DINÁMICA DE LA ATRAZINA Y EFECTOS DE SU APLICACIÓN EN SUELOS AGRÍCOLAS DE GALICIA

Memoria que, para optar al grado de Doctor, presenta

JORGE MAHÍA SAAVEDRA

Santiago de Compostela, 2012

MONTSERRAT DÍAZ RAVIÑA, INVESTIGADOR CIENTÍFICO DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS, ADSCRITA AL INSTITUTO DE INVESTIGACIONES AGROBIOLÓGICAS DE GALICIA (IIAG-CSIC)

CERTIFICA:

Que el presente trabajo de investigación, titulado "*Dinámica de la atrazina y efectos de su aplicación en suelos agrícolas de Galicia*", que, para optar al grado de Doctor presenta D. Jorge Mahía Saavedra, ha sido realizado en el Departamento de Bioquímica del Suelo del Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC), bajo mi dirección, y que, considerando que representa trabajo de Tesis, autorizo su presentación en la Facultad de Biología de la Universidad de Santiago de Compostela.

Y para que así conte, a efectos oportunos, firmo el presente certificado en Santiago de Compostela a 9 de marzo de 2011.

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CERTIFICA:

Que considerando que la presente Memoria titulada "*Dinámica de la atrazina y efectos de su aplicación en suelos agrícolas de Galicia*" constituye trabajo de Tesis Doctoral, autoriza su presentación en el Departamento de Edafología y Química Agrícola de la Universidad de Santiago de Compostela a fin de que pueda ser juzgada por el Tribunal correspondiente.

Y para que así conste a los efectos oportunos, firma la presente en Santiago de Compostela a 9 de marzo de 2012.

Fdo. Felipe Macías Vázquez

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ANEXOS

Esta tesis está basada en los siguientes artículos:

I Mahía J., A. Martín, T. Carballas y M. Díaz-Raviña. 2007. Atrazine degradation and enzyme activities in an agricultural soil under two tillage systems. *The Science of Total Environment*, 378: 187-194.

II Mahía J., A. Martín y M. Díaz-Raviña. 2008. Extractable atrazine and its metabolites in agricultural soils from the temperate humid zone. *Environmental Geochemistry Health*, 30: 147-152.

III Mahía J. y M. Díaz-Raviña 2007. Atrazine degradation and residues distribution in two acid soils from temperate humid zone. *Journal of Environmental Quality*, 36: 826-831.

IV Mahía J., A. Cabaneiro, T. Carballas y M. Díaz-Raviña 2008. Microbial biomass and C mineralization in agricultural soils as affected by atrazine addition. *Biology and Fertility of Soils*, 45: 99-105.

V Mahía J., S. J. González-Prieto, A. Martín, E. Bååth y M. Díaz-Raviña. 2011. Biochemical properties and microbial community structure of five different incubated soils untreated and treated with atrazine. *Biology and Fertility of Soils,* 47: 577-589.

1.- Introducción

La atrazina (2-cloro-4-etilamina-6-isopropilamina-s-triazina), herbicida perteneciente al grupo de las triazinas, es considerada como un contaminante químico de persistencia moderada en el ambiente, con una vida media de varios días a meses (Khan y Saidak, 1981; Jones *et al.*, 1982), dependiendo de las propiedades del suelo, la historia previa de aplicación del herbicida y las prácticas agrícolas (Koskinen y Clay, 1998). Sin embargo, residuos del compuesto original y de sus productos de degradación son detectados en los suelos y en las aguas superficiales y subterráneas como consecuencia de la aplicación de atrazina (Schiavon, 1988; Sánchez-Camazano *et al*., 2005). La principal ruta de descomposición de la atrazina en la mayoría de los suelos es la degradación biótica (Kaufman y Kearney, 1970; Cook, 1987; Barriuso y Houot, 1996); sin embargo, la decloración de la atrazina a hidroxiatrazina (hidrólisis química) también es una importante vía de desaparición de atrazina en suelos ácidos (Da Silva *et al*., 2000; Pimentel y Rosim, 2000). Por tanto, la degradación de la atrazina en los suelos ocurre tanto por procesos químicos como biológicos, dando lugar a la formación de metabolitos, tales como la hidroxiatrazina, la desetilatrazina y la desisopropilatrazina, cuya movilidad y toxicidad es diferente a la de la atrazina y, por consiguiente, tienen diferente potencial contaminante (Barrett, 1996). La presencia de estos compuestos xenobióticos puede modificar drásticamente las comunidades microbianas del suelo, alterando así el funcionamiento de los ecosistemas edáficos, y, en consecuencia, tiene importantes repercusiones en la fertilidad y calidad del suelo (Voets *et al.*, 1974; Greaves, 1982; Schäffer, 1993).

