

The Role of the Bacterial Community of an Agroecosystem in Simazine Degradation

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Received: 2 December 2006. Accepted: 2 January 2007

Abstract

The use of pesticides and fertilizers in agricultural practice is the main source of soil and groundwater contamination. S-Triazines are among the most used herbicides in the world for selective weed control in several types of crops. The homeostatic capability of an agroecosystem to remove a triazinic herbicide, simazine, was assessed in microcosms treated with the herbicide in presence/absence of urea fertilizer. The latter, as well as a fertilizer, is also one of the last by-products before simazine mineralization. The biodegradation, in terms of disappearance of 50% of the initial concentration (DT_{50}), was compared to the degradation and metabolite formation occurring in sterilized soil. Moreover, the bacterial community response was assessed in terms of abundance and community structure by the epifluorescence direct count method and fluorescence *in situ* hybridization. The results show that the microbial community has a primary role in simazine degradation and that this process is due to the presence of a microbial pool working in succession and of which the metabolism may be modulated by exogenous sources of nitrogen, like urea. The latter influences the degradative pathway with a greater formation and accumulation of the desethyl-simazine metabolite, which is a hazardous contaminant of soil and groundwater ecosystems, as well as its parent compound.

Key-words: simazine, desethyl-simazine, bacterial community, degradation.

1. Introduction

Simazine is a herbicide used widely both in Italy and worldwide. Simazine and its deethylated metabolite, desethyl-simazine, are frequently found in groundwater above the legal limit ($> 1 \mu\text{g L}^{-1}$) (Funari et al., 1995). Both compounds are toxic (Edwards, 2002), and for this reason simazine is included in the EU Priority Pollutant List. Simazine has two main metabolites, 2-hydroxy-simazine (OH-Sim), which can be formed via both abiotic and biotic processes, and desethyl-simazine (DES) formed only via biotic transformation (Di Corcia et al., 1999). DES maintains the phytotoxic properties of its parent compound (Winkelmann and Klaine, 1991). The formation of cyanuric acid as an intermediate, and then its transformation to biuret, was found to be the common step before mineralization, although the sequence of steps

varies among degraders and depends on the availability of carbon and nitrogen sources in the environment (Radosevich et al., 1995; Struthers et al., 1998; Gebendinger and Radosevich, 1999; Singh et al., 2004). Since triazine herbicides are often applied to soils receiving fertilizers it is important to know if simazine biodegradation can be influenced by urea. Knowledge of this process may support the development of management strategy for preventing or remedying soil and water contamination in agricultural areas. The main objective of this study was in fact to assess the homeostatic capacity of the microbial community of an agricultural soil to remove simazine and whether the presence of urea might affect its biodegradation. Urea is not only a fertilizer, but also one of the last metabolites before simazine mineralization. Laboratory experiments were

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therefore performed in order to evaluate the biodegradation in terms both of DT_{50} of the initial simazine concentration and formation of the main metabolites in presence/absence of urea. Moreover, soil samples were incubated in liquid cultures with simazine as carbon source, in order to isolate the bacterial populations involved in the degradation.

2. Materials and methods

2.1 Soil characteristics

Soil samples were collected from the Ap1 (10YR4/2) horizon (0-20 cm depth) of an agricultural area in which simazine and urea are being applied. The soil was 49.5% sand, 32.6% loam, 17.9% clay, the pH was 5.9, the of organic C 0.8% and 0.1% the total N. It was classified as a Haplic Acrisols (WRB classification) and Ultic Haplustalf, fine loamy over sandy, mixed, super active, mesic (USDA, 1994).

2.2 Soil degradation experiment

The soil degradation experiment was conducted in duplicate according to SETAC procedures (Lynch, 1995) and previous experiments (Fava et al., 2001; Barra Caracciolo et al., 2005a,b). Some soil samples (200 g) were treated with simazine at a concentration of 1.5 mg kg⁻¹ (Sim), others were amended with both the herbicide and urea at a rate of 500 mg kg⁻¹ (Sim + Urea) and finally some samples were treated with simazine after a previous sterilization (Sterile). Moreover, some non-treated samples were used as a microbiological control (Control). All samples were incubated at 21 °C (± 0.5) in the dark. At fixed intervals (0-77 days) soil sub-samples (1g) were collected from each microcosm for the chemical (2 replicates) and microbiological analyses (4 replicates).

