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Effects of wood amendments on the degradation of terbuthylazine and on soil microbial community activity in a clay loam soil

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Abstract The herbicide terbuthylazine is widely used within the EU; however its frequent detection in surface and groundwater, together with its intrinsic toxicological properties, may pose a risk both for human and environmental health. Organic amendments have recently been proposed as a possible herbicide sorbent in soil, in order to limit herbicide movement from soil to water. The environmental fate of terbuthylazine depends not only in its mobility, but also its persistence. The latter is directly dependent on microbial degradation. For this reason the effects of pine and oak residues on terbuthylazine soil microbial community functioning and on the potential of this community for terbuthylazine degradation were studied. For this purpose, degradation kinetics, soil dehydrogenase activity and the number of live bacteria were assessed in a clay-loam soil treated with terbuthylazine and either amended with pine or oak wood or unamended (sterilized and non-sterilized). At day 65, 85% of the herbicide applied still persisted in the sterile soil, 73% in the pine-amended one and 63% in the oak-amended and unamended ones. Pine residues increased the sorption of terbuthylazine to soil and hampered microbial degradation owing to its high terbuthylazine sorption capacity and a decrease in the bioavailability of the herbicide. On the contrary, in the presence of oak residues the herbicide sorption did not increase significantly. The overall results confirm the active role of the soil microbial community in terbuthylazine degradation in amended and unamended soils and in a liquid enrichment culture performed using an aliquot of the same soil as the inoculum. In this clay loam soil, in the absence of amendments, the herbicide was found to be quite persistent ($t_{1/2}$ > 95 days), while in the enrichment culture the same natural soil bacterial community was able to halve terbuthylazine in 24 days. The high terbuthylazine persistence in this soil was presumably ascribable to its texture and in particular to the mineralogy of the clay fraction.

Keywords Terbuthylazine, Degradation, Soil texture, Organic amendments, Pine and oak residues, Microbial community activity.

1 Introduction

Terbuthylazine is an *s*-triazine herbicide widely used in agriculture to control grass and broad-leaved weeds in a variety of crops. In Italy terbuthylazine is used in maize and sorghum (Fait et al. 2010), and in Spain is used also in olive tree cultures (Cabrera et al. 2007, 2008).

The fate and behaviour of terbuthylazine in soil have raised environmental concern because, together with its metabolite desethylterbuthylazine (DET), it has been frequently found in surface water and groundwater at levels above 0.1 $\mu g\ L^{-1}$, which is the limit established in the EU for individual pesticides in drinking water (Guzzella et al. 2006; Hildebrandt et al. 2006).

European Food Safety Authority (EFSA) has recently reported that terbuthylazine poses high long-term risks for mammals, aquatic organisms, non-target plants, earthworms (EFSA 2001) and can have genotoxic effects (Mladinic et al. 2012). The fact that it has been recently (16 August 2011) re-evaluated and its placing in the EU market approved until 2021 by Commission Implementing Regulation 820/2011 makes its environmental occurrence, together with its toxicological relevant metabolite, DET, a risk both for the environment and human health.

Terbuthylazine degradation depends on both abiotic and especially biotic processes, which are responsible for its complete degradation. The more it is degraded in soil, the less the likelihood of it being leached to groundwater or run off to surface water. Biodegradation and mineralization of *s*-triazines have been shown to be carried out by bacterial consortia and by strains isolated from contaminated sites (Grenni et al 2009a; Barra Caracciolo et al. 2010). 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 pathway steps varied among degraders (Santiago-Mora et al. 2005; Barra Caracciolo et al. 2010). Degradation rates in agricultural soils may depend on the history of terbuthylazine treatment, which may increase the

soil self-remediation potential (Rhine et al. 2003) and on the specific soil characteristics (soil depth, pH, temperature, water content, presence of exogenous nitrogen, organic matter content and texture) which can directly or indirectly influence the degradation process (Di Corcia et al. 1999; Barra Caracciolo et al. 2010; Kodešová et al. 2011).

In soil, one of the primary mechanisms of its transformation is a biotic oxidative N-deethylation with the formation of desethylterbuthylazine, DET (Di Corcia et al. 1999). Monitoring data show that DET is frequently present in groundwater and its concentration is often higher than its parent compound; this phenomenon is due to the intrinsic characteristics of DET (e.g. water solubility and soil organic carbon partition coefficient) which determine its lower adsorption and higher mobility in soils (Bottoni et al. 1996; Guzzella et al. 2003; Barra Caracciolo et al. 2005a; EFSA 2011; FOOTPRINT, 2011).

Point-source contamination by pesticides has been identified as a major concern contributing significantly to the deterioration in the quality of natural water resources. Indeed, monitoring studies have clearly shown that pesticide point-source contamination produced by improper pesticide handling before or after their field application (e.g. spills, uncontrolled disposal, equipment washing water, etc.) has resulted in the frequent detection of high concentrations of pesticides in natural water resources (De Wilde, 2007; Fait et al. 2010; Kravvariti et al. 2010). The addition of exogenous organic matter of different origin, including wastes, may prevent the mobility of pesticides released in soil from point as well as from non-point sources of contamination and enhance their biodegradation (Rodríguez-Cruz et al. 2007a; Delgado-Moreno and Peña 2009). In recent years different low-cost sorbent systems (biobed, biomassbed, biofilter) have been developed to minimize point sources of pesticide pollution. These systems consist of a mixture of different organic biomaterials and soil which can retain and degrade pesticides (Kravvariti et al. 2010; Castillo et al. 2008). The addition of organic amendments to soil can affect the biodegradation of pesticides owing to the application of an

93 additional source of organic matter and sometimes microorganisms (Briceño et al. 2007; Kan et
94 al. 2007) with the result of accelerating the degradation of pesticides (Kravvariti et al. 2010;
95 López-Piñeiro et al. 2011). In other cases, the addition of an organic residue to soil can lead, by
96 decreasing the bioavailability of pesticides owing to their increased sorption capacity, to a
97 decrease in pesticide degradation (Moorman et al. 2001; Briceño et al. 2007; Grenni et al 2009a;
98 Kravvariti et al. 2010).

99 Pine and oak wood residues have recently been shown to be effective low-cost sorbents
100 of the herbicide linuron in a sandy-loam soil (Grenni et al 2009a). The greater adsorption of
101 linuron to pine than oak was related to its higher lignin content, the hydrophobic wood
102 component (Rodríguez-Cruz et al. 2007b). However, the influence of the addition to soil of these
103 wood residues on the adsorption and degradation of the herbicide terbuthylazine has not been
104 studied so far.

