba, and its incorporation was as described for the herbicide (Table 1). In the NB treatments, the application of the bioremediation tool was replaced by the same volume of artificial rain, while in the controls both the spraying of herbicide and the bioremediation tool were replaced by artificial rain. The total amount of artificial rain added to each treatment at day 0 (for herbicide and

Table 1. Summary of the experimental design to evaluate the efficacy of a bioremediation tool to clean up soils contaminated with 10 times the recommended dose of Atrazerba under three different worst-case scenarios of pesticide contamination toward soil habitat and retention functions[a], aquatic organisms due to surface runoff[b], and aquatic organisms due to leaching[c,d]

	Treatment	Sampling time (days)					
Experiment (simulator scheme)	Atrazerba dose + bioremediation tool	0	1	2	3	4	7
A	$0 \times RD + NO$ (Ct)	SCA					SCA SC
B							SCA RS
C			LS[e]			LC[f,g]	SCA
A	$10 \times RD + NO$ (NB)	AS SCA					SCA SC
B							SCA RS
C			LS[e]			LC[f,g]	SCA
A	$10 \times RD + YES$ (B)	AS BS SCA SMA	SMA	BS[h] SMA	SMA		SCA SMA SC
B							SCA SMA RS
C			LSe[e]			LC[f,g]	SCA SMA

[a] Experiment A, slope of 0% representing flat fields.
[b] Experiment B, slope of 42% representing fields with marked slope.
[c] Experiment C, slope of 0 and 42% representing fields with marked slope near flat areas.
[d] Evaluation included atrazine analysis (on soil, eluates, runoff, and leachates), microbial analysis (on *Pseudomonas* sp. ADP viability), and toxicity tests (on soil with plants and on eluates, runoff, and leachates with microalgae).
[e] Daily from days 1 to 6.
[f] Daily from days 4 to 7.
[g] Samples used for chemical analysis and microalgae tests.
[h] *P.* ADP spraying.
AS = atrazine spraying; BS = bioremediation tool (*P.* ADP + CIT) spraying; SCA = soil sampling for chemical analysis; SMA = soil sampling for microbiological analysis; SC = soil collection (for eluate preparation and soil toxicity tests); RS = runoff simulation; LS = leachate simulation; LC = leachate collection.

P. ADP + CIT incorporation) corresponded to 1.8 h of rain during the rainiest day of October 2005 in Coimbra (26.2 mm [29]). The total amount of aqueous solutions added per simulator was adjusted to obtain an initial soil moisture of approximately 60% of water holding capacity.

At day 2, a second application of the same amount of *P.* APD (no CIT incorporated) was performed, following the same procedures of day 0. In a previous study at a microcosm scale under laboratory-controlled conditions, a single soil application of *P.* ADP at day 0 proved to be effective to clean up soil contaminated with this same dose of Atrazerba (10 times RD) [23]. In the present study, the second application of the bacteria intended to minimize a possible decrease in its efficacy under semifield exposure conditions. The natural fluctuations of environmental variables (e.g., temperature, rainfall, wetting–drying cycles), as well as the presence of indigenous communities of soil microorganisms (that may act as competitors) and soil micro- and mesofauna (that may act as predators/grazers), may diminish the number of viable cells of the biodegradative bacteria and/or the atrazine–bacteria contact area and hence its atrazine degradation activity [30,31]. For these reasons, the experimental period selected in the present study to evaluate the efficacy of the bioremediation tool was 7 d instead of the 5 d reported in Chelinho et al. [23]. Composite soil samples (three per treatment, top 5 cm) were collected at the beginning and end of each experiment to determine soil atrazine concentrations. In aqueous samples, concentrations were determined (in the unique sample of each) only at day 7 (Table 1). For microbial analysis, B treatments were sampled on days 0, 1, 2, 3, and 7 (Table 1).