En los últimos años muchos países de Europa han restringido o incluso prohibido el uso de la atrazina como herbicida debido a su persistencia en el ambiente y a sus propiedades toxicológicas. La influencia de la atrazina sobre los microorganismos del suelo ha sido

estudiada detalladamente. La microbiota edáfica y los procesos en los que interviene son esenciales para la sostenibilidad a largo plazo de los sistemas agrícolas dado que controlan el ciclo de la materia orgánica y, por consiguiente, también los flujos de C y nutrientes a través de los procesos de descomposición, mineralización e inmovilización (Jenkinson, 1988; Pankhurst *et al*., 1997); existe, por tanto, un gran interés en el estudio de los efectos de los herbicidas sobre la biomasa y actividad microbianas, con el fin de preservar la salud del suelo. Diversos estudios han demostrado que la atrazina puede afectar a la dinámica de determinados grupos microbianos (Voets *et al.*, 1974; Ostrofsky *et al.*, 1997; Rhine *et al.*, 2003; Ross *et al*., 2006); sin embargo, la respuesta de la biomasa microbiana y de la actividad global de la comunidad microbiana a la adición o presencia de atrazina en el suelo es poco conocida (Wardle y Parkinson, 1990; Ghani *et al.*, 1996; Lin y Brookes, 1999; Accinelli *et al.*, 2002). La aplicación de herbicidas a los suelos induce la inhibición (por ejemplo, a través de los efectos sobre los microorganismos) o la estimulación (por ejemplo, a través de los microorganismos que usan los herbicidas como fuente de C y nutrientes) de estos parámetros microbianos. Se observó un efecto variable dependiendo de las propiedades del suelo, de la dosis del herbicida y del tiempo transcurrido desde su aplicación (Haney *et al.*, 2002; Moreno *et al.*, 2007). La mayoría de estas investigaciones son experiencias de laboratorio que estudian el efecto a corto plazo de una única adición de atrazina a dosis muy altas en suelos sin aplicación previa del herbicida (suelos no adaptados). Sin embargo, en el campo, el herbicida en general es aplicado anualmente a dosis bajas. Esto explicaría por qué el impacto de la atrazina sobre la biomasa y actividad microbianas es poco conocido dado que es difícil extrapolar los resultados de estos estudios de laboratorio a las condiciones de campo.

2.- Objetivo

En Galicia (N.O. de España), hay un alto riesgo potencial de contaminación de suelos y acuíferos con compuestos xenobióticos del grupo de las *s*-triazinas (anillos aromáticos heterocíclicos con 3 átomos de N en posiciones alternas), tales como la atrazina, debido al uso generalizado de estos compuestos en suelos agrícolas, a las condiciones climáticas (abundantes precipitaciones y bajas temperaturas) y a las propiedades de los suelos (textura arenosa, pH ácido, elevado contenido de materia orgánica y baja actividad microbiana). Sin embargo, a pesar de su interés, no hay información sobre el impacto de las s-triazinas sobre estos ecosistemas terrestres. El objetivo de esta investigación es estudiar, tanto en experiencias de laboratorio como en condiciones naturales mediante una experiencia de campo, el impacto de la aplicación de atrazina en suelos agrícolas de Galicia, determinando la dinámica de la atrazina (mineralizada, extraíble y no extraíble) y midiendo diversos parámetros, tales como la biomasa microbiana, la respiración del suelo, la mineralización del nitrógeno, las actividades enzimáticas específicas de los ciclos del C (ß-glucosidasa) y del nitrógeno (ureasa) y la diversidad o estructura de la comunidad microbiana (ácidos grasos de los fosfolípidos, PLFA). Para el seguimiento de la evolución de la atrazina en el suelo, se utilizará atrazina marcada en el C (U-^{13}C-atrazina) y en el N (etilamina-^{15}N-atrazina).

3.- Material y métodos

Las experiencias de laboratorio fueron realizadas utilizando 5 suelos agrícolas (P, G, E, M y C), 4 Cambisoles Húmicos y un Cambisol Gleico (G), desarrollados sobre diferentes tipos de roca (granito, sedimentos, granito, esquistos ácidos y anfibolita, respectivamente) con un historial de 10-40 años de cultivo de maíz y aplicación de atrazina (P, 1964-2004; G, 1994-2004; E, 1964-1994; M, 1984-2004; C, 1996-2004). El pH (H_2O) de estos suelos es similar (5,6 a 5,9) pero otras propiedades (C orgánico, N total y textura) varían entre diferentes rangos (P: 24,84 g C kg^{-1}; 2,39 g N kg^{-1}; areno francoso: 12 % arcilla, 14 % limo, 74 % arena; G: 41,12 g C kg^{-1}; 2,79 g N kg^{-1}; franco arcillo arenoso: 27 % arcilla, 9 % limo, 64 % arena; E: 41,9 g C kg^{-1}; 3,14 g N kg^{-1}; areno francoso: 13 % arcilla, 12 % limo, 75 % arena; M: 16,51 g C kg^{-1}; 1,51 g N kg^{-1}; franco limoso: 19 % arcilla, 51 % limo, 30 % arena; C: 26,41 g C kg^{-1}; 2,20 g N kg^{-1}; franco: 19 % arcilla, 41 % limo, 40 % arena).

Muestras representativas de estos 5 suelos, recogidas de los 0-15 cm superiores del horizonte Ap, antes de la siembra del maíz y de la aplicación de atrazina, fueron adicionadas o no con U-^{13}C-atrazina a la dosis de 5 mg kg^{-1} e incubados, bajo condiciones controladas de temperatura y humedad (28ºC, 85 % de la capacidad de campo), durante un período de 3 meses. El contenido de las diferentes fracciones de la atrazina (mineralizada, extraíble y no extraíble), así como diversas propiedades bioquímicas (C de la biomasa microbiana, respiración del suelo, carbohidratos solubles, actividades de las enzimas β-glucosidasa y ureasa) y microbiológicas (diversidad o estructura de la comunidad microbiana), se midieron a diferentes intervalos de tiempo durante la incubación.

La experiencia de campo fue realizada en un Cambisol Gleico de textura franco arcillo arenosa (pH 5,16-5,85; 40,35-55,55 g C kg^{-1}; 2,71-3,70 g N kg^{-1}) y 8 años de historial de aplicación de atrazina, cultivado con maíz bajo dos sistemas de cultivo, siembra directa (NT) y cultivo convencional (CT). Se utilizó un diseño de bloques al azar con 4 repeticiones por tratamiento, parcelas de 20 m x 5 m. En cada época de muestreo se recogieron de las filas centrales entre las plantas de maíz, muestras de suelo representativas de cada parcela. Las medidas del contenido de atrazina y de sus metabolitos y de las actividades enzimáticas (β-glucosidasa, ureasa) se realizaron en todas las muestras de suelo recogidas antes de la siembra (tiempo 0) y a diferentes intervalos de tiempo durante la cosecha de maíz a lo largo de dos años consecutivos (2002, 2003).

Los análisis químicos y bioquímicos se realizaron con suelo tamizado (<4 mm) y homogenizado. El contenido de atrazina (A) y de sus metabolitos (desisopropilatrazina, DIA; desetilatrazina, DEA; hidroxiatrazina, HA) se determinó en extractos de metanol/agua (70/30, v/v) por HPLC (Ghani *et al.*, 1996). Los valores de ^{13}C se obtuvieron, usando muestras de suelo molidas (<100 μm), con un analizador elemental acoplado "en línea" con un espectrómetro de masas isotópico. La atrazina residual se calculó a partir de la ecuación: ^{13}C residual de la atrazina = ^{13}C en muestras de suelo adicionadas con atrazina– ^{13}C en muestras de suelo no adicionadas con atrazina. El porcentaje de atrazina mineralizada se calculó usando la siguiente fórmula: % atrazina mineralizada = 100 - % atrazina residual en el suelo. La atrazina no extraíble se determinó restando la fracción extraíble de la fracción residual de ^{13}C-atrazina. El C microbiano se midió por el método de fumigación-extracción (Basanta *et al.*, 2002) y el C de la respiración se determinó a partir del CO_2 desprendido de los suelos no adicionados y adicionados con atrazina a lo largo de una incubación aeróbica de 84 días (Díaz-Raviña *et al.*, 1988). El N mineralizado se determinó por el método de extracción-

difusión, como diferencia entre el contenido de N inorgánico total (amoniacal + nítrico), determinado a diferentes intervalos de tiempo durante la incubación, y el contenido de N inorgánico inicial en el tiempo 0 (antes de la incubación), siguiendo el procedimiento descrito por Couto-Vázquez y González-Prieto (2006). Los carbohidratos solubles se determinaron colorimétricamente en extractos de agua caliente (Puget *et al.*, 1999) por el método de la antrona (Doutre *et al*., 1978), y las actividades β-glucosidasa y ureasa se analizaron, siguiendo los procedimientos descritos por Eivazi y Tabatabai (1988) y Kandeler y Geber (1988), respectivamente. La estructura o diversidad de la comunidad microbiana se determinó mediante análisis del perfil de ácidos grasos de los fosfolípidos (PLFA), siguiendo el procedimiento y la nomenclatura descrita por Frostegård *et al.* (1993). Una descripción detallada de los suelos, del diseño experimental y de la metodología usada puede encontrarse en los artículos de Mahía y Díaz-Raviña (2007) y Mahía *et al*. (2007, 2008a, 2008b, 2011).

4.- Resultados

Experiencias de campo

La distribución de la atrazina extraíble y sus metabolitos y las actividades enzimáticas en las diferentes muestras de suelo recogidas a distintos intervalos de tiempo durante 2 años consecutivos en las parcelas sometidas a cultivo convencional (CT) y siembra directa (NT) se muestran en las Figuras 1 y 2, respectivamente.

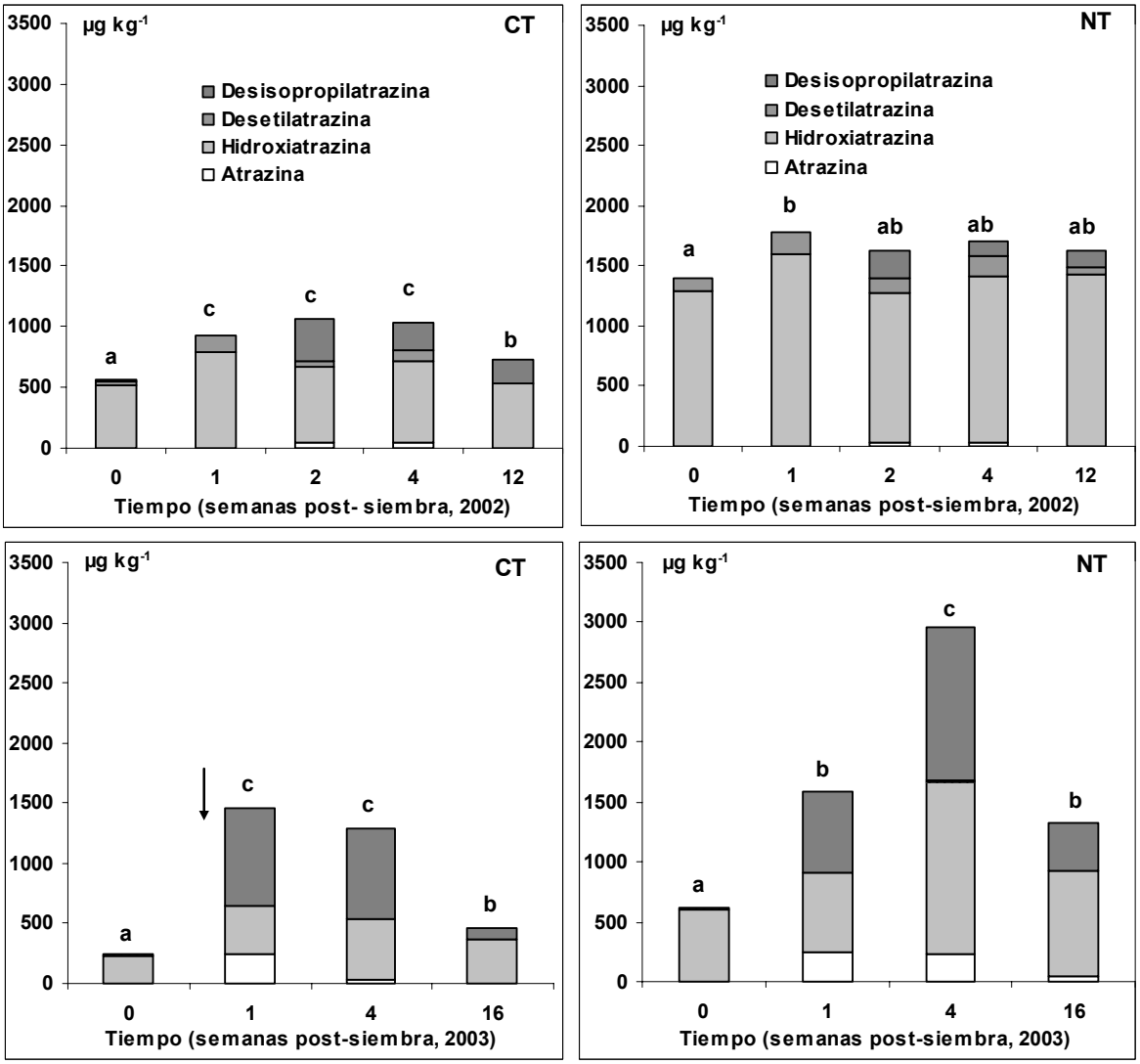

Figura 1. Concentraciones de atrazina y sus metabolitos en la capa de 0-5 cm de un suelo bajo laboreo convencional (CT) y no laboreo o siembra directa (NT) en los diferentes tiempos de muestreo (0, 1, 2, 4, 12 y 16 semanas) después de la siembra de maíz y aplicación de atrazina (↓). Letras diferentes indican diferencias significativas (p<0.05) en el contenido de compuestos extraíbles. (Datos tomados de Mahía *et al.*, 2007).

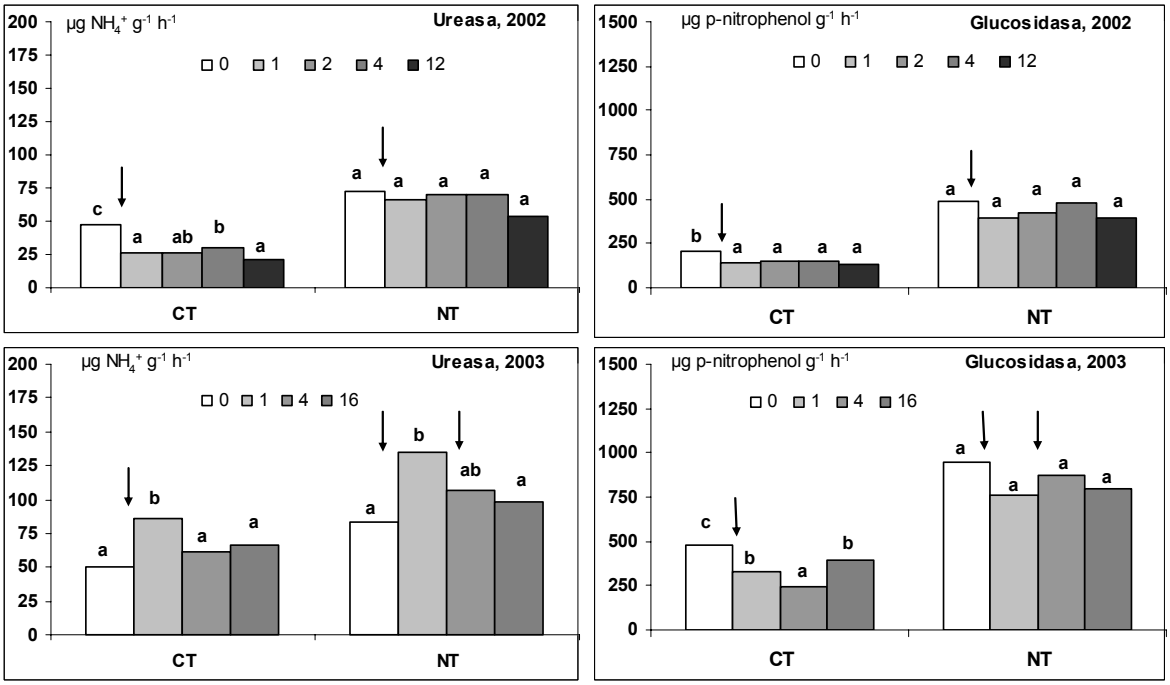

Figura 2. Actividades enzimáticas en la capa de 0-5 cm de un suelo cultivado bajo laboreo convencional (CT) y no laboreo o siembra directa (NT) a los diferentes tiempos de muestreo (0, 1, 2, 4, 12 y 16 semanas) después de la siembra del maíz y aplicación de atrazina (↓). Para cada tipo de manejo letras diferentes indican diferencias significativas (p<0.05). (Datos tomados de Mahía *et al*., 2007).

Experiencias de laboratorio

La Figura 3 muestra los resultados de la distribución de las diferentes fracciones de la atrazina (mineralizada, extraíble y no extraíble) obtenidos para las distintas muestras de los suelos P y G durante el experimento de incubación. La evolución de la atrazina extraíble (A), y de sus metabolitos declorados (HA) y dealquilados (DIA y DEA) en estos mismos suelos se muestran en la Figura 4. La evolución de la mineralización de C, biomasa microbiana y coeficiente metabólico en las muestras de los suelos P y G no adicionados y adicionados con atrazina se muestran en las Figuras 5 y 6. Las Figuras 7, 8 y 9 muestran los valores de las propiedades bioquímicas analizadas (actividades enzimáticas, carbohidratos, mineralización de N) durante la experiencia de incubación de las muestras no tratadas y tratadas con atrazina en los cinco suelos estudiