

Biochemical properties and microbial community structure of five different soils after atrazine addition

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Abstract Atrazine is one of the most used herbicides worldwide; however, consequences of its long-term agricultural use are still unknown. A laboratory study was performed to examine changes in microbial properties following ethylamino-¹⁵N-atrazine addition, at recommended agronomic dose, to five acidic soils from Galicia (NW Spain) showing different physico-chemical characteristics, as well as atrazine application history. Net N mineralization was observed in all soils, with nitrate being the predominant substance formed. The highest values were detected in soils with low atrazine application history. From 2% to 23% of the atrazine-¹⁵N was found in the soil inorganic-N pool, the highest values being detected after 9 weeks in soils with longer atrazine application history and lower indigenous soil N mineralization. The application of atrazine slightly reduced the amount of soil N mineralized and microbial biomass at short term. Soluble carbohydrates and β -glucosidase and urease activity decreased with incubation time, but were not significantly affected by the single application of atrazine. Microbial community structure changed as consequence of both soil type and incubation time, but no changes in the phospholipid fatty acid (PLFA) pattern were detected due to recent atrazine addition at normal doses. The saturated 17- to 20-carbon fatty acids had higher relative abundance in soils

with a longer atrazine history and fungal biomass, as indicated by the PLFA 18:2 ω 6,9, decreased with the incubation time. The results suggested that the PLFA pattern and soil N dynamics can detect the long-term impact of repeated atrazine application to agricultural soils.

Keywords Atrazine · N mineralization · Enzyme activities · Phospholipid fatty acid pattern

Introduction

Most agricultural soils in Western Europe are treated with herbicides at least once a year. Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] is one of the most widely used herbicides for controlling broadleaf and grassy weeds in agriculture and forestry. Consequently, atrazine and its degradation products are the most frequently detected herbicide contaminants in surface and ground waters (Capriel et al. 1985; Herwig et al. 2001), and therefore, there is concern for negative effects of these compounds on both humans and the environment. Atrazine is subjected to abiotic and biotic degradation with reported half lives in soils ranging from a few days to several months depending on site conditions, application history, and agricultural practices (Erickson and Lee 1989; Assaf and Turco 1994; Koskinen and Clay 1998). In recent years, soils showing a potential for rapid mineralization of atrazine have been identified throughout the world (Barriuso and Houot 1996; Yassir et al. 1999). However, residues of both the parent compound and its degradation products have been detected in soils years after application (Schiavon 1988; Mahía et al. 2007). These findings are being taken into account by regulatory bodies in EU countries, leading to increased restrictions on the use of atrazine.

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Microbial degradation is the dominant way of atrazine attenuation in soils (Abdelhafid et al. 2000a). There are several microorganisms that partially transform atrazine by N-dealkylation or dehalogenation reactions, but relatively few bacteria have been reported that completely mineralize the triazine ring (Kaufman and Kearney 1970; Mandelbaum et al. 1995; Bouquard et al. 1997). Degradation of pesticides is often slow because low supply and/or availability of the compound or essential nutrients. In the case of atrazine, oxidation of the alkali side chains provides the only source of C and energy available to microorganisms since the ring C atoms are fully oxidized and thus are not incorporated into microbial biomass (Cook 1987; Yanze-Kontchou and Gschwind 1994). Atrazine can be utilized as a sole C source by some mixed cultures and bacterial isolates (Behki and Khan 1986; Mandelbaum et al. 1993, 1995; Yanze-Kontchou and Gschwind 1994). Side-chain and ring-N atoms can be utilized as an N source by some microorganisms (Cook and Hütter 1981; Mandelbaum et al. 1995; Radosevich et al. 1995). Various organic and inorganic amendments have been shown to affect herbicide degradation. The addition of alternative N sources, resulting in a low C/N ratio, can inhibit atrazine mineralization (Cook and Hütter 1981; Alvey and Crowley 1995; Abdelhafid et al. 2000a, b) and the inhibitory effect increased with soil N mineralization rate; by the contrary, in soils with a high C/N ratio, atrazine mineralization may be enhanced as a result of limited soil N availability (Alvey and Crowley 1995; Topp et al. 1997).

The influence of *s*-triazines on soil microorganisms has been intensively investigated. These studies have shown that while some microbial groups can use an applied herbicide as a source of energy and nutrients, the herbicide may be toxic to other groups. The presence of these herbicides can modify the soil microbial communities thus altering the normal functioning of terrestrial ecosystems, which in turn might have implications for soil fertility and quality (Voets et al. 1974; Greaves 1982; Schaëfer 1993). In general, the impact of *s*-triazines on soil microbial communities has often been studied by conventional methods based on cultivation of soil microorganisms or on measurements of their microbial biomass and metabolic activities (Davies and Greaves 1981; Ghani et al. 1996; Sannino and Gianfreda 2001; Accinelli et al. 2001; Moreno et al. 2007; Hussain et al. 2009). To overcome the drawbacks of the culture-dependent methods, interest is currently focused on the use of molecular techniques, allowing the analysis of microorganisms in their natural habitats. In this context, phospholipid fatty acid (PLFA) pattern and DNA/RNA analysis have been successfully applied in the last decade to characterize soil microbial communities following atrazine addition (Ostrofsky et al. 2002; Rhine et al. 2003; Ross et al. 2006).