After 7 d, soil and aqueous samples were collected from each treatment to assess the efficacy of the bioremediation tool toward soil and aquatic organisms, specifically two terrestrial plants and one aquatic microalgal species, respectively (see *Ecotoxicological tests* section). For experiment A, the top 3 cm of soil were sampled (as a single composite sample) to evaluate

the soil habitat and retention functions, the latter through the preparation of soil eluates (Table 1). For experiment B, surface runoff was obtained by simulating a heavy rainfall with artificial rain (Table 1). The precipitation values used were those of a tropical country (Brazil), where atrazine is extensively used [7] and where the ecological risks of pesticide runoff into adjacent water basins are especially high [7,19]. Ten liters of artificial rain per simulator were used, corresponding to 9 h of rain on the rainiest day of October 2007 (66 mm) in an intensive agricultural region in central São Paulo state (meteorological station of CRHEA/SHS/EESC/USP, Itirapina, SP, Brazil; 22°10′13.53″, –47°53′58.12″). In experiment C, the collection of leachates comprised the simulation of a continuous period of rainfall, daily from day 1 to 6 (Table 1), using artificial rain and corresponding to 1.2 h of rain during the rainiest day of October 2005 in Coimbra (26.2 mm [29]). Because the soil achieved its maximum water holding capacity, leachate could be collected into glass vials attached to the taps of the simulators. Although leachate samples were obtained already at day 2, only samples collected on the last 4 d (days 4–7) were used for toxicity testing, to give time for the bacteria to work, because results from a previous microcosm study [23] indicated a period of 5 to 7 d to obtain an effective cleanup of the dose of Atrazerba used in the present study. All samples were stored either at 4°C in the dark until use (within 24 h or 15 d, for the plant and microalgal tests, respectively) or at –20°C for chemical analysis of atrazine.

The mean temperature values were 22 ± 3.7, 18 ± 4.7, and 18 ± 2.7°C during the performance of experiments A, B, and C, respectively (mean ± standard deviation, $n = 336$), whereas the corresponding humidity values were 68 ± 16, 52 ± 20, and $85 \pm 6.6\%$ ($n = 336$). Water losses by evaporation were estimated every two days by weighing a vessel containing a 15-cm layer of moist soil (~5 kg dry wt), extrapolated to the amount of soil in the simulators and replenished using artificial rain.

Bioaugmentation agent

A spontaneous rifampicin-resistant (Rif^r) mutant of *P.* ADP was used. This mutant can mineralize atrazine with efficiencies equivalent to the wild type [32]. The cell suspension used as inoculum was prepared from a late exponential culture of *P.* ADP Rif^r, grown as previously described [24]. The mixture sprayed onto soil consisted of a concentrated solution of CIT, to amend the soil with 1.2 mg trisodium citrate/g of soil dry weight, corresponding to a C_s to N_{atz} ratio of 50 [22,24], mixed with a concentrated inoculum suspension ($2.8 \pm 0.5 \times 10^{10}$ colony-forming units [CFUs] of *P.* ADP/ml).

Test organisms

Oat (*Avena sativa*) and rape (*Brassica napus*) were selected as model plants. They are part of a list of nontarget species for use in standardized plant toxicity tests [33] and known to be sensitive to atrazine [34]; seeds were obtained from a commercial supplier (Hortícola). The microalgae *Pseudokirchneriella subcapitata* (strain no. WW 15-2521; Carolina Biological Supply) was chosen as a model aquatic organism, as it has historically been recommended for freshwater toxicity studies and standard guidelines are available [35,36]. Cultures were maintained under nonaxenic conditions as previously outlined [37].

Ecotoxicological tests

To evaluate the impacts of atrazine on the aquatic compartment, toxicity tests with the microalgae *P. subcapitata* were carried out on soil eluate, runoff, and leachate samples originating from the simulators (Table 1). Eluates were prepared following standard methods [38] as previously described [23]. The runoff samples were centrifuged following the same procedures used for the eluate preparation (at 3,370 *g* for 20 min) to remove excess suspended particles. Whereas for the NB treatments a series of five dilutions was prepared and tested (100, 50, 25, 12.5, and 6.25%), for the control and B treatments only the original sample (100% dilution) was tested. The 72-h *P. subcapitata* growth tests were performed according to standard guidelines [35,36] on 24-well sterile microplates, at 21 to 23°C, and under continuous cool white fluorescent light (100 μE/m^2/s). Three 900-μl replicate cultures per each eluate, runoff, and leachate sample and six control (standard medium, also used to prepare NB dilutions) replicates were set up and inoculated with 100 μl of the algal inoculum. For further details on testing procedures, see Rosa et al. [37]. At the end of the 72-h exposure, algal growth was estimated as the mean specific growth rate per day. Conductivity and pH were measured at the start of the test. Measured levels were comparable across treatments and not expected to have deleterious effects on the test organisms [35,36].

To evaluate the bioremediation efficacy on soil habitat function, plant germination, and growth were evaluated following the ISO guideline [33]. For each treatment and each plant species, the soil was carefully mixed and distributed among four replicates (six for the control) that consisted of plastic boxes ($12 \times 9 \times 6$ cm; width × length × height, respectively) filled with 250 g (dry wt) of soil. After this, 10 seeds of *A. sativa* or *B. napus* were buried into the soil (~1 cm deep). Tests were run at controlled temperature (21°C), photoperiod (16:8-h light:dark, 100 μE/m^2/s), and relative humidity (70%). To provide suitable soil moisture during the tests, each box was perforated at the bottom and connected to another box by a glass fiber wick, filled with deionized water. Fourteen days after the emergence of more than 50% of the seeds in the controls, the aboveground part of the plants was cut, dried (for 16 h at 80°C), and weighed to estimate growth as shoot dry weight per emerged seed.

Microbiological analysis

To determine the number of *P.* ADP Rif^r viable cells (expressed as CFU/g of soil dry wt), soil samples (mean ± SD SD of 1.3 ± 0.3 g wet wt) were diluted in saline solution (0.9% w/v NaCl) and serial dilutions were spread plated onto selective medium (agarized Lennox broth supplemented with rifampicin [50 mg/L] and cycloheximide [100 mg/L]) [22]. Colonies were counted after 72 h of incubation at 30°C.

Chemical analysis

Soil samples (~20 g dry wt) were extracted with ethylacetate (3×10 ml) using a Liarre 60 ultrasonic apparatus (20 min, frequency 28–34 kHz), centrifuged (15 min, 2,500 pm), and analyzed for atrazine by gas chromatography/electron impact mass spectrometry, as previously described [24].

Atrazine from eluate, runoff, and leachate samples (~250 ml each) was extracted with the automated system ASPEC XL (Gilson) at neutral pH. Oasis 60-mg HLB cartridges were conditioned with 6 ml of dichloromethane, 6 ml of acetonitrile, and 6 ml of high-performance liquid chromatography water. Samples were percolated through the cartridge (flow rate of 6 ml/min), which was subsequently rinsed with 1 ml of high-performance liquid chromatography water (flow rate of 30 ml/min); after that, the adsorbent was dried with nitrogen for 30 min. Elution was performed with 2.5 ml acetonitrile:

dichloromethane (1:1, v:v) and 3 ml of dichloromethane. The final extract was concentrated to 200 μl with a gentle nitrogen flow.

The gas chromatography/electron impact mass spectrometry analyses were performed with Perkin-Elmer Model Clarus 500. The mass spectrometer was operated in the electron impact ionization mode with an ionizing energy of 70 eV and FV (Varian)-5MS (30 m × 0.25 mm i.d. with 0.25 μm film thickness) programmed from 50°C (1 min) to 150°C at 10°C/min, 150 to 240°C at 4°C/min, and to 270°C at 15°C/min, keeping this temperature for 2 min. Helium was used as the carrier gas at 30 ml/min, under the splitless mode and using 1 μl of injection volume. Chromatograms were recorded under time-scheduled selected ion monitoring (SIM). Full scan conditions (50–450 amu) were also used. All extracts were injected in SIM, for quantitative purposes and by scan mode, to confirm the presence of the analyte. The quality control comprised the use of control standards and the performance of recovery tests. Recovery ranged between 85 and 100%. Limits of quantification were 0.1 μg/g of soil dry weight or 0.1 μg/L.

Data analysis

In accordance with the study previously conducted at a microcosm scale [23], statistical analysis was carried out to answer three major questions: (1) Was the 10 times RD of Atrazerba toxic to the test organisms? (2) Was there bioremediation; that is, did the addition of *P.* ADP + CIT to the B treatments cause a decrease in toxicity compared to the correspondent NB treatments? and (3) What was the efficacy of the bioremediation tool; that is, what was the performance of the organisms in the B treatment compared to that in the control? All three questions were answered by comparing organism responses through a two-sample, one-tailed *t* test between NB versus control, NB versus B, and B versus control treatments for questions 1, 2, and 3, respectively. Normality and homoscedasticity were checked using Shapiro-Wilk's and Levene's tests, respectively. Whenever these assumptions were violated, even after data transformation, the equivalent nonparametric one-tailed Mann-Whitney test was used.

Additionally, to fully characterize the ecotoxicological potential of the aqueous samples from NB soils, microalgal growth responses in the control and tested dilutions (100, 50, 25, 12.5, 6.25, and 0%) were fitted to a logistic model using the least squares method [39], to estimate the effective concentrations inducing 20% (EC20) and 50% (EC50) of growth inhibition relative to the control, and respective 95% confidence limits. The growth inhibition caused by a 10% dilution of the aqueous extracts was also quantified as this corresponded roughly to the spraying of the RD of Atrazerba. All analyses were performed using Statistica 7.0 (http://www.statsoft.com/).

RESULTS

Survival of P. *ADP and atrazine biodegradation*

The quantification of the viable cell numbers of the bioaugmentation bacteria during the course of the three experiments is shown in Figure 2. A decline in the survival of the bacteria was observed during the first 2 d following soil bioaugmentation with *P.* ADP Rif^r and biostimulation with CIT. Notwithstanding, with the second soil inoculation with the atrazine-mineralizing bacteria, viable cell numbers higher than 3×10^8 CFU/g of soil were achieved during at least 1 d more, after which a progressive drop in bacterial survival occurred (Fig. 2).

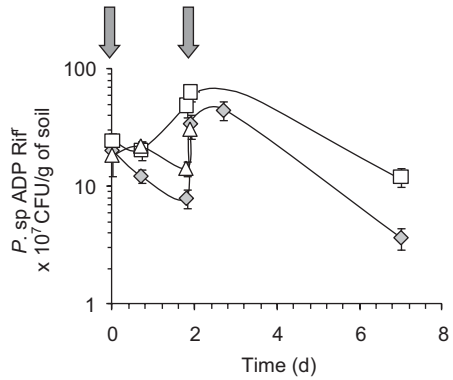

Fig. 2. Time-course variation of the concentration of viable cells of *Pseudomonas* sp. ADP Rif^r in the simulators contaminated with 10 times the recommended dose of Atrazerba and subsequently sprayed with the bioremediation tool *P.* ADP + CIT at day 0 plus the same amount of *P.* ADP at day 2 (gray arrows) during the 7-d experiments representing worst-case scenarios of pesticide contamination (see *Experimental design* section and Table 1 for details). Experiments A (◆), B (□), and C (Δ). Error bars indicate ± standard deviation.