2.3 Chemical analysis

Concentrations of simazine and its main metabolites (OH-Sim and DES) were measured by Liquid Chromatography – Electrospray Mass Spectrometry (LC-ES/MS), until a reduction of about 80% of the initial value. Pesticide and metabolite extraction was performed in an Accelerated Solvent Extractor, as reported in detail in previous works (Di Corcia et al., 1999; Barra Caracciolo et al., 2005a).

2.4 Analysis of bacterial community by epifluorescence methods

The bacterial community was studied by using epifluorescence microscope methods, such as direct count and fluorescence *in situ* hybridization (FISH). The bacterial abundance (N. bacteria g⁻¹ soil) was measured by using DAPI as the DNA fluorescent agent (Barra Caracciolo et al., 2005a,b). The phylogenetic characterization was performed by FISH, estimating the cells binding the fluorescent probes as a proportion of the total DAPI-positive cells. Sample pre-treatment and 16S RNA oligonucleotide probe hybridization were performed in accordance with the protocol of Pernthaler et al. (2001) and adapted by us for soil samples (Barra Caracciolo et al., 2005c). The probes used, sequences, target sites and taxa identified are shown in Table 1.

The slopes of the linear equations obtained transforming the chemical data in ln were compared applying the ANCOVA (Analysis of covariance) test. The bacterial abundances were correlated with the simazine degradation.

2.5 Enrichment culture on simazine

Soil samples (2 replicates for each condition) were inoculated in a liquid medium, following the procedure described in Sanchez et al. 2005, with the simazine (5 ppm) as the C source, in presence (Sim + Urea) or in absence of urea (Sim). At day 20 sub-aliquots of the liquid cultures (100 μ L) were collected and plated on a solid medium with simazine in presence/absence of urea. After several platings, the colonies isolated were inoculated in a liquid medium with the herbicide in order to assess their capability to grow on simazine. The bacterial growth was measured in terms of optical density (OD) and their degradation capability in terms of the herbicide concentration, as described above.

3. Results

3.1 Soil degradation experiment

The percentage of simazine vs time in the different conditions is shown in Figure 1. The disappearance time of 50% of the initial concentration of the herbicide (DT_{50}) was 36 days ($R^2 = 0.98$) in presence of simazine (Sim), 32 days ($R^2 = 0.99$) in co-presence of both simazine and urea (Sim + Urea) and 90 days ($R^2 = 0.86$) in

Table 1. Fluorescent probes, sequences, target sites and corresponding taxa identified.

Probe	Sequence from 5' to 3'	Target molecule and position	Taxa
EUB338	GCT GCC TCC CGT AGG AGT	16S rRNA 338-355	Bacteria
EUB338 II	GCA GCC ACC CGT AGG TGT	16S rRNA 338-355	Bacteria
EUB338 III	GCT GCC ACC CGT AGG TGT	16S rRNA 338-355	Bacteria
ALF1B	CGT TCG YTC TGA GCC AG	16S rRNA 19-35	α -Proteobacteria
BET42a	GCC TTC CCA CTT CGT TT	23S rRNA 1027-1043	β -Proteobacteria
GAM42a	GCC TTC CCA CAT CGT TT	23S rRNA 1027-1043	γ -Proteobacteria
Pla46	GAC TTG CAT GCC TAA TCC	16S rRNA 46-63	Planctomycetes
Pla886	GCC TTG CGA CCA TAC TCC C	16S rRNA 886-904	Planctomycetes
HGC69A	TAT AGT TAC CAC CGC CGT	23S rRNA 1901-1918	Actinobacteria
Nso190	CGA TCC CCT GCT TTT CTC C	16S rRNA 189 - 207	Nitrosomonadaceae
Nsr1225	CGC CAT TGT ATT ACG TGT GA	16S rRNA 1225-1244	Nitrospira

sterile soil (Sterile). The degradation was significantly greater in both microbiologically active soils (Sim, Sim + Urea) than in the sterile one (test ANCOVA, $P < 0.01$).

3.2 Metabolites

The herbicide was transformed mainly into two metabolites: hydroxy-simazine (OH-Sim) and desethyl-simazine (DES). OH-Sim was the most abundant and detected from the first day in the sterile soil as well (Fig. 2A); on the contrary, DES was found only in the microbiologically active soils and in a greater amount in Sim + Urea than in Sim (Fig. 2B). After two weeks the detection of other metabolites (data not shown), such as bis-desethyl-simazine and hydroxyl-desethyl-simazine, indicated that the OH-Sim and DES were further transformed.

3.3 Bacterial community

Bacterial abundance (N. bacteria g^{-1} soil) is shown in Figure 3. The number of bacteria not only was not affected by the herbicide, but also increased in the Sim + Urea between 7-21 days

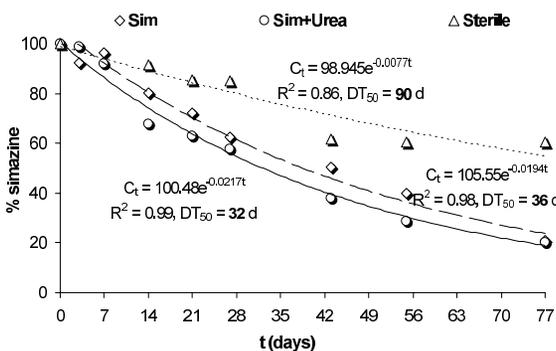


Figure 1. Decrease (%) of simazine vs time in the three different conditions (Sim, Sim + Urea, Sterile).

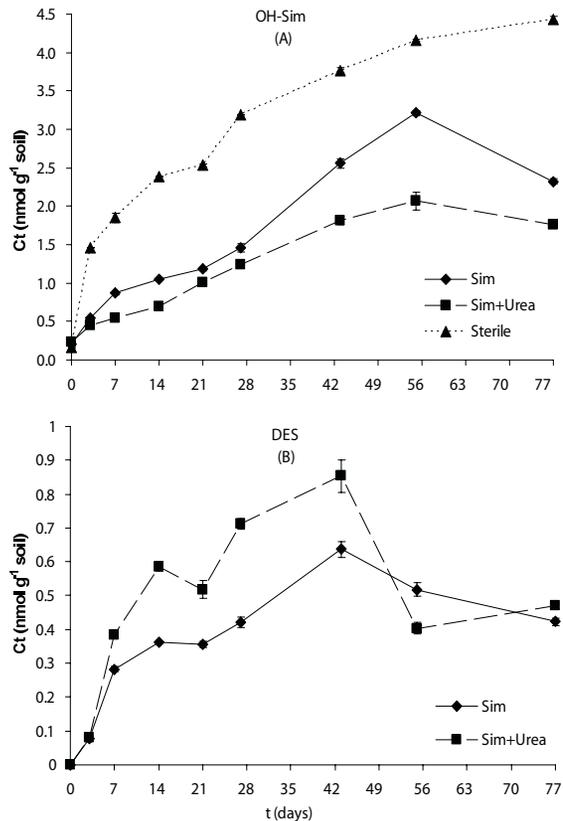


Figure 2. Amount ($nmol\ g^{-1}soil$) of the two main metabolites formed in the three different conditions (Sim, Sim+Urea, Sterile). (A) hydroxy-simazine, OH-Sim; (B) desethyl-simazine, DES. Vertical bars represent standard errors.

and in Sim between 21-28 days; in the latter condition the increase in number was also correlated to the decrease of the herbicide ($r = 0.97$, $p < 0.01$). The presence of urea presumably affected all the bacterial populations positively, including those not involved in the herbicide

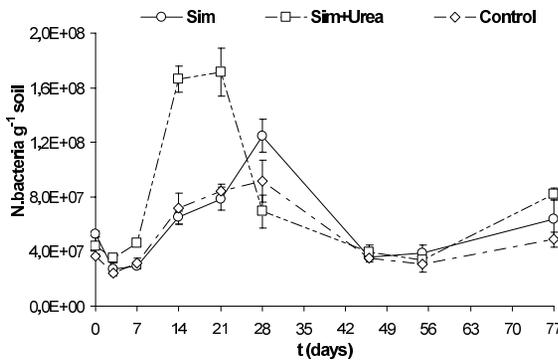


Figure 3. Bacterial abundance (N. bacteria g^{-1} soil) vs time detected by DAPI in the treated (Sim, Sim + Urea) and non-treated (Control) soils. Vertical bars represent standard errors.

degradation. The application of FISH permitted the phylogenetical identification and classification of the bacterial populations. Data are expressed as percentages of total DAPI-stained cells. At day 0 with an initial abundance of about $4 \cdot 10^7$ cell g^{-1} soil the fluorescence in situ hybridization detected about 50% of cells positive to Bacteria domain probes (EUB338/II/III), but the percentage in treated soils increased between 7 and 14 days to values of 80-90%. Cells positive to all the oligonucleotidic probes used were found, indicating the active presence of bacteria belonging to the α -, β -, γ -Proteobacteria, Planctomycetes, Actinobacteria, Nitrospira and Nitrosomonas groups. Among all these groups, α -, β -, γ -Proteobacteria were the most affected by the treatment, with changes in their relative percentages (Tab. 2). The α -Proteobacteria increased in Sim between 0 and 21 days and then their presence was similar in all the three experimental conditions. The percentage of β -Proteobacteria increased both in Sim and Sim + Urea, while that of γ -Proteobacteria increased at day 7 in Sim + Urea, reaching a peak (22%) five times greater than at day 0; in Sim the increase was observed between 14 and 21 days.

3.4 Enrichment cultures with simazine as carbon source

Since the microbial pool was able to degrade the herbicide, showing a similar DT_{50} in presence/absence of urea (Fig. 4A, B), the simazine was used as a carbon source. The DT_{50} values greater than those found in the soil microcosms

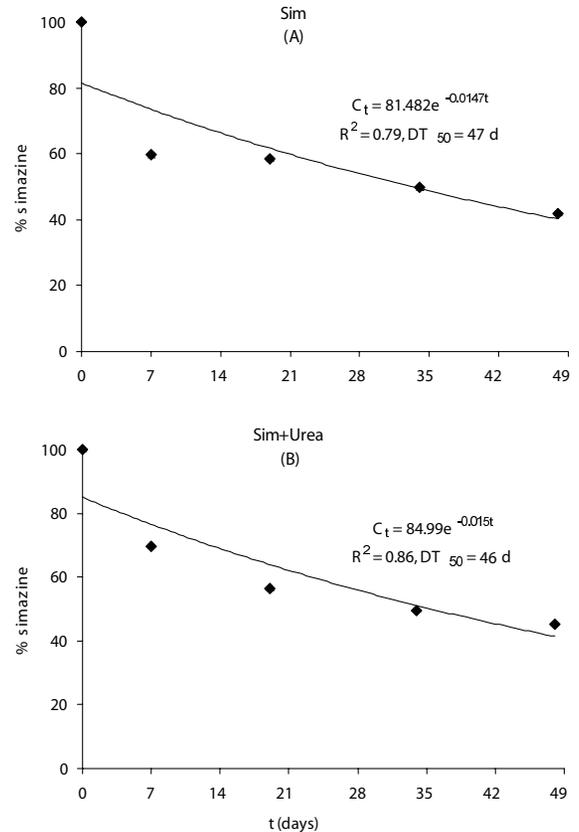


Figure 4. Degradation of simazine in enrichment cultures with simazine as the C source. (A) Sim and (B) Sim + Urea.

(46-47 days vs 32-36 days) show that the cultivation on laboratory media is not always an optimal condition for bacterial growth. The desethyl-simazine was detected from day 0 in both conditions (Sim e Sim + Urea); however a peak (0.6 mg L^{-1} , about 12% initial simazine) was observed at day 7 in the presence of urea. Moreover, the mineralization experiment performed with [U-ring ^{14}C] simazine, (as reported in Sanchez et al., 2005) at the University of Madrid, showed that in 28 days the same microbial pool was able to mineralize about 3% of the initial herbicide, while in the presence of urea the mineralization decreased to 0.8%.

3.5 Bacterial strains isolated

Two morphotypes (Fig. 5) were isolated on culture media from the microbial pool culture. The capability of the two strains to degrade simazine was tested by inoculating a colony of each strain into a liquid culture with the herbicide. Although the culture media were found to become turbid after about 48 hours, indicating that the

Table 2. Average percentage (%) and standard errors of total DAPI stained cells positive to the oligonucleotide probes in the three different conditions, Sim: A, Sim + Urea: B and Control: C. Each number is obtained by counting at least 1,000-1,200 bacteria cells.

days	Bacteria			α -Proteobacteria			β -Proteobacteria			γ -Proteobacteria		
	A	B	C	A	B	C	A	B	C	A	B	C
0	50±7.7	50±7.7	50±7.7	23±0.1	23±0.1	23±0.1	9±1.0	9±1.0	9±1.0	4±1.3	4±1.3	4±1.3
7	75±6.4	83±4.5	20±0.5	33±0.4	17±1.6	13±1.2	16±4.0	22±2.2	11±1.8	2±0.9	22±0.9	4±2.4
14	84±3.6	82±1.0	50±10	30±6.9	9±2.8	6±1.5	27±3.1	22±2.2	15±0.3	7±1.2	9±1.2	10±4.0
21	76±0.9	72±8.7	54±0.0	50±3.9	14±2.0	18±6.5	17±0.5	19±2.0	14±3.2	15±0.0	13±0.0	9±0.0
28	66±14.9	63±4.9	59±1.4	6±1.6	18±2.3	10±0.0	20±4.8	25±1.5	8±0.8	3±1.3	9±1.3	4±1.3
43	57±4.3	75±16	40±13	9±4.5	10±2.0	15±8.7	7±0.5	17±2.0	18±3.8	5±0.6	5±1.8	2±0.6
55	63±17.6	58±10	50±4.4	14±1.6	10±0.4	14±2.7	30±1.4	9±1.5	12±3.0	1±0.3	5±0.1	2±0.3
77	40±16.7	48±2.2	40±5.5	15±4.4	14±1.7	15±0.6	21±0.9	31±1.8	6±0.3	5±0.2	7±4.8	4±0.2
Av	62±5.8	65±5.8	46±4.7	22±5.2	15±2.4	14±1.6	18±3.0	19±3.1	13±1.5	5.4±1.6	9.3±2.1	5.1±1.1

days	Planctomycetes			Actinobacteria			Nitrosomonadaceae			Nitrospira		
	A	B	C	A	B	C	A	B	C	A	B	C
0	4±1.0	4±1.0	4±1.0	4±0.2	4±0.2	4±0.2	4±1.0	4±1.0	4±1.0	6±1.0	6±1.0	6±1.0
7	13±1.9	7±0.7	15±1.3	6±0.5	6±1.3	8±1.9	9±1.6	7±1.1	5±0.1	14±1.3	5±0.2	5±1.8
14	6±1.8	4±1.1	8±0.2	5±0.3	5±0.0	5±0.1	4±0.2	3±0.0	4±0.4	9±0.6	9±1.8	7±1.6
21	29±7.6	13±1.3	22±2.5	18±2.9	12±4.5	16±5.1	20±3.7	6±2.0	2±0.0	10±0.0	10±0.0	5±0.0
28	13±7.6	9±0.0	11±0.2	14±2.9	12±2.4	5±0.6	1±0.6	1±0.0	0±0.0	1±0.3	4±1.9	2±0.2
43	9±2.5	12±1.1	10±2.8	12±2.0	6±0.6	6±4.1	4±0.5	6±2.0	2±1.0	3±0.7	6±2.0	4±0.9
55	6±0.0	5±1.3	3±0.0	10±0.9	8±1.3	4±0.0	2±0.1	5±1.0	2±0.3	1±0.4	2±0.3	1±0.2
77	6±0.0	2±0.1	2±1.0	1±0.1	7±1.1	4±0.3	2±0.2	2±0.5	1±0.2	2±0.2	9±1.5	5±0.1
Av	11±2.9	7±1.4	9±2.5	9±2.0	8±1.1	7±1.4	6±2.0	4±0.9	2±0.6	7±2.5	7±2	5±1

A = Sim; B = Sim + Urea; C = Con; Av = average

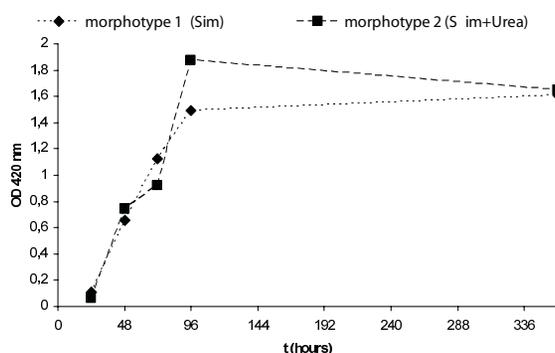


Figure 5. Growth of morphotypes 1 and 2 in liquid cultures, measured in terms of optical density, OD (420nm).

two bacterial populations were growing (Fig. 5), the chemical analyses showed that their overall herbicide degradation capability was very low. This result was confirmed by the fact that the same strains were able to mineralize just 0.1-0.3% of the initial herbicide concentration at day 28. Finally, the DNA sequencing (Gibello et al., 1999) and a comparison with the GenBank database permitted the identification of the morphotype 1 with the *Burkholderia* genus, (Class β -Proteobacteria), and

the morphotype 2 with the *Acinetobacter iwof-fii* species (Class γ -Proteobacteria); the latter result was interesting as it confirmed that within these two classes there are bacteria able to degrade the herbicide.

4. Discussion

The degradation in the sterile conditions was due to the formation of OH-Sim, since the herbicide hydrolyzation may occurs both via biotic and abiotic processes; however the results in the microbiologically active soils show a key role of the soil bacterial community in simazine degradation. The bacterial populations showed a significant degradative capability with DT₅₀ values in line with the lowest reported in literature (Garcia-Valcarcel and Tadeo, 1999), indicating an adaptation acquired from previous exposure (Moran et al., 2006). The urea affected the degradation pattern and favoured a greater formation of desethyl-simazine, presumably because the ethylic chain of the herbicide was used mainly as a C source. However, further DES mineralization was less favoured by the pres-

ence of the fertilizer, as confirmed by the lower mineralization observed (0.8% vs 3%). The latter result is ascribable to the fact that the final part of simazine degradation (cyanuric acid, biuret and urea formation) is due mainly to bacteria which use the metabolites as N sources; consequently the degradation is less favoured in soil treated with an exogenous source of N, like urea, as found in other works (Struthers et al., 1998; Gebendinger and Radosevich, 1999; Strong et al., 2002; Barra Caracciolo et al., 2005a).

The presence of the herbicide in combination with urea therefore stimulated the presence of some bacterial populations more than others, as confirmed by different percentages of α -, β -, γ -Proteobacteria detected in the microcosms. Moreover, the enrichment culture results indicated that the degradation was due mainly to a microbial pool working in succession than to a single bacterial strain, as shown by the low degradative capability found in the isolated strains.

5. Conclusions

The presence of urea affected the biodegradation of simazine with more formation and less mineralization of its most hazardous metabolite, DES. Consequently, in agricultural areas in which simazine is used together with urea there is more likelihood that desethyl-simazine will be found as a contaminant of soil and groundwater.

Acknowledgements

The authors thank Prof. Margarita Martin and Prof. A. Gibello of the Complutense University of Madrid for the mineralization tests and the DNA sequencing of the isolated strains.

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