Most atrazine studies focused on short-term application of the herbicide at large concentrations; although it is well

known that under field conditions, the herbicide is often applied annually at low dose for many successive years in the same soil. The concentration of atrazine in soils is an important factor which affects biodegradation and the microbial response (Dzantor and Felsot 1991; Gan et al. 1996). In Galicia (NW Spain), there is a widespread use of atrazine in agricultural soils and a high potential risk of surface and groundwater contamination due to abundant precipitations and sandy textured soils. However, information concerning the impact of *s*-triazine compounds on microorganisms of these acidic soils with relatively high organic matter content is scarce. The purpose of this research was to evaluate the microbial response to atrazine at recommended agronomic dose in soils from the temperate humid zone with different histories of atrazine application and contrasting physico-chemical properties. PLFA analysis and several biochemical properties (N mineralization, urease activity, β -glucosidase activity, soluble carbohydrates) were used to characterize the soil microbial communities. Thus, the microbial variables included biomass, activity and community structure measurements, as well as enzyme activities connected to the C- and N-cycles. The kinetics of atrazine degradation was also examined. This is one of the few studies that combine microbial community changes induced by both recent and repeated herbicide application at field doses and herbicide dissipation.

Material and methods

Soils Five contrasting agricultural soils (P, E, M, G, and C), developed over different parent material and with a history of 10–40 years of annual maize cropping and atrazine application, located in the temperate humid zone (Galicia, NW Spain) were used (Table 1). It should be noticed that only four soil types were considered since P and E samples were collected in the same agricultural experimental area from plots with different previous exposure to herbicide. Soil subsamples, collected randomly in spring 2005 before maize planting, from the top 15 cm of the A horizon, were mixed, sieved (< 2 mm), and thoroughly homogenized.

Experiment conditions Ethylamino-chain- ^{15}N -labeled-atrazine (99 atom% ^{15}N in excess, purity >98.5) was purchased from Isotec (USA). Fresh soils (aliquots equivalent to 50 g of dry weight) were incubated under controlled conditions (28°C, 85% of water holding capacity, darkness). The soils were amended with atrazine at the recommended agronomic dose (8 ml of an atrazine solution of 30 mg L $^{-1}$ to give a final concentration of 5 mg herbicide per kilogram soil). The same volume of distilled water was added to the unamended control soils. Biochemical properties and

Table 1 Main characteristics of the agricultural soils studied

Soil	Parent material	Atrazine application history (year)	pH water	C g kg ⁻¹	N g kg ⁻¹	Inorganic N mg kg ⁻¹	Clay%	Silt%	Sand%
P	Granite	1964–2004	5.60	24.8	2.39	7.4	12	14	74
E	Granite	1964–1994	5.73	41.9	3.14	6.8	13	12	75
M	Acid schists	1984–2004	5.79	16.5	1.51	27.1	19	51	30
G	Sediments	1994–2004	5.88	41.1	2.79	7.7	27	9	64
C	Amphibolite	1996–2004	5.88	26.4	2.20	13.1	19	41	40

community structure were measured after 3, 6, 9, and 12 weeks of incubation. Sampling was destructive with three replicates used for each treatment-time combination, resulting in a total of 24 replicates of each soil (12 untreated and 12 treated with atrazine).

Biochemical properties and community structure Inorganic N (NH₄⁺ and NO₃⁻) was analyzed with an extraction–diffusion method at room temperature, the evolved NH₃ being trapped in 10 ml of 0.005 M H₂SO₄ and measured by back titration with 0.01 M NaOH (Couto-Vázquez and González-Prieto 2006). The resulting (NH₄)₂SO₄ solutions were then acidified with 1 ml 0.005 M H₂SO₄ and evaporated to obtain (NH₄)₂SO₄ salts, whose ¹⁵N/¹⁴N ratio was measured in an elemental analyzer (EA) coupled on-line with an isotopic ratio mass spectrometer (Finnigan Mat, delta C, Bremen, Germany). Traceability of results was verified by including, in each series of ten analyses, an isotopic certified standard (ammonium sulfate IAEA-305a and IAEA-305b, alternately). For each soil and sampling date: (a) net soil N mineralization was calculated as the variation in N content (total inorganic N, NH₄⁺-N, NO₃⁻-N) during the incubation subtracting the initial N content, and (b) the soil inorganic N content and its atom% ¹⁵N in excess (compared with the natural abundance in the corresponding control soil not added with atrazine) were used to calculate the net N mineralization rate of the added ¹⁵N-labeled atrazine. All samples were analyzed in duplicate.

The β-glucosidase activity was measured following the procedure of Eivazi and Tabatabai (1988), which determines the released *p*-nitrophenol after incubation of the soil with *p*-nitrophenyl glucoside solution for 3 h at 37°C. Urease activity was estimated by incubating soil samples with an aqueous urea solution and extracting the NH₄⁺ with 1 M KCl and 0.01 M HCl followed by colorimetric NH₄⁺ determination by a modified indophenol reaction (Kandeler and Gerber 1988). Soluble carbohydrates content was estimated colorimetrically by the anthrone method after extraction with distilled water (1:10 *w/v*) at 80°C for 24 h (Puget et al. 1999).

The microbial community structure was determined by PLFA analysis using the procedure and nomenclature

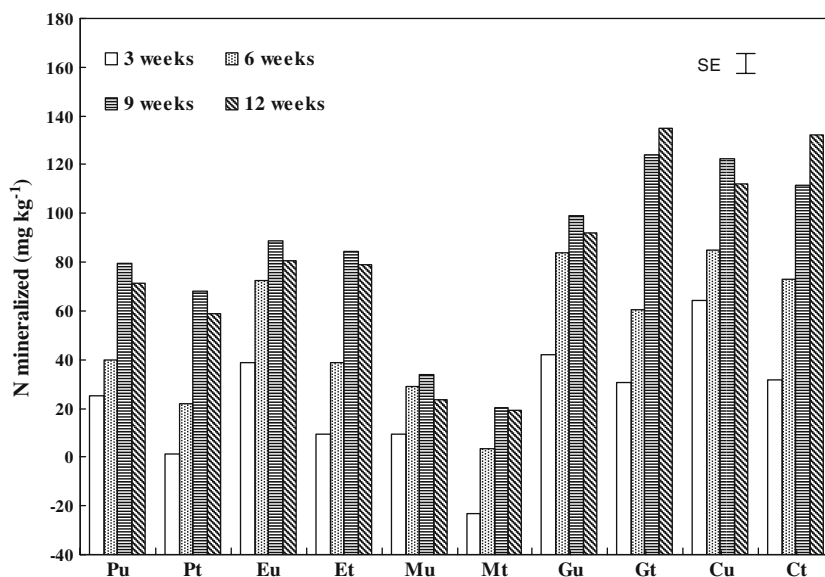
described by Frostegård et al. (1993). The total microbial biomass was estimated as the sum of total PLFAs (totPLFAs). The sum of the PLFAs considered to be predominantly of bacterial origin was used as an index of the bacterial biomass (bactPLFAs), and the quantity of the PLFA 18:2ω6,9 was used as an indicator of the fungal biomass (fungPLFAs) (Frostegård and Bååth 1996). The PLFAs i14:0, i15:0, i16:0, and 10Me18:0 are predominantly found in gram-positive (G⁺) bacteria and the PLFAs cy17:0, cy19:0, 16:1 ω7c, and 18:1ω7 characterize gram-negative (G⁻) bacteria (Zelles 1999).

Statistical analysis All results were obtained by triplicate determinations and were expressed on the basis of oven-dry (105°C) weight of soil. The data were analyzed by a three-way analysis of variance (ANOVA) to determine the percentage of the variation attributable to the factors soil type, incubation time, and single atrazine addition. For each incubation time, the effect of atrazine addition was examined by the standard unpaired *t* test comparing the data of some parameters analyzed in the untreated and atrazine-treated samples of the same soil (*n*=3) and by the paired *t* test comparing the untreated and atrazine-treated samples of five soils (*n*=5). For each variable, the standard error of the difference between means was calculated by one-way ANOVA. Data corresponding to the concentrations of all the individual PLFAs, expressed in mole percent and logarithmically transformed, were subjected to principal component analysis (PCA) to elucidate the main differences in the PLFA patterns. All statistical analyses were made using SPSS 15.0 statistical package.

Results

Net N mineralization after 12 weeks of aerobic incubation varied from 24 to 112 mg kg⁻¹ soil and from 19 to 135 mg kg⁻¹ soil in the untreated and atrazine-treated samples, respectively (Fig. 1). The net N mineralization values were significantly affected by soil type, incubation

Fig. 1 Net soil N mineralization (ammonification plus nitrification) in samples non-added (*u*) and added (*t*) with ^{15}N -ethylamino-atrazine in the soils studied (*P*, *E*, *M*, *G*, and *C*) at different incubation times. Mean values of three incubation replicates



time, and atrazine treatment; the interaction between these factors also being significant (Table 2). The soil type and incubation time explained most of the variance (47% and 36%, respectively), whereas atrazine treatment account for only 1.8% of variance, and the interaction between these factors explained a further 9%. Nitrogen mineralization increased significantly with the incubation time. During the whole incubation time, M soil had the lowest N mineralization capacity, followed by P and E soils with similar values and G and C soils that exhibited the highest N mineralization values. Similar results were obtained for the fraction of total soil N that was mineralized during the incubation, with values following the order: M (-1.05% to 2.22% of total N) < E = P (0.04% to 3.30% of total N) < G (1.1% to 4.83% of total N) < C (1.44% to 6.0% of total N).

For all soils and most incubation times, the net mineralized N in atrazine-treated samples was slightly lower than in the corresponding untreated samples, although differences were not statistically significant at some incubation times. Thus, the application of 5 mg atrazine per kilogram soil resulted in an N mineralization reduction of 1.6–34 mg N kg⁻¹, the highest values being detected after 3 or 6 weeks of incubation. However, significant increases (unpaired *t* test, $P < 0.005$) in N mineralization, ranging from 25 to 43 mg N kg⁻¹, were also observed as consequence of atrazine addition in G and C soils at 9–12 weeks of incubation. Thus, compared with those of the respective untreated soils, the inorganic-N pool of all atrazine-treated soils was significantly reduced at 3 and 6 weeks of incubation (paired Student's *t* test, $P < 0.005$), while an inconsistent and non-significant atrazine effect was observed after both 9 and 12 weeks of incubation.

Since atrazine was labeled in the ^{15}N -ethylamino-side chain, the N mineralization data also provided information on atrazine dynamics in these agricultural soils, as well as on

its contribution to the soil N labile pool. $^{15}\text{NHCH}_2\text{CH}_3$ and $^{15}\text{NH}_3$ are released in the intermediate processes of the ^{15}N -ethylamino-atrazine degradation pathway; thus the presence of $\text{NH}_4^+ \text{-}^{15}\text{N}$ and $\text{NO}_3^- \text{-}^{15}\text{N}$ in the KCl extracts of atrazine-added soils showed that ethylamine side chain of atrazine had been degraded by soil microorganisms. Figure 2 shows the percentage of added ^{15}N -ethylamino-atrazine found in the soil inorganic-N pool at each incubation time. After 3 weeks of incubation in P, E, G, and M soils, the ^{15}N mineralization values ranged from 5% to 19%, then increased until maximum values at 9 weeks (8–23%), and finally, they decreased significantly during the last 3 weeks of incubation. In contrast, ^{15}N mineralization values increased progressively in C soil from 3 weeks (2%) until the end of the incubation (10%). In all soils and incubation times, less than 0.7% of the total N mineralized was derived from ^{15}N atrazine. The atrazine degradation rate followed the order soil M > P > E > G > C. A significant negative relationship between net N soil mineralized and ^{15}N -atrazine mineralized was observed at all incubation times (3 weeks, $r = -0.931$, $P < 0.005$; 6 weeks, $r = -0.866$, $P < 0.0005$; 9 weeks, $r = -0.749$, $P < 0.002$; 12 weeks, $r = -0.864$, $P < 0.005$).

The enzyme activities and soluble carbohydrate concentrations are shown in Fig. 3. Average values considering all incubation times and both untreated and atrazine-treated samples of five different soils were 114 ± 53 μg *p*-nitrophenol per gram soil per hour, 19 ± 12 μg N-NH₄⁺ per gram soil per hour, and 47 ± 25 μg C per gram soil, for β -glucosidase, urease, and soluble carbohydrates. The atrazine treatment hardly affected these variables, whereas incubation time, explaining 18–64% of variance, and soil type, accounting for 30–75% of variance, had a pronounced influence on these properties, the interaction between these factors also being significant (Table 2).

Table 2 Three-way analysis of variance of the effect of soil type (*S*), incubation time (*T*), and single atrazine addition (*A*) on soil biochemical and microbiological properties analyzed

Variable	Source	Variance explained (%)	F ratio
Soil N mineralized	Soil (S)	47	156***
	Incubation time (T)	36	161***
	Atrazine addition (A)	1.8	23***
	S × T	1.7	3***
	S × A	3.5	4***
	A × T	3	14***
Carbohydrates	Soil (S)	75	338***
	Incubation time (T)	18	104***
	S × T	5	7***
Urease activity	Soil (S)	31	443***
	Incubation time (T)	55	1,055***
	Atrazine addition (A)	0.1	17***
	S × T × A	0.1	2**
Glucosidase activity	Soil (S)	30	504***
	Incubation time (T)	64	1,448***
	S × T	4.6	26***
	S × T × A	0.5	3***
TotPLFA	Soil (S)	37	34***
	Incubation time (T)	14	18***
	Atrazine addition (A)	1.3	5*
	T × A	9.6	12***
	S × T × A	11	3***
FungPLFA	Soil (S)	8.5	10***
	Incubation time (T)	45	67***
	S × T	9	3***
	T × A	8.8	13***
	S × T × A	10	4***
BactPLFA	Soil (S)	43	42***
	Incubation time (T)	17	13***
	T × A	9.7	13***
	S × T × A	9.6	3***
Gram-positive	Soil (S)	50	69***
	Incubation time (T)	21	40***
	S × T	2.3	1***
	T × A	5.9	11***
	S × T × A	6.1	3**
Gram-negative	Soil (S)	32	24***
	Incubation time (T)	17	17***
	Atrazine addition (A)	4.5	14***
	S × T	8.7	2**
	T × A	3.6	4**
	S × T × A	7.3	2*

Only significant factors are included

* $P < 0.05$, ** $P < 0.01$,

*** $P < 0.001$

There was a significant reduction ($P < 0.05$) of these variables between 3 and 6 weeks of incubation and then values hardly changed (enzyme activities) or slightly tend to recover (soluble carbohydrates) until the end of the incubation (Fig. 3). In general, during the whole incubation, G, E, and M soils exhibited the highest values and C

and P soils the lowest. When the incubation time was excluded and the data were analyzed independently for each incubation time, these biochemical parameters were not significantly different in untreated and atrazine-treated samples (paired Student's *t* test, $P > 0.05$ at 3, 6, 9, and 12 weeks).

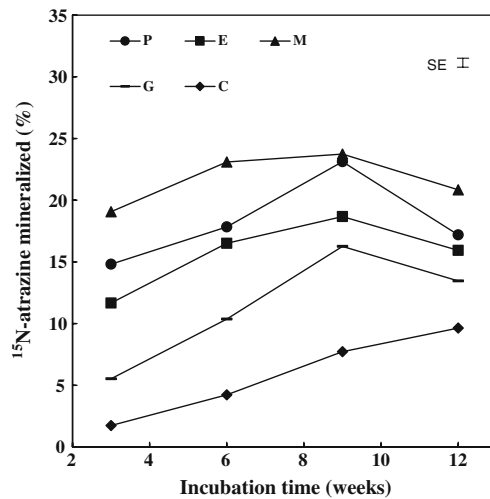


Fig. 2 Atrazine-derived inorganic-N ($\text{NH}_4^+ + \text{NO}_3^-$) expressed as percentage of added ^{15}N -ethylamine-atrazine in the soils studied (P, E, M, G, and C) at different incubation times. Mean values of three incubation replicates

The microbial biomass, estimated as totPLFAs, ranged from 1.3 to 3.6 $\mu\text{mol g}^{-1}$ organic C; the highest values found in G and C soils, followed by M, P, and E soils (Table 3). The amounts of PLFAs that were chosen to represent bacteria and fungi PLFAs varied between 440 and 1,410 and 17 and 72 nmol g^{-1} organic C, respectively and comprised 29–40 and 1–2.4 mol% of the total amount of PLFAs. The amount of PLFAs representative of G^+ bacteria and G^- bacteria ranged from 220 to 590 nmol g^{-1} organic C and 120 to 420 nmol g^{-1} organic C, respectively, and comprised 15–21 and 7–18 mol% of the total amount of PLFAs. The ANOVA indicated that the total biomass and the biomass of specific groups were significantly affected by soil type and incubation time and the interaction between these factors was also significant (Table 2). In general, soil type explained most of variance (32–50%) and the incubation time accounted for a 14–21%; however, for fungPLFA values, the importance of incubation time increased notably (45% of variance) as compared with the soil effect (8% of variance). The recent atrazine addition only showed a significant influence on total and G^- bacteria, explaining 1–4% of variance.

The PCA performed with the whole data set showed that the main differences in the PLFA pattern were due to soil type (Fig. 4a). The first component, differentiating between C and G soils (amphibolites and sediments, last 8–10 years of annual atrazine application), E soil (granite, 30 years of atrazine application, last 10 years without application) and M and P soils (acid schist and granite, last 20–40 years of annual atrazine application history), explained 34% of variation, while the third component, mainly differentiating samples according to the incubation time, only explained 11% of the variation. The second component, explaining

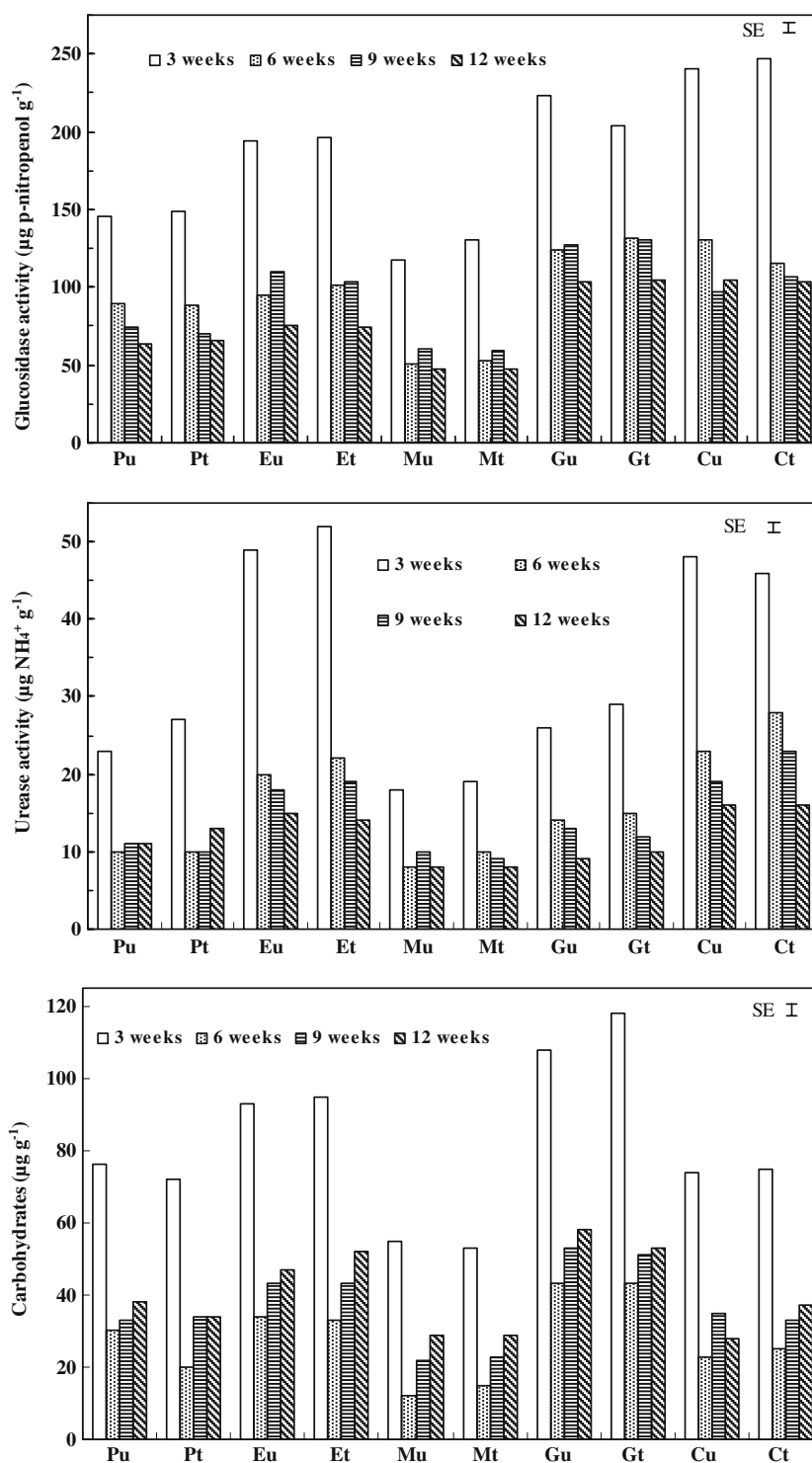
13% of variance, was not correlated to any of the factors studied. The C and G soils (having positive values along PC1) were mainly characterized by high concentrations of 14 to –16 carbon-saturated fatty acids (i16:0, a15:0, i15:0, 14:0) and monounsaturated fatty acid 16:1 ω 7c and 16:1 ω 5, while the M and P soils (with negative values along PC1) were characterized by high concentrations of the saturated 17 to 20 carbon fatty acids (Fig. 4b). The PLFAs decreasing with the incubation time (with positive values along PC3) were mainly of fungal (18:2 ω 6,9, 18:1 ω 9) and bacterial (18:1 ω 7, i14:0) origin. The distribution of samples in PCA also indicated that for the same incubation time, differences among untreated and recently atrazine-added samples were detected; however, a non-consistent pattern was observed for the five studied soils. Furthermore, changes produced were negligible compared to those provoked by soil type and incubation time.

Discussion

Most biochemical properties analyzed were significantly affected by soil type and incubation time after atrazine addition, and not significantly or only slightly affected by the recent atrazine addition at normal dose. A similar pattern concerning both recent herbicide and incubation effects was observed independently of soil studied and parameter measured. Thus, except few cases (see below) the biochemical parameters were similar in the two different treatments (untreated and treated with 5 mg atrazine per kilogram soil), indicating that atrazine at normal doses had little effect on the soil microorganisms.

The data of the present experiment clearly showed that atrazine, if used as recommended, does not lead to significant effects on urease and β -glucosidase activities and consequently suggest that the herbicide does not cause short-time effects on soil microbial activity. In most cases, little effect of atrazine application at field rates on several enzymes activities have also been reported in literature (Voets et al. 1974; Gianfreda et al. 1994; Sannino and Gianfreda 2001). Under laboratory conditions, Perucci et al. (2000) found that herbicide addition to the soil at field rate did not affect phosphatase activity, but this was greatly enhanced by applying the herbicide at ten times the field rate. Kruglow et al. (1974) reported that urease was unaffected by atrazine application at 3 kg ha^{-1} for up to 3 years and Davies and Greaves (1981) showed that the effects of 2–9 kg atrazine per hectare on dehydrogenase, phosphatase, and urease activities were not statistically significant. Recently, Moreno et al. (2007) found that high concentrations of atrazine added to soil (250–1,000 mg kg^{-1}) increased notably the hydrolytic activity of urease after 45 days of incubation with the herbicide.

Fig. 3 Soil biochemical properties in the studied soils (*P*, *G*, *E*, *M*, and *C*) untreated (*u*) and treated (*t*) with atrazine at different incubation times. Mean values of three incubation replicates



This could, however, be due to the high application rate with atrazine functioning as substrate for the soil microorganisms. In agreement with these laboratory studies, no effect and sometimes inconsistent small stimulatory or adverse effects of atrazine application at agronomic doses on urease and β-glucosidase activities were also observed under field conditions (Mahía et al. 2007). The results

supported that it is difficult to understand the impact of pesticides on soil enzyme activities because there are direct and indirect effects (Schäfer 1993; Nannipieri 1994).

Since short-term (3–6 weeks) N mineralization values were significantly reduced by herbicide application, the results indicate that measurements of net N mineralization is a more sensitive indicator of atrazine impact on these

Table 3 Total biomass (*TotPLFA*), bacterial (*BactPLFA*), fungal (*FungPLFA*), gram-positive bacteria PLFA (*Gram⁺ bact*) and gram-negative bacteria PLFA (*Gram⁻ bact*) in the studied soils (*P, E, M, G, and C*) untreated (*u*) and treated (*t*) with atrazine at different incubation times

		Incubation time (weeks)			
		3	6	9	12
Total PLFA (nmol g ⁻¹ C)	Pu	2,412±33	1,728±32	2,022±186	1,607±40
	Pt	2,135±159	1,838±41	1,677±42	1,868±244
	Eu	2,171±66	1,308±34	1,775±188	1,621±175
	Et	1,899±40	1,586±52	1,559±52	1,667±34
	Mu	3,036±71	1,851±74	1,879±33	2,265±19
	Mt	2,135±101	2,596±131	1,990±108	1,689±323
	Gu	3,604±216	1,847±173	2,977±328	2,448±81
	Gt	2,684±165	2,831±141	2,068±144	2,847±198
	Cu	2,751±45	2,607±204	2,171±236	2,285±116
	Ct	2,351±73	2,326±85	2,133±204	2,457±61
Fung PLFA (nmol g ⁻¹ C)	Pu	45±3	26±3	24±1	17±1
	Pt	42±6	26±3	26±1	20±2
	Eu	43±2	24±1	31±2	25±2
	Et	36±1	27±1	31±3	22±1
	Mu	72±1	21±2	26±1	35±6
	Mt	50±3	53±1	29±3	22±4
	Gu	57±1	22±4	32±1	31±4
	Gt	37±1	34±2	26±2	31±3
	Cu	48±2	39±5	38±9	25±6
	Ct	42±2	38±1	25±2	25±2
Bact PLFA (nmol g ⁻¹ C)	Pu	785±17	508±11	711±106	528±17
	Pt	720±59	590±10	573±20	613±66
	Eu	759±30	437±13	641±111	548±66
	Et	660±23	551±32	513±16	578±14
	Mu	1,063±139	586±26	601±7	783±9
	Mt	693±55	822±3	673±44	566±111
	Gu	1,411±38	661±80	861±59	847±21
	Gt	983±31	1,134±65	727±63	1,077±87
	Cu	1,091±25	1,013±101	821±116	853±41
	Ct	879±30	898±42	842±113	940±25
Gram-positive (nmol g ⁻¹ C)	Pu	385±14	283±8	313±10	279±9
	Pt	414±6	329±10	287±13	276±20
	Eu	340±11	219±6	277±11	269±25
	Et	342±14	280±8	263±7	247±6
	Mu	516±20	351±16	374±1	409±3
	Mt	438±32	497±3	389±21	310±57
	Gu	585±19	319±27	402±16	405±21
	Gt	461±16	484±22	357±18	413±29
	Cu	563±9	497±37	412±29	403±20
	Ct	524±9	484±9	387±30	440±15
Gram-negative (nmol g ⁻¹ C)	Pu	270±18	305±44	239±41	279±9
	Pt	202±20	236±30	139±8	276±20
	Eu	213±6	146±7	184±37	269±25
	Et	189±7	156±7	120±7	152±7
	Mu	307±47	209±9	181±17	409±3
	Mt	203±14	172±6	158±5	118±25

Table 3 (continued)

	Incubation time (weeks)			
	3	6	9	12
Gu	416±9	226±30	246±17	405±21
Gt	308±14	313±21	189±19	298±29
Cu	299±5	339±51	231±34	403±21
Ct	252±9	229±18	244±41	250±5

Mean values±SE of three incubation replicates

agricultural soil ecosystems than β -glucosidase and urease activity assays were. Pesticides can both stimulate and inhibit N mineralization (Chen et al. 2001; Haney et al. 2002). We found no consistent effects of the atrazine addition on N mineralization values after a longer incubation time (9–12 weeks), indicating only a temporal and non-persistent influence of a single application of herbicide at normal doses. Likewise, Hart and Brookes (1997) also showed that pesticides at the recommended rates had no long-term harmful effects on soil microbial activity as assessed by N mineralization.

The presence of isotopically labeled inorganic N in the KCl extracts clearly demonstrated that the side-chain atrazine was utilized/mineralized by atrazine-degrading microorganisms, particularly in soils with low N availability. This is in agreement with many studies showing that N status may alter microbial processes and the use of added N sources and thus influence rates of herbicide degradation (Cook and Hütter 1981; Entry et al. 1993; Mandelbaum et al. 1995; Radosevich et al. 1995; Bichat et al. 1999). The significant negative relationship found between net soil N mineralization and ^{15}N -atrazine mineralized is consistent with results showing lower atrazine mineralization rates when soil N availability increased as a consequence of organic and inorganic amendments (Alvey and Crowley 1995; Abdelhafid et al. 2000b; Haney et al. 2002) and higher percentages of atrazine mineralized when soil N availability decreased (Bichat et al. 1999; Abdelhafid et al. 2000b).

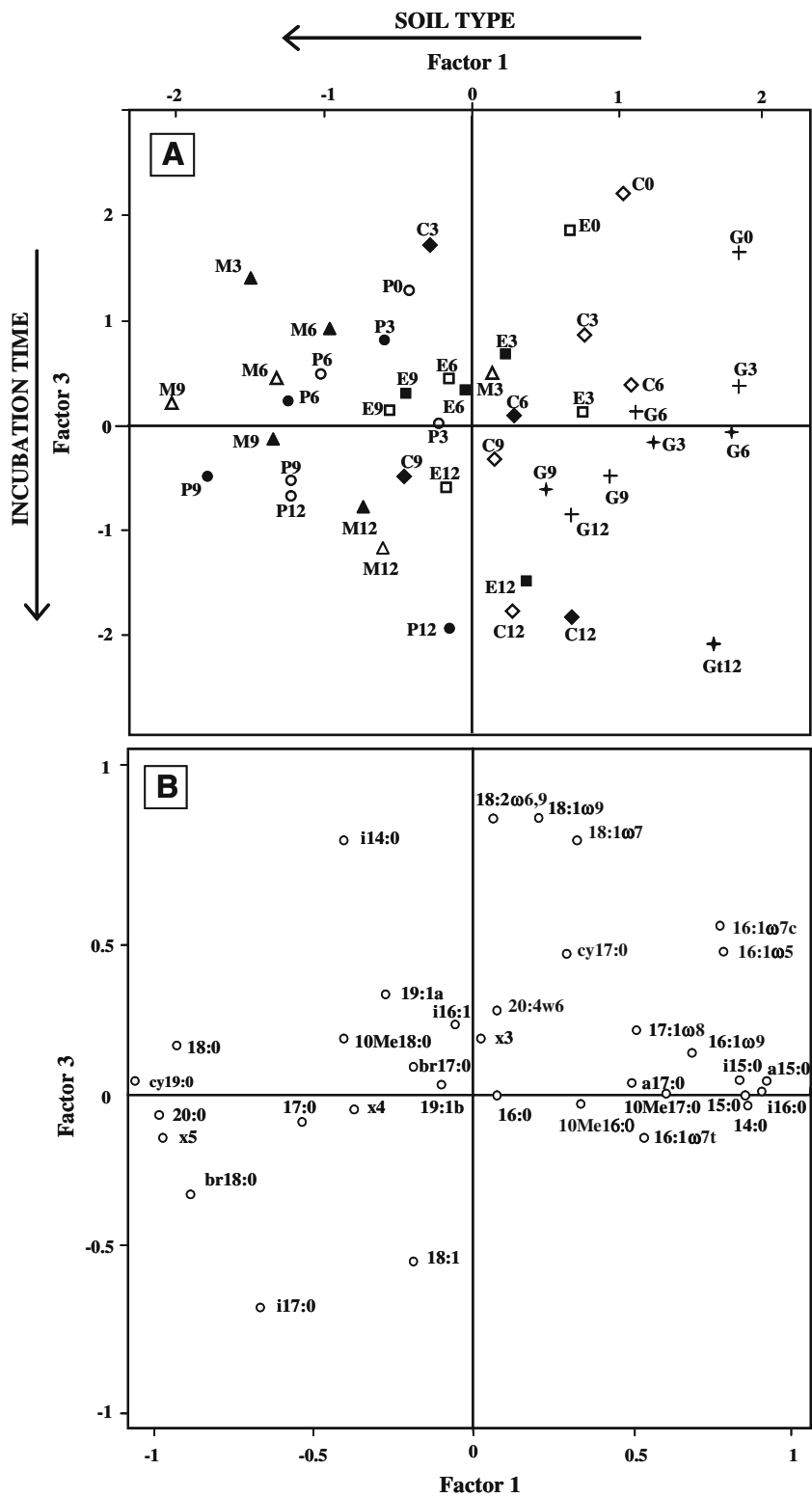
Atrazine application history appeared also to be a factor which determined the mineralization of the atrazine side chain of these soils (Table 1 and Figs. 1 and 2), with increasing mineralization with time of atrazine application. Rousseaux et al. (2001), in a 131-day incubation study with 12 soils, clearly grouped the soils according to their ability to mineralize atrazine; a first group included soils with a long atrazine application history that showed enhanced degradation of side-chain atrazine (52–61% of that initially added) and a second group included soils without adaptation to atrazine addition with lower degradation values (19–38% of that initially added). Yassir et al. (1998) reported

that after a 60-day incubation period with labeled atrazine, the cumulative degradation never exceeded 10% of the initial amount added for the ^{14}C -ethylamine-atrazine and 3% for the ^{14}C -isopropylamine-atrazine in a soil with no history of atrazine treatment. It is well known that dynamics of C cycle differs notably from that of N cycle; therefore, although the values obtained in the present study (^{15}N -ethylamino-atrazine) cannot be directly compared with those obtained by Yassir et al. (1998) (^{14}C -ethylamine-atrazine), similar results showing a marked influence of previous exposure to atrazine on kinetics of atrazine degradation were clearly observed independently of the label of the atrazine compound used in our two experiments. The results obtained here are also consistent with other studies showing that previous exposure of atrazine is a determinant factor governing atrazine biodegradation in agricultural soils from the temperate humid zone (Mahía and Díaz-Raviña 2007; Mahía et al. 2008a, b).

Thus, atrazine degradation and ^{15}N mineralization in the soils studied here could be explained by a combination of N availability and history of atrazine application. The P and M soils (20–40 years history of atrazine application) probably had a very active community of atrazine degraders due to repeated annual atrazine application. Despite of shorter atrazine history, the M soil exhibited higher percentage atrazine degradation than the P soil, probably due to the lower soil N levels. The E soil, with the highest initial inorganic N levels and intermediate net N mineralized values, had been treated with atrazine during 30 years, but had not received the herbicide in the last 10 years; therefore, its capacity to degrade atrazine could have considerably decreased compared to soils with recent applications. The G and C soils exhibited the lower percentages of ^{15}N -ethylamine-atrazine degradation, which can be explained by a shorter atrazine application history (8–10 year) and high soil inorganic N levels.

PLFA analysis indicated that the range of values obtained for total biomass and the biomass of specific groups were in the reported range for agricultural soils (Frostegård and Bååth 1996) and were mainly determined by the soil type and incubation time. As occurred for N mineralization, a minor

Fig. 4 Score and loading plots from principal component analysis performed on the PLFAs of the samples untreated (*open symbols*) and treated (*filled symbols*) with atrazine from the studied soils (*P, E, M, G, and C*) at different incubation times. Factors 1 and 3 accounted for 34% and 11% of the variance, respectively



negative and significant atrazine effect was detected on most biomass parameters during the first 3 weeks of incubation, whereas no effect was observed over the rest of incubation period. Thus, also the PLFA pattern was little affected by recent atrazine addition at normal rates.

The PLFAs 18:2 ω 6,9 and 18:1 ω 9, characteristic of fungi, and 18:1 ω 7, i14:0, characteristic of bacteria, displayed the largest decreases in concentration with the incubation time. Prolonged incubation of soils often results in a decrease in fungal PLFAs (Frostegård et al. 1996;

Bååth et al. 2004), although these studies were made in forest soils. The decrease in fungal PLFAs was then ascribed to ectomycorrhizal fungi dying off when deprived of their plant host. In the present study, agricultural soils were used, and thus, no ectomycorrhiza were present. Instead, the soil incubation could have altered the competitive strength between fungi and bacteria. However, since the effect of incubation time was found in a separate principal component from that due to soil/atrazine application, the incubation time did not confound the study of atrazine effects.

The PCA analysis clearly showed that the main differences in the community structure were due to soil type and to a lesser extent to incubation time, whereas no changes were detected as consequence of recent atrazine application. The fact that individual PLFAs along PC1 in Fig. 4a discriminated between soils with lower (C and G), intermediate (E) and higher (M and P) application history and between samples of same soil with different previous exposure to herbicide (P and E) could suggest that atrazine application history rather than soil type is determinant for soil microbial composition of the 0–15 cm layer of these agricultural soils under continuous maize cultivation. Effects of atrazine application on the microbial community composition have earlier been found (Chang et al. 2001; Rhine et al. 2003; Seghers et al. 2003; Ross et al. 2006). The results of Rhine et al. (2003) indicated that repeated exposure to atrazine provided sufficient selective pressure to cause shifts in fatty acid composition of the soil microbial communities, but specific groups of microorganisms affected were not identified. Likewise, Seghers et al. (2003) indicated that the long-term use of the atrazine (20 years) at a normal agronomic dose resulted in an altered soil microbial community, in particular for the methanotrophic bacteria, characterized by the group-specific polymerase chain reaction-denaturing gradient gel electrophoresis. Chang et al. (2001) and Ross et al. (2006) also found differences in bacterial community structure determined by PCR-DGGE technique, in short-term laboratory experiments at high concentrations of atrazine. It should be noticed, however, that changes observed under field conditions were much less marked than those under laboratory conditions, which can be due to the low atrazine doses, time passed after application, and the influence of other factors (environmental conditions, soil properties, management practices, plant, etc.) that confounded the atrazine effect. In our case, a residual herbicide effect on PLFA pattern seem to be detected as consequence of previous repeated annual application of herbicide under field conditions, indicating that microbial communities structure measured by the PLFA pattern can be a useful tool to detect the long-term impact of atrazine application to agricultural soils.

Conclusion

It can be concluded that atrazine not only affect the target organisms (weeds) but also the microbial community present in these agricultural soils from temperate humid zone with 8–40 years of annual herbicide application history. However, recently added atrazine at agronomic dose did not substantially alter most soil biochemical properties analyzed (β -glucosidase, urease, soluble carbohydrates) and only slightly decreased soil N mineralization and microbial biomass values. The soil PLFA pattern indicated that the response was affected by previous exposure to atrazine, which seems to indicate that atrazine should be applied with precaution since repeated herbicide application during 8–40 year can induce changes in microbial community structure. Our results also showed that soil N dynamics can be influenced by annual and repeated application of atrazine. Future work should be concentrate on the mechanisms of the induced microbial changes as consequence of recent and repeated herbicide addition, as well as how rapid the effects will disappear after herbicide application ceases.

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