Initial atrazine concentrations (overall values between 7.60 and 15.7 μg/g of soil dry wt) were strongly reduced in the B soils (by 98%, declining to less than 0.17 μg/g of soil dry wt) but not in the NB soils (~32–100% of the initial atrazine remained in the soil) after the 7-d treatments (Table 2). In the control soils (without application of Atrazerba), atrazine was always below the detection limits (data not shown). Consistent with the decrease in atrazine concentrations in the B soils, concentrations in all the aqueous extracts (eluates, runoff, and leachates) were considerably lower than in the corresponding extracts from NB soils after the 7-d bioremediation period (by at least 98%), even though atrazine concentration in NB leachate was approximately 100-fold lower than in NB eluate or runoff (Table 2).

Table 2. Mean atrazine concentrations in soil (μg/g of soil dry wt) and in the respective aqueous extracts[a] at the beginning and the end of 7-d treatment of soil contaminated with 10 times the recommended dose (RD) of Atrazerba and subsequently sprayed with (B) and without (NB) the bioremediation tool for the three experiments representing worst-case scenarios of pesticide contamination[b,c]

		Treatment			
		Soil (μg ATZ/g soil dry wt)		Aqueous extract (μg ATZ/L)	
Experiment	Day	B	NB	B	NB
(A) Soil + eluates	0	15.7 ± 3.05	18.0 ± 6.24	n.a.	n.a.
	7	0.120[d]	8.23 ± 2.64	10.1 ± 3.52	652[f]
(B) Runoff	0	14.7 ± 4.72	10.1 ± 1.66	n.a.	n.a.
	7	0.165 ± 0.0919[e]	10.5 ± 0.923	1.20	445
(C) Leaching	0	7.60 ± 0.854	10.4 ± 1.91	n.a.	n.a.
	7	<0.1	3.30 ± 0.100	<0.1	4.70

[a] Eluates, runoff, and leachates; μg/L.
[b] Soil and eluate concentrations represent mean ± standard deviation of three replicates and subreplicates, respectively, while runoff and leachate concentrations represent a single sample.
[c] See *Experimental design* section and Table 1 for details.
[d] Two replicates with concentrations below the detection limit.
[e] One replicate with concentration below the detection limit.
[f] Two subreplicates with concentrations below the detection limit.
ATZ = atrazine; n.a. = not applicable.

Efficacy of the bioremediation tool on soil

In both plant tests, the validity criterion of more than 70% seed emergence in the controls [33] was surpassed. A highly significant inhibition ($p < 0.001$) in shoot dry weight due to Atrazerba spraying in NB soil relative to the control was observed for both *A. sativa* (by 70%) and *B. napus* (by 88%) (Fig. 3A and B); evident signals of leaf chlorosis and necrosis, especially in *B. napus*, were also noted. Regarding seed emergence, no effects were observed for *B. napus* ($p > 0.05$), while for *A. sativa* the percentage of emerged seed was lower than in the control ($p < 0.001$) (Fig. 3A and B). The bioremediation of the atrazine-contaminated soil during 7 d resulted in an increase in shoot dry mass relative to the respective NB soil for both plant species ($p < 0.001$) (Fig. 3A and B); seed emergence of *A. sativa* was also higher in the B than in the NB soil ($p < 0.003$) (Fig. 3A). Comparisons between results from the B soil relative to those of the control showed that germination of *A. sativa* was lower in the former treatment ($p < 0.05$), while its aerial biomass was enhanced ($p < 0.001$) (Fig. 3A). For *B. napus*, plant biomass produced in B soil was lower than in the control ($p < 0.05$) and no significant effects on seed germination were observed ($p > 0.05$) (Fig. 3B).

Efficacy of the bioremediation tool for soil aqueous extracts

The validity criteria established for the algal growth in the standard control (cell density increase of at least 16-fold and coefficient of variation of mean growth rate $\leq$20%) were always fulfilled [35,36]. Microalgal growth was inhibited by at least 90% in eluate and runoff obtained from NB soils compared to the respective control ($p < 0.001$) (Fig. 4). Similar results were observed for the leachate samples, although growth was inhibited by only 18% ($p < 0.01$) (Fig. 4). In accordance, EC20 and EC50 values (and respective 95% CIs) for eluate (3.3% [2.2–4.4] and 7.6% [6.3–8.9]) and runoff (3.2% [2.9–4.3] and 9.8% [8.8–10.9]) were very close, whereas respective values for leachate could not be determined due to a low growth inhibition. Furthermore, exposing *P. subcapitata* to aqueous extracts resulting from the hypothetical soil application of the RD of Atrazerba (10% of 10 times RD) could cause a growth inhibition of 61, 51, and 0% for eluate, runoff, and leachate samples, respectively.

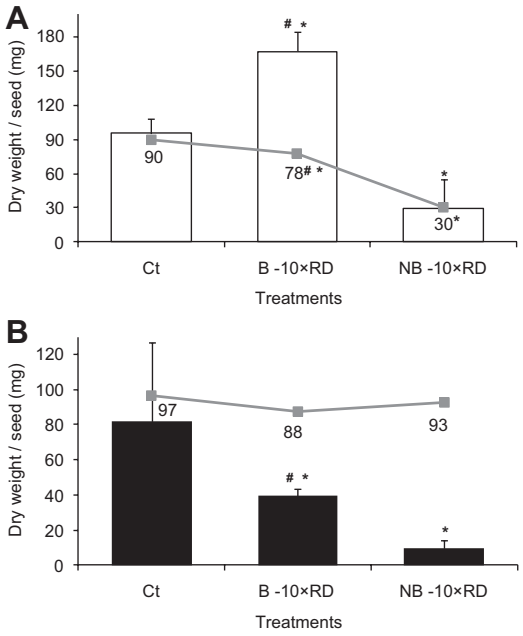

Fig. 3. Mean seed germination (in %, gray line) and growth (shoot dry wt/emerged seed, bars) of *Avena sativa* (**A**) and *Brassica napus* (**B**) in soil collected from the simulators contaminated with 10 times the recommended dose (RD) of Atrazerba and subsequently sprayed with (B) and without (NB) the bioremediation tool. Error bars indicate ± standard deviation, * Mean statistically different from control. # Mean statistically different from the NB treatment.

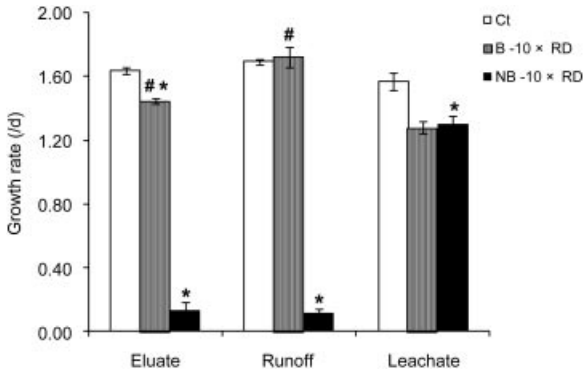

Fig. 4. Mean 72-h growth rate of *Pseudokirchneriella subcapitata* on eluates, runoff, and leachates collected after 7 d from the simulators contaminated with 10 times the recommended dose (RD) of Atrazerba and subsequently sprayed with (B) and without (NB) the bioremediation tool for the three experiments representing worst-case scenarios of pesticide contamination (see *Experimental design* section and Table 1 for details). Error bars indicate ± standard deviation. * Mean statistically different from control. # Mean statistically different from the NB treatment.

The growth rate of *P. subcapitata* in eluate and runoff was higher in samples obtained from B than from NB soils ($p < 0.001$) (Fig. 4). In contrast, no differences were found between NB and B leachates ($p > 0.05$) (Fig. 4). Microalgal growth in runoff samples collected from B soil was similar to that in control soil ($p > 0.05$). On the other hand, growth in eluate samples from B soil was still lower than that from control soil ($p < 0.001$), although this difference was merely 11% (Fig. 4) and thus considered not to represent a toxic effect.

DISCUSSION

Biodegradative performance of P. *ADP + CIT at semifield scale*

In the present study, effective removal of atrazine was achieved within a period of 7 d at a semifield scale. These results are similar to those previously reported for laboratory microcosms [23,24], where there were more controlled and presumably more favorable conditions for bioremediation. A treatment comprising double bioaugmentation with *P.* ADP, within a 2-d interval, and a single initial amendment with CIT of soil previously contaminated with 10 times RD of an atrazine commercial formulation promoted the biodegradation of more than 98% of the initially applied atrazine. The two successive inoculations of soils with *P.* ADP apparently contributed to the delayed decline in bacterial survival that occurred in a natural soil 1 to 2 d following soil bioaugmentation [23,24]. The large number of physiologically active *P.* ADP maintained in the soil ($>10^8$ CFU/g of soil dry wt on average) may have contributed to the extent and fast degradation of atrazine at the semifield scale. Nevertheless, in soils that did not receive the bioremediation treatment, a moderate decrease of atrazine was also observed during the 7 d: 50% in experiment A, 68% in experiment C, and no decrease in experiment B. Despite this difference and since intrinsic biodegradation is not likely to occur in this soil [24], it

seems conceivable that abiotic degradation [40], probably accompanied by the formation of nonextractable residues [41,42], also occurred in the soil herein used [23]. Because atrazine biodegradation was equally effective for the three independent experiments carried out, it can be suggested that with the amendment of the soil with the cleanup tool, small variations in the natural environmental conditions (e.g., temperature, humidity, and light) may not significantly affect the rate and extent of atrazine biodegradation under field conditions.

Ecotoxicological monitoring of the efficacy of the bioremediation tool

Soil. The herbicidal properties of atrazine caused severe toxic effects on both *A. sativa* and *B. napus*, though different sensitivities were observed depending on the endpoint measured. For *A. sativa* phytotoxicity was observed during seed germination and plant growth, while for *B. napus* only the aerial biomass production was negatively affected. Despite this, *B. napus* seemed to be most sensitive since shoot dry mass in NB soils was reduced by 88% relative to the control, while for *A. sativa* the same parameter was reduced by 70%. The present results are in agreement with the notion that dicotyledonous species appear to be more sensitive to atrazine than monocotyledonous species [43,44]. They also highlight the potential risks of atrazine applications on nontarget plants that may exist in the surrounding fields and that may be exposed to herbicide by spray drift or accidental spillage [2].

According to the chemical analysis, at the time of the collection of soil samples in the plant tests (day 7), at least 99% of the initial atrazine on the bioremediated soil was removed; thus, theoretically, atrazine toxicity would be strongly diminished. However, results of plant tests in this soil showed that bioremediation was not 100% effective, especially in the case of *B. napus*, because plant growth was reduced by 48% compared to control soil. This fact suggests that this plant species is highly susceptible to atrazine, even at low soil concentrations. The observed toxicity in the present study may have been due to the presence of atrazine soil bound residues, which might still be bioavailable [45]. The highest efficacy of the bioremediation tool in reducing atrazine's toxic effects to plants was observed for *A. sativa*: 86% effective for seed germination and 146% for plant growth, the last value traducing a boosting effect probably due to the addition of *P.* ADP + CIT to the soil. Some strains of *Pseudomonas* may act as plant-growth promoters, while others play the opposite role [46]; but this feature has not been reported for the strain used in the present study. Wenk et al. [47] also reported successful atrazine biodegradation and restoration of normal plant growth (*Nasturtium officinale* and *Solanum nigrum*) due to the amendment of soil contaminated with the herbicide (0.06–4 ppm) with an atrazine-degrading *Pseudomonas* strain different from the one herein used, under both laboratory and greenhouse conditions.

Soil aqueous extracts. As expected for an herbicide, atrazine significantly inhibited the growth of *P. subcapitata* exposed to eluates and runoff, though a slight toxicity was observed for leachates collected from NB soils.

Moreover, according to the results of the present study, misapplications of the herbicide atrazine are potentially toxic to phytoplankton. Applying atrazine at recommended label rates might also represent a risk, as indicated by the EC20 and EC50 values for both eluates and runoff. Indeed, the derived values were always below 10% of 10 times RD of Atrazerba, and inhibition of at least 50% in microalgal growth was observed in eluates and runoff estimated to have such an RD. However, as the percentage of pesticide loss by runoff is influenced by pesticide application rates [48], these results should be validated using the same experimental scheme described in the present study but applying the recommended label rates.

According to the literature, among aquatic organisms, phytoplanktonic species such as *P. subcapitata* are more susceptible to atrazine contamination than other organisms from higher trophic levels [49,50].

The lower toxicity observed for leachates in NB soils was likely related to the timing of the collection, at the end of the experiments (days 4–7 as the aim of the present study was to evaluate the efficacy of the cleanup tool in a period of at least a 4-d bioremediation treatment). Thus, during the first days of artificial rain (day 0–3), a great amount of atrazine may have leached from the topsoil layer. Despite this, concentrations of 4.7 μg/L of atrazine (such as those found in the leachates collected in the present study from nonbioremediated soils and causing an 18% inhibition in microalgal growth) might even have deleterious effects on phytoplankton (single-species tests with *P. subcapitata*; for a review see [50]) and corroborate the high leaching potential of atrazine reported in previous works [7,51].

Thus, according to the results of the present study, misapplications of the herbicide atrazine (and, theoretically, recommended label rates) may pose a risk to aquatic producers when soils have low ability to retain atrazine and its metabolites, thus potentiating its mobilization into groundwater [25] and/or when their mobilization by surface runoff from adjacent fields takes place [7,50,52]. The addition of the bioremediation tool to atrazine-contaminated soils significantly enhanced the removal of the herbicide in the B soils. Consistently, the respective eluates and runoff water were significantly less toxic to the microalgae than those obtained from NB soils. In comparison with the control soil, no toxicity was observed for the runoff obtained from B soils, while for eluates, the divergence of only 11% between the B treatment and control suggests an almost complete bioremediation in soil.

Therefore, these results indicate an effective detoxification of the water compartment as a result of the bioremediation treatment of soil as atrazine (and possible metabolites) in eluates and runoff were presumably reduced to essentially nontoxic levels for the microalgae.

CONCLUSIONS

Under semifield conditions, applying the bioremediation tool comprising soil amendment with *P.* ADP + CIT was clearly effective at reducing the potential environmental risks of atrazine misuse applications for both soil and aquatic compartments in just 7 d. Indeed, besides the improved extent of herbicide removal from soil (>98% of the initial concentration), an effective cleanup of soil was long established by the results from ecotoxicological monitoring of the bioremediation treatment. This decontamination of the bioremediated soil and of the aqueous extracts collected from it (namely, runoff and eluates) was revealed by the decreased or annulled toxicity of atrazine to two plant (*A. sativa* and *B. napus*) and one microalgal (*P. subcapitata*) species compared to that observed in samples collected from nonbioremediated soils. Thus, the present study also indicates that the application of this technology in real field scenarios of atrazine contamination might be feasible in a short time span, although its performance in different soil types as well as with other atrazine formulations needs to be evaluated.

Also, the integrated approach used here (namely, evaluation of the bioremediation efficacy under different worst-case scenarios of atrazine contamination, gathering chemical and ecotoxicological information) proved to be a robust and relevant method that may be transposable to other situations of bioremediation of contaminated soils.

In addition, a cost-effective laboratory simulator of pesticide applications, enabling runoff and leaching scenarios, is now available.

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