105 In the present work the degradation of terbuthylazine was evaluated in an agricultural
106 clay loam soil, where the groundwater beneath is found to be chronically contaminated by this
107 herbicide and its metabolite DET (Barra Caracciolo et al., 2010). In order to assess the
108 applicability of pine and oak amendments for the immobilization of herbicide in this soil, an
109 experimental set-up, consisting of soil microcosms treated with terbuthylazine and either
110 amended with pine or oak residues or unamended, was performed. The ability of
111 microbiologically active soils (amended or unamended) to degrade the herbicide was evaluated
112 by comparing the half-lives ($t_{1/2}$) in microcosm studies in the various scenarios (pine-amended,
113 oak-amended, and unamended) to that in sterile soil. Moreover, the effects of these amendments
114 on soil bacterial community activities, such as dehydrogenase and viability, were also assessed.

115 Finally, an enrichment culture was set up with terbuthylazine as the sole carbon source,
116 using aliquots of the same soil as the inoculum. This experiment was performed in order to

evaluate the capability of this soil microbial community to degrade the herbicide and to grow on it in a liquid culture.

2 Material and Methods

2.1 Experimental site

The criteria used for the selection of the site (located near Assisi, Central Italy) were the presence of intensive agriculture with previous terbuthylazine application and a shallow alluvial aquifer (water table at 12 m depth, alkaline-bicarbonate geochemical facies) vulnerable to herbicide contamination (Daly et al. 2002). According to the Umbria Regional Environmental Agency's monitoring surveys (2000–2010), terbuthylazine and its metabolite, desethylterbuthylazine, are commonly found in this groundwater (> 0.1 μg L^{-1} parametric value). It is also common to find significant nitrate contamination at this site (> 100 μg L^{-1}).

2.2 Soil and wood samples

Soil samples were collected from the surface layer (0-20 cm depth) and left to dry at room temperature, then sieved (< 2 mm) and analysed for their physiochemical characteristics. The soil was classified according to F.A.O. World Soil Classification as Calcaric Cambisol (Giovagnotti and Calandra 1994) and the soil texture was classified as clay loam according to USDA (22.9% sand, 43.2% silt and 33.9% clay). The organic carbon and nitrogen content were 1.87% and 0.13%, respectively, and the pH 7.7. The clay minerals in the soil were montmorillonite (9.25%), illite (20.6%) and kaolinite (4.01%).

Pine and oak wood residues (< 1 mm) were selected as the organic soil amendments because of their different lignin contents of 24.4% and 18.2%, respectively (Rodríguez-Cruz et al. 2007b). They were obtained from a local company in Salamanca (Spain). The amended soils were prepared by uniformly mixing soil with oak or pine (5% w/w).

The organic carbon and the pH of wood residues, determined in a previous work (Rodríguez-Cruz et al. 2007b) are reported in Table 1. The wood amended occurrence increased the organic carbon content of the soil and did not affect the soil pH (Table 1).

2.3 Chemicals

Terbuthylazine (N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine) and its main metabolites desethylterbuthylazine (DET) and desethyldebutylterbuthylazine (DEDT), were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) (> 98.0% purity). Terbuthylazine is a colourless powder with a water solubility of 8.5 mg L^{-1} at 20°C and log K_{ow} of 3 (Tomlin et al. 2003; Rodríguez-Cruz et al. 2007b).

2.4 Laboratory degradation experiments with unamended and amended soils

The herbicide degradation experiment was conducted in duplicate microcosms for each different treatment in accordance with SETAC guidelines (Lynch 1995) and some previous experiments (Grenni et al 2009a; Barra Caracciolo et al. 2005a; 2005b). Terbuthylazine was added to unamended or amended soil (200 g) to obtain a final concentration of 1.5 mg kg^{-1}. Initially some soil samples were sterilized twice (autoclaved 120 ± 2°C, 20 min) and then treated with terbuthylazine (SST); other soil samples were only treated with terbuthylazine (ST); others were treated with both terbuthylazine and pine (SPT) or oak (SOT) sawdust (5% w/w); lastly,

166 microbiological control soils (S) were prepared with only water and with water and pine (SP) or
167 oak (SO) sawdust. All soils were thoroughly stirred with a sterilized spatula and the water added
168 was in all cases sterilized by filtration (0.22 μm). The final moisture content was adjusted to 60%
169 of the maximum soil water holding capacity (WHC_{max}).

170 The soils were maintained in beakers closed with a sterilized cotton plug wrapped in
171 gauze to allow air exchange. The soil moisture was kept constant during the entire period of the
172 experiments by periodically weighing and replacing any losses with sterile water. Samples were
173 incubated at 20 ± 2°C in the dark. Solutions and instruments were sterilized and all steps were
174 performed in a sterile cabinet. The overall experimental set consisted of 14 microcosms (two for
175 each of the 7 different treatments, S, ST, SST, SOT, SPT, SO, SP). For each chemical or
176 microbiological analysis we collected 2 sub-samples from each of the two replicate microcosms.
177 Consequently, each value reported is the average of a total of four data. Sampling was performed
178 at different times (0, 6, 12, 20, 33, 49, and 64 days) for both the chemical and microbiological
179 analyses.

180

181 2.5 Enrichment culture on terbuthylazine

182

183 An enrichment culture experiment was performed in order to evaluate the occurrence of natural
184 bacterial populations able to degrade the herbicide and to grow on it as the sole carbon source.
185 Three soil samples (3.5 g each) were inoculated into 30 mL of a liquid medium MB 1×
186 (K_2HPO_4, 1.6 g L^{-1}; KH_2PO_4, 0.4 g L^{-1}; $CaSO_4 \times 2H_2O$, 0.1 g L^{-1}; $MgSO_4 \times 7H_2O$, 1.0 g L^{-1};
187 $FeSO_4 \times 7H_2O$, 0.02 g L^{-1};$(NH_4)_2SO_4$, 2g L^{-1}) with the herbicide terbuthylazine at a
188 concentration of 2 mg L^{-1}, as performed in similar works (Sánchez et al. 2005; Grenni et al.
189 2009b). Two other flasks containing a previously sterilized liquid medium (MB1×) and the

herbicide at the same concentration (2 mg L^{-1}) were used as controls and monitored for chemical analysis until 85 days.

The experimental set consisted of flasks (50 mL capacity) which were incubated at 20°C in the dark and gently shaken. Samples for chemical and microbiological analysis were collected at selected times until the degradation of at least 80% of the initial concentration was reached. The concentrations of the terbuthylazine and its metabolite DET were measured immediately after the treatment and at different times (0, 3, 8, 15, 23, 30, 37, 44, 51, and 58 days). The bacterial growth of the soil microbial pool was monitored during the incubation period by the epifluorescence direct count method.

2.6 Sorption studies

The sorption of terbuthylazine both to wood residues and to wood-amended soils was determined using a batch equilibrium method described in detail in Rodriguez-Cruz et al. (2007b). Briefly, triplicates 100 mg wood samples or triplicates 5 g unamended and wood-amended (5 %) soils were equilibrated with 10 mL of an aqueous solution of terbuthylazine at an initial concentration (Ci) of 7 mg L^{-1}**.** Preliminary experiments pointed out that a contact for 24 h was long enough for the equilibrium to be reached. The pesticide amount adsorbed (Cs) was considered to be the difference between that initially present in the solution (Ci) and that remaining after equilibration (Ce) with the wood or wood-amended soil. Sorption distribution coefficients, Kd, were calculated from the relationship between Cs and Ce (Kd = Cs/Ce), and were considered a measure of pesticide adsorption capacity by the wood or wood-amended soils. All measurements were carried out in duplicate. The quantification of terbuthylazine was performed by HPLC-MS in a Waters chromatograph (Waters Assoc., Milford, MA) equipped with a model e2695 multisolvent delivery and autosampler system attached to a ZQ mass spectrometer detector

215 (MS), and Empower software as the data acquisition and processing system. A Waters Symmetry
216 C18 (75 mm x 4.6 mm I.D., 3.5 μm) column was used at ambient temperature. The mobile phase
217 was 80:20 methanol/water in a 0.1% formic acid solution. The flow rate of the mobile phase was
218 0.3 mL min^{-1} and the sample injection volume was 20 μL. Detection by HPLC/MS to quantify
219 terbuthylazine was monitoring the positive molecular ion (m/z) 230. The quantification of
220 terbuthylazine was done with the external standard method using the calibration curves obtained
221 by the injection of standard solutions at a concentration range between 0.05 and 1 μg mL^{-1}
222 (r^2>0.99).
223
224 2.7 Herbicide analysis
225
226 Soil sub-samples (1 g) were taken from each microcosm and shaken at 60 rpm with 6 mL of
227 methanol for 24 h at 20°C for residue analysis. Samples were centrifuged and 4 mL of each
228 supernatant were evaporated at 30°C under nitrogen stream (Concentrator EVA VLM-EC-2V-
229 130, Germany) and re-dissolved in 0.5 mL of methanol for analysis.
230 Quantitative determination of terbuthylazine and its main metabolite DET and its further
231 transformation product desethyldebutylterbuthylazine (DEDT) in soil and in enrichment culture
232 samples was performed by GC-MS in a 7890A Agilent gas chromatograph coupled to a 5975C
233 Agilent mass spectrometer (Agilent Technologies, Avondale, USA) with an Agilent 7683
234 autosampler. Chromatographic separation was performed on a 30 m × 0.25 mm I.D, 0.25 μm
235 film thickness HP-5MS capillary column. The carrier gas was helium at a rate of 1 mL min^{-1}. A
236 split/splitless injector was used in the split-less mode. A sample volume of 0.2 μL was injected
237 in the splitless mode with an injector temperature of 225ºC. The following temperature program
238 was used: the temperature was increased from 100ºC to 150ºC at 50ºC min^{-1} and maintained for
239 1 min, then at 5ºC min^{-1} to 200ºC and finally increased to 290ºC at 30ºC min^{-1} and maintained

240 for 1 min. The quadrupole mass spectrometer was operated in the electron impact ionization (EI)
241 mode at 70 eV. The transfer line and the injector were set up at 250ºC and the source and the
242 quadrupole were at 230°C and 150ºC, respectively. Measurements in the GC-MS were
243 performed in the single-ion monitoring (SIM) mode. The more abundant ions were chosen for
244 quantification (terbuthylazine m/z 214, DET m/z 186 and DEDT m/z 173). The quantification
245 was carried out by double injection. Recoveries for terbuthylazine, DET and DEDT were 90%,
246 80%, 72% respectively. Samples were extracted and analysed in duplicate. The quantification of
247 terbuthylazine and its metabolites was performed by the external standard method using the
248 calibration curves obtained by the injection of standard solutions at a concentration range
249 between 0.1 and 1 μg mL^{-1} (r^2>0.99).
250
251 2.8 Soil dehydrogenase activity, total cell number and cell viability
252
253 At different times (0, 6, 12, 20, 33, 49, and 64 days) after herbicide application, the
254 dehydrogenase activity, total cell number and cell viability were assessed. Soil dehydrogenase
255 activity was determined following the method described by Tabatabai (1994). The method is
256 based on extraction and colorimetric determination of the intensely coloured 2,3,5-triphenyl
257 formazan (TPF) produced from the reduction of colourless 2,3,5-triphenyltetrazolium chloride
258 (TTC) in soils after an 24 h incubation at 37°C in the dark. Results were expressed as μg TPF g^{-1}
259 dry soil. Measurements were performed in duplicate for each microcosm.
260 The total cell number (No. bacteria g^{-1} dry soil) was assessed (in duplicate for each
261 microcosm) in 1 g of fixed soil with the epifluorescence direct count method, using 4',6-
262 diamidino-2-phenylindole (DAPI) as the DNA fluorescent agent, as reported in detail in previous
263 works (Barra Caracciolo et al. 2005a; 2005b).

264 Cell viability (% Live/Live+Dead) was measured in 1 g of fresh soil (in duplicate for
265 each microcosm) using two fluorescent dyes, SYBR Green II and propidium iodide (Sigma-
266 Aldrich, Germany) in order to distinguish between viable (green) and dead (red) cells under a
267 fluorescence microscope (Leica DM 4000B Leica Mycrosystems GmbH, Wetzlar, Germany), as
268 reported in a previous work (Grenni et al. 2009a).
269
270 2.9 Statistical analysis
271
272 Analysis of variance (two-way analysis of variance) was used to determine the significant
273 differences ($p < 0.05$) in the dehydrogenase activity, bacterial number and viability among the
274 different soil treatments during the experimental period, using the Statistical software
275 SIGMASTAT (version 3.0).
276
277 **3 Results**
278
279 3.1 Degradation of terbuthylazine in soil microcosm experiments
280
281 The decrease in the herbicide concentrations (expressed in percentages of residual
282 terbuthylazine) over a period of 64 days in unamended and sterile soil (SST), unamended soil
283 (ST) and soils amended with oak (SOT) or pine (SPT) is shown in Fig. 1. Degradation of
284 terbuthylazine was fitted first-order kinetics: $C_t = C_0\, e^{-kt}$, where C_t is the concentration at time t,
285 C_0 is the initial concentration at t=0, and k is the constant rate. In the ST and SOT treatments
286 there was a phase of slow degradation at the beginning of the incubation. The theoretical half-life
287 values ($t_{1/2}$) calculated from the corresponding exponential equations, obtained from the
288 regressions between concentrations and time, were: 257± 27 d (r^2= 0.90, $p < 0.01$) in SST < 161

289 ± 38 d (r^2 = 0.75, $p < 0.05$) in SPT < 105 ± 10 d (r^2 = 0.94, $p < 0.01$) in ST < 95 ± 7 d (r^2 = 0.93,
290 $p < 0.01$) in SOT. These values suggest that the herbicide was quite persistent in the soil studied.
291 At the end of the experiment (day 64) the lowest herbicide concentrations observed were in the
292 unamended (ST) and oak-amended (SOT) soils and about 40% of the initial concentrations were
293 degraded.

294 DET and its further metabolite DEDT were found in microbiologically active soils (ST,
295 SOT, SPT) during the degradation process and the amounts of these metabolites generally
296 increased over time, as shown in Fig. 2. Both metabolites were found in higher concentrations in
297 SOT and ST from day 49 to day 64, in line with a higher terbuthylazine degradation observed in
298 these treatments at the end of the incubation.

299

300 3.2 Microbiological analysis: soil dehydrogenase activity, total cell number and cell viability

301

302 The herbicide effects on soil dehydrogenase activity, total cell number and cell viability were
303 studied in all herbicide-treated soils and compared with non-treated ones. Fig. 3 shows soil
304 dehydrogenase activity (μg TPF g^{-1} dry soil) in relation to time in the terbuthylazine treated soils
305 (A) and in the control ones (B).

306 A significant difference ($p < 0.05$) in dehydrogenase activity was observed among the
307 different soil treatments. Dehydrogenase activity was significantly higher in all the soils
308 amended with pine or oak than in unamended ones. After 28 days in the SOT treatment a
309 significant increase ($p < 0.05$) of dehydrogenase activity was observed compared to the other
310 treatments.

311 The initial total cell numbers (No. bacteria g^{-1}dry soil) obtained by DAPI counts were
312 higher ($p < 0.05$) in all the amended soils (SP: $4.8 \times 10^7 \pm 3.2 \times 10^6$; SO: $4.0 \times 10^7 \pm 2.4 \times 10^6$).

However, cell numbers were subsequently not significantly different in the various treatments (data not shown).

The cell viability values (% Live/Live+Dead) are reported in Fig. 4. In the presence of the amendments a transient decrease in viability at day 6 was observed; this was particularly evident in the SOT and SPT treatments.

3.3 Degradation of terbuthylazine in the enrichment culture

The soil microbial pool in the enrichment culture was able to degrade the terbuthylazine with a $t_{1/2}$ of 24 ± 2 days ($r^2 = 0.99$) (Fig. 5A on the left axis). In contrast, after 85 days in the sterile medium more than 98% of the initial concentration of the herbicide was still present. The total cell number (No. bacteria mL^{-1}) of the microbial pool was assessed during the experimental time. A positive correlation ($p < 0.05$) was found between the terbuthylazine concentration (% of TBA applied) and the cell number (Fig. 5A), indicating that the soil bacterial populations were able to grow using the herbicide as a carbon source.

The metabolite DET was immediately detected and it was exclusively found in the presence of the soil microbial pool (Fig. 5B). Its formation was correlated ($p < 0.05$) to the terbuthylazine degradation.

3.4 Sorption of terbuthylazine by wood residues and soil

The sorption of terbuthylazine by the woods used and the unamended and wood-amended soils are reported in Table 1. The Kd value for the sorption of terbuthylazine by pine was much higher than for oak. Similarly, the sorption of terbuthylazine by the pine amended soil (11.7±2.63) was

337 higher than the oak amended one (5.3 ±0.38). However, the latter Kd value was not significantly
338 different from the unamended soil (4.27 ±1.08).
339
340 **4 Discussion**
341 The overall results show that the microbial community had a significant role in the
342 terbuthylazine degradation, as shown when comparing the degradation results for sterile soil and
343 microbiologically active soil in both the soil and enrichment culture experiments. DET was
344 found as the main metabolite in accordance with other studies (Navarro et al. 2003; Barra
345 Caracciolo et al. 2005a; Delgado-Moreno and Peña 2007). The slight decrease in terbuthylazine
346 concentration in the sterile conditions (both in soil and MB medium of the enrichment
347 experiment) was presumably due chemical hydrolysis (Fig. 1A and Fig. 5A). DET and DEDT
348 were not detected in sterile soil (data not shown) in line with the fact that their formation is
349 reported to occur exclusively via biotic transformations (Di Corcia et al. 1999; Barra Caracciolo
350 et al. 2005a; 2010).
351 The results of the microbiological analysis indicate that the presence of oak and pine
352 amendments, rich in labile carbon fractions, stimulated soil dehydrogenase activity during the
353 experimental period (Fig. 3). The positive influence of organic amendments on the
354 dehydrogenase activity of the overall microbial community was found in several works
355 (Moorman et al.2001; Delgado-Moreno and Peña 2007; 2009) and in our previous experiment
356 using the same pine and oak residues (Grenni et al. 2009a). Terbuthylazine did not negatively
357 affect bacterial community functioning in terms of dehydrogenase activity (in the case of SOT
358 the activity even increased), presumably because it was adapted to its presence (Fig. 3A).
359 The initial bacterial numbers were higher in all the amended soils than in the unamended
360 ones and this was due to the fact that with pine and oak both organic matter and microorganisms
361 were added to the soil (Briceño et al. 2007). However, since this difference was limited to the

362 start of the experiment, the allochthonous bacterial populations introduced by the residues were
363 presumably not able to survive in the soil and they were both excluded competitively by the
364 autochthonous populations and also affected negatively by the herbicide in the case of the treated
365 soils. This hypothesis is confirmed by the cell viability values (% Live/Live+Dead) reported in
366 Figure 4. In fact a transient decrease in viability was observed in all amended soils at day 6 and it
367 was particularly evident in the SOT and SPT treatments.

368 The bacterial viability trend can be linked to the activation of bacterial populations
369 involved in the herbicide degradation and this is particularly evident in the ST. In fact, in the
370 control soil S (non-terbuthylazine treated soil) the overall cell viability tended to decrease during
371 the experimental period. The $t_{1/2}$ values of TBA were related to the Kd values found in the
372 different treatments (Fig. 1A and Table 1); therefore the adsorption phenomena affected the
373 amount of herbicide bioavailable for degrading populations.

374 The higher sorption of terbuthylazine by pine-amended than oak-amended soil is in line
375 with its higher lignin content (24.4% in pine vs 18.2% in oak) and its greater organic carbon
376 content (41.5% in pine vs 38.5% in oak). The Kd coefficients obtained in this work are higher
377 than those found in a previous work (Rodríguez-Cruz et al. 2007b) for the sorption of linuron,
378 alachlor and metalaxyl herbicides. The latter result is ascribable to the higher hydrophobicity of
379 terbuthylazine (log Kow = 3.21) compared to other non-ionic pesticides (log Kow range 1.75-
380 3.09) (Rodríguez-Cruz et al. 2007b). The Kd value obtained in the SPT is comparable with that
381 obtained by Cabrera et al. (2008) in a soil amended with alperujo. The initial decrease of TBA
382 concentration in SPT can be explained by the degradation of the limited bioavailable fraction
383 (20%) occurring in the amended soil; the pine sorption capability then presumably increased
384 with the incubation time and no significant amount was further degraded during the experimental
385 period. With the increase in incubation time ageing phenomena, which imply the formation of
386 bound-residues or strong immobilization of pesticide residues in non-amended or amended soils

387 are commonly found (Gevao et al. 2000). The addition of organic amendments (urban sewage
388 sludge, poultry compost and alperujo), increasing terbuthylazine sorption to soil, has been found
389 to retard its degradation by other authors (Navarro et al. 2003; Cabrera et al. 2007; 2008;
390 Dolaptsoglou et al. 2007; Sayara et al. 2010).

391 In the presence of oak amendment the herbicide sorption did not increase significantly
392 and therefore did not substantially hamper the biodegradation, with the TBA decrease in
393 concentration being just slightly delayed by about 12 days; however, at the end of the experiment
394 the residual herbicide concentration was identical (60% of the initial concentration) to that in the
395 unamended soil.

396 The enrichment culture experiment confirms the fact that the capability of the same
397 herbicide degrading populations was hampered in different ways by adsorption phenomena. In
398 fact, it demonstrates that the autochthonous soil microbial pool was able to degrade the
399 terbuthylazine in the liquid culture ($t_{1/2}$ = 24 days, Figure 5A) significantly better than in its
400 original soil ($t_{1/2}$ = 105 days in ST, Figure 1A) and to grow on the herbicide as the sole carbon
401 source. This result not only confirms that terbuthylazine biodegradation can be carried out by
402 bacterial consortia (de Souza et al. 1998; Grenni et al. 2009b) but it also demonstrates how the
403 same microbial pool was prevented in the original clay loam soil from performing the
404 degradation efficiently.

405 In fact in this soil, without any amendments, the herbicide terbuthylazine was quite
406 persistent ($t_{1/2}$ 95-105 days). The TBA degradation rate is indeed reported highly variable, from a
407 few weeks to more than 200 days (Di Corcia et al. 1999; Barra Caracciolo et al. 2005a;
408 Kravvariti et al. 2010) and it depends on bacterial activity and on abiotic factors that directly or
409 indirectly influence the degradation rate. Abiotic factors, such as temperature, soil moisture,
410 organic carbon content and pH, have been known to significantly influence the degradation
411 process of terbuthylazine; however, the soil intrinsic characteristics (such as texture and

412 mineralogy) have been taken into consideration only very recently (Vischetti et al. 2010). In this
413 context, the relatively high half-life values (95-105 days) found in this clay loam soil could be
414 ascribed to its fine texture and in particular to its clay mineral montmorillonite fraction which
415 has a great capacity to adsorb organic matter (Arnarson 2000), including triazine herbicides,
416 decreasing their bioavailability for degradation (Bailey et al. 1986). In fact in our previous
417 microcosm studies, performed with terbuthylazine in the same laboratory conditions but on
418 different soils (silty-loam and sandy-loam), we observed a $t_{1/2}$ of 22 days and 30 days,
419 respectively (Barra Caracciolo et al. 2001; 2005a).

420 The slow degradation rate found in the unamended soil was due firstly to the intrinsic
421 characteristics of its clay, such as texture and mineralogy (e.g. the montmorillonite fraction),
422 which made the herbicide less available for both abiotic and biotic degradation processes.

423 Moreover, although the soil studied had a fine texture, which is known to limit the
424 diffusion and transport of contaminants, the groundwater beneath has been found to be
425 contaminated by triazines. This fact can be ascribed to preferential flow pathways (Flury et al.
426 1996; Kördel et al. 2008) occurring when large and discontinuous macropores operate and cause
427 rapid movement of chemicals through the unsaturated zone. The transport of pesticides via
428 macropores has been frequently found in fine textured soils and herbicides may contaminate
429 groundwater especially if the degradation phenomena in surface soil do not significantly reduce
430 their concentration (Guzzella et al. 2003, 2006), as in the case of our soil.

431 The evaluation of the environmental fate of terbuthylazine in clay soil requires the
432 knowledge of how its persistence and movement can be affected by the possible interactions
433 with its fine texture. Owing to the high adsorption of terbuthylazine to this soil, its leaching is
434 not likely to occur through micropore flow, but through a preferential flow. The occurrence of
435 terbuthylazine and DET in the groundwater beneath can therefore be explained by their transport
436 by macropore flow.

Particular attention has therefore to be paid to clay soils because their structure can strongly affect both the transport and the degradation of soil contaminants and consequently makes it difficult to forecast their fate and control their mobility. The latter statement has to be taken in consideration for a better risk management of pesticide use in line with the requirements of the recent EU Directive 2009/128/EC and Regulation (EC) No. 1107/2009.

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Figure captions

Fig. 1. (A) Percentage of residual terbuthylazine in unamended soils. (*SST*, sterile soil; *ST*, non-sterile soil) and soil amended with oak (*SOT*) or pine (*SPT*) vs time. *Vertical bars* represent standard errors. **(B)** Terbuthylazine structure (on the *left*) and images of oak and pine wood residues (on the *right*).

Fig. 2. Metabolites detected over time in unamended (*ST*) and soil amended with oak (*SOT*) or pine (*SPT*) **(A)** Desethylterbuthylazine (DET); **(B)** Desethyldebutylterbuthylazine (DEDT). *Bars* represent standard errors.

Fig. 3. Soil dehydrogenase activity (μg TPF g^{-1} dry soil) detected over time in the soils **(A)** treated with terbuthylazine (*SPT*, terbuthylazine + pine; *SOT*, terbuthylazine + oak; *ST*, terbuthylazine) and **(B)** in the control ones (*SP* amended with pine; *SO*, amended with oak; *S*, unamended). *Bars* represent standard errors.

Fig. 4. Cell viability (% Live/Live+Dead) vs time **(A)** in the soils treated with terbuthylazine (*SPT*, terbuthylazine + pine; *SOT*, terbuthylazine + oak; *ST*, terbuthylazine) and **(B)** in the control ones (*SP* amended with pine; *SO*, amended with oak; *S*, unamended). *Bars* represent standard errors.

Fig. 5. Terbuthylazine degradation, desethylterbuthylazine (DET) formation and total cell number in an enrichment culture. **(A)** Degradation (%) of terbuthylazine (TBA) vs time; **(B)** Formation of the metabolite desethylterbuthylazine (DET). *Bars* represent standard errors.

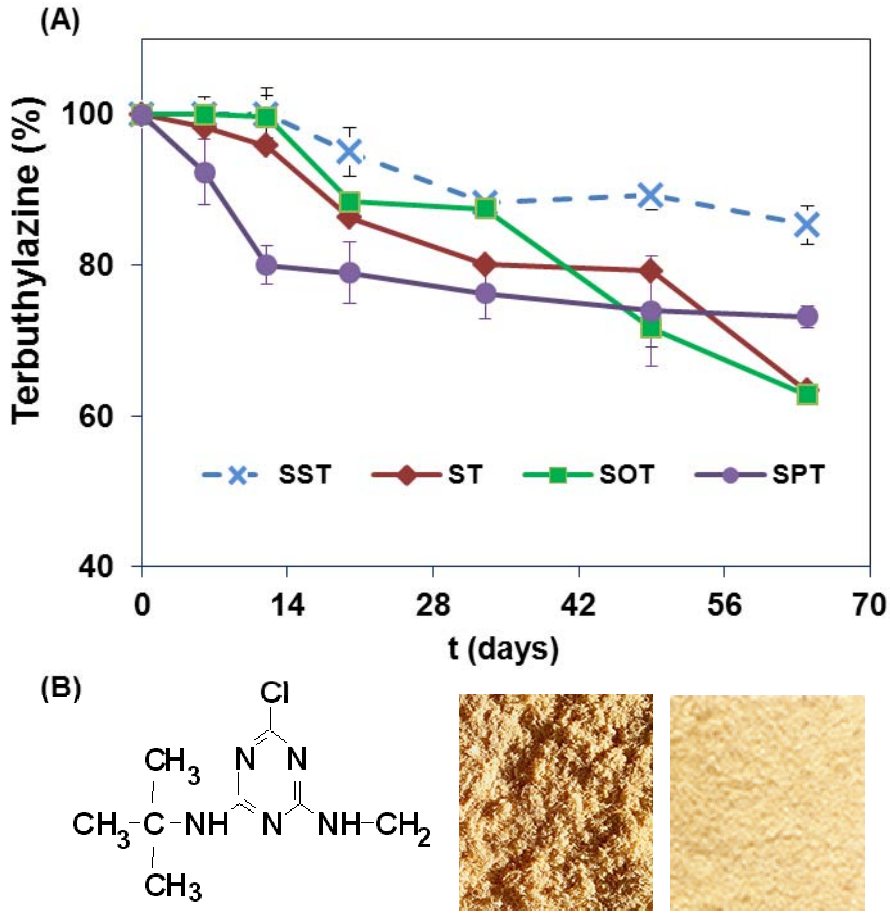
(A)
Terbuthylazine (%)
100
80
60
40
0
14
28
42
56
70
t (days)
SST
ST
SOT
SPT
(B)
Cl
CH3
N
N
CH3-C-NH
N
NH-CH2
CH3

Fig. 1.

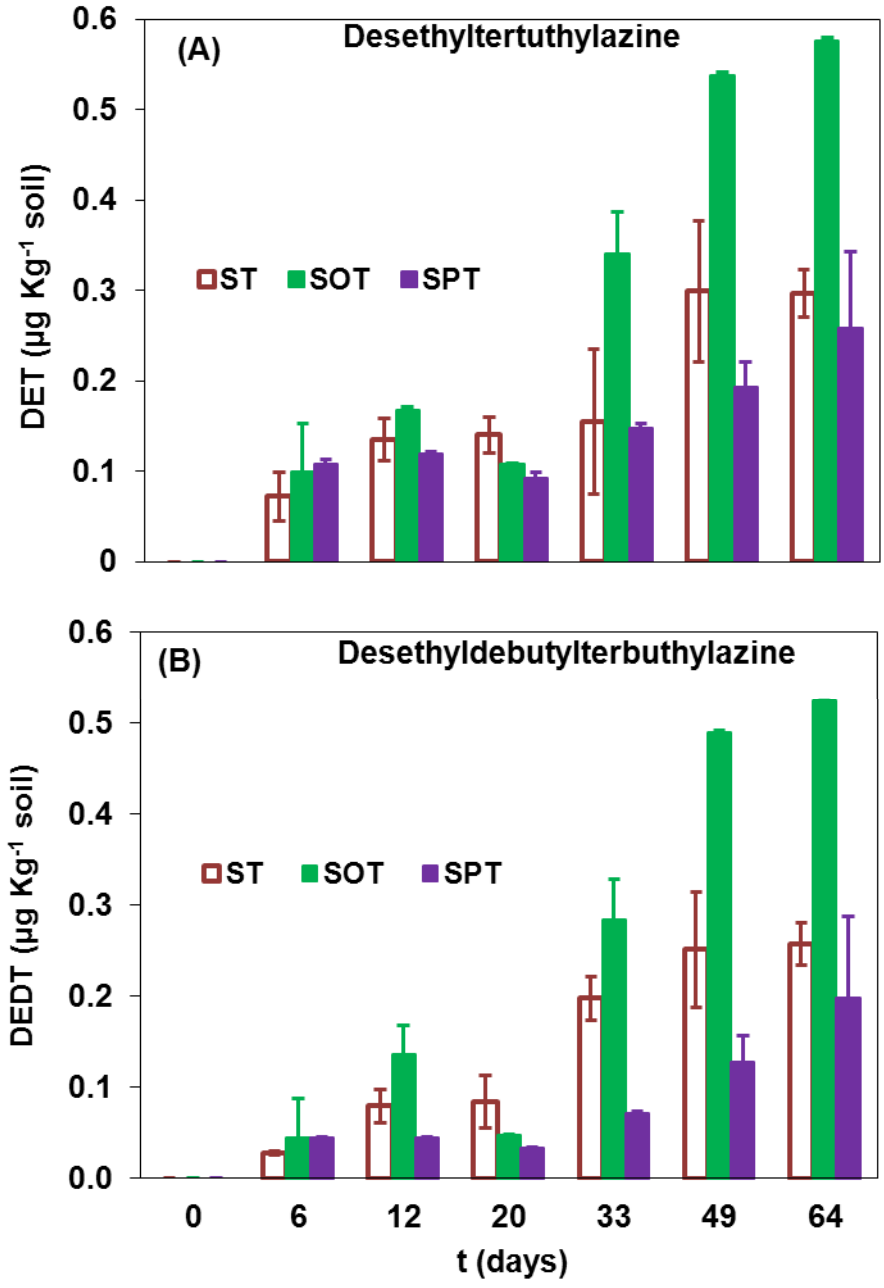
(A)
Desethyltertuthylazine
DET (µg Kg-1 soil)
ST
SOT
SPT
0.6
0.5
0.4
0.3
0.2
0.1
0
(B)
Desethyldebutylterbuthylazine
DEDT (µg Kg-1 soil)
ST
SOT
SPT
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
20
33
49
64
t (days)

Fig. 2.

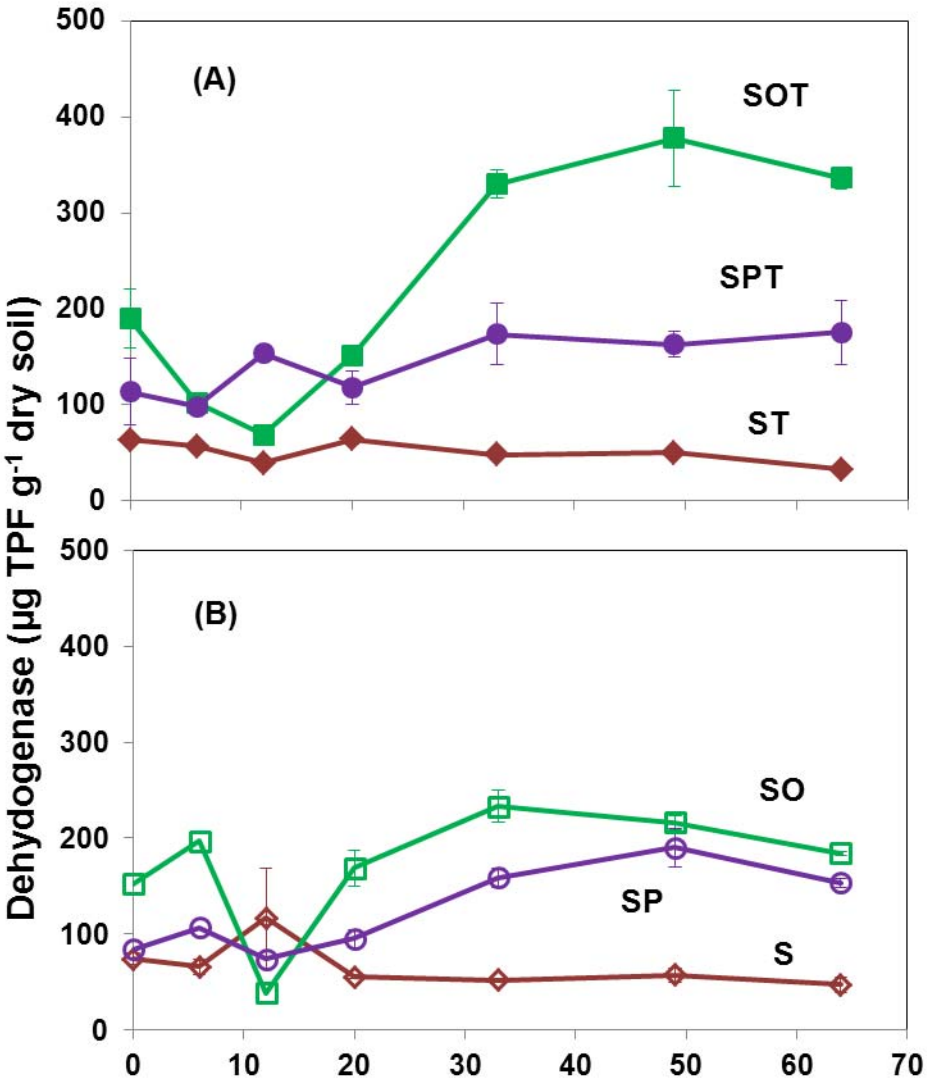
(A)
SOT
SPT
ST
(B)
SO
SP
S
Dehydogenase (µg TPF g-1 dry soil)
500
400
300
200
100
0
0
10
20
30
40
50
60
70

Fig. 3.

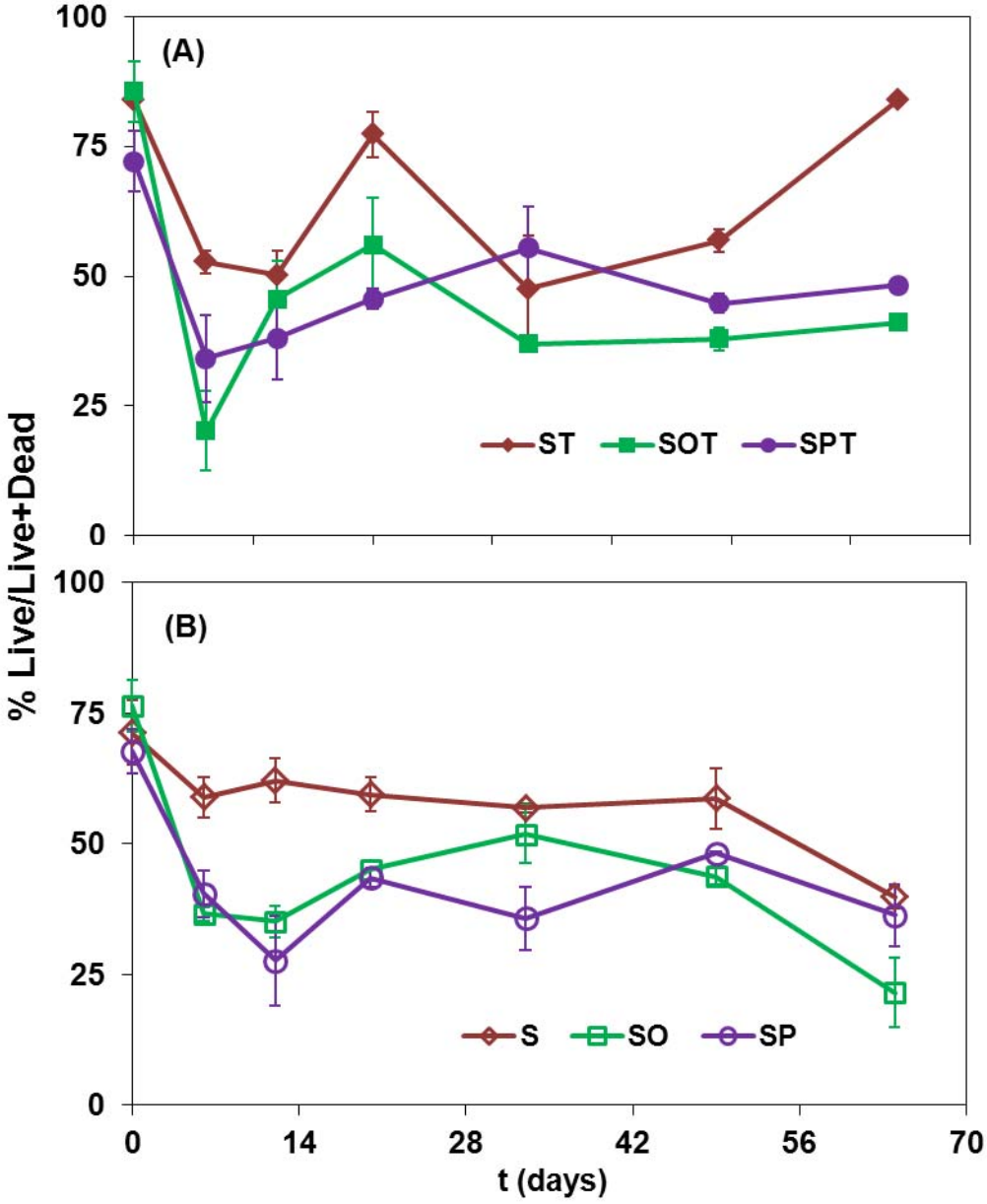
(A)
(B)
ST
SOT
SPT
S
SO
SP
% Live/Live+Dead
t (days)
0
25
50
75
100
0
14
28
42
56
70

Fig. 4.

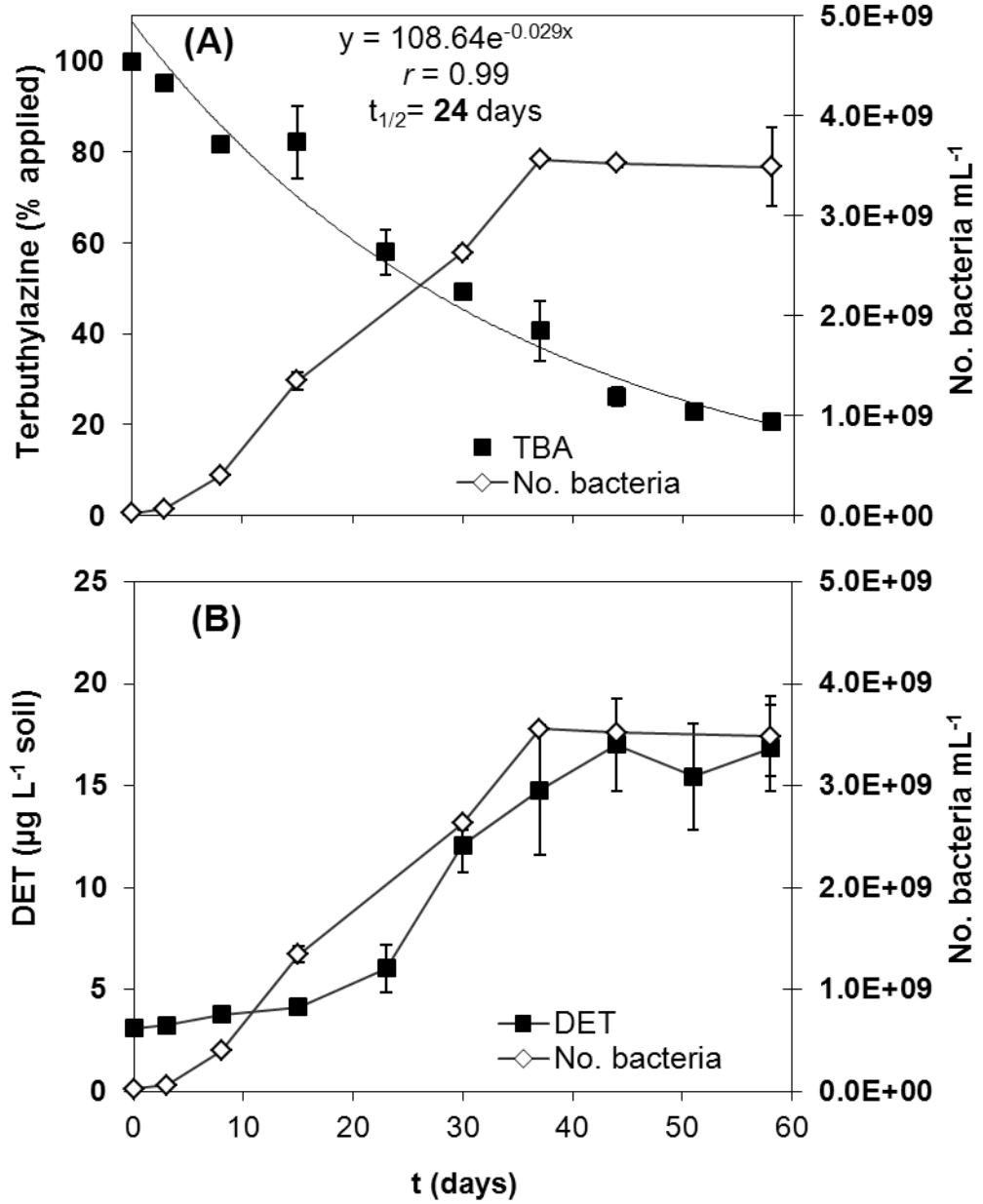
(A)
y = 108.64e-0.029x
r = 0.99
t1/2= 24 days
Terbuthylazine (% applied)
No. bacteria mL-1
TBA
No. bacteria
(B)
DET (µg L-1 soil)
DET
t (days)

Fig. 5.

Table 1 Organic carbon (OC %), pH and Sorption distribution coefficient (K_d) of terbuthylazine by the wood residues (pine and oak), unamended (soil) and amended soils (soil + pine, soil + oak).

	OC %	pH	Kd (mL g^{-1}) ± SD
Pine	41.5	5.0	1856 ±46.4
Oak	38.5	4.0	71.2 ±1.18
Soil	1.87	7.7	4.27 ±1.08
Soil + Pine	4.21	7.7	11.7 ±2.63
Soil + Oak	4.09	7.8	5.30 ±0.38

SD standard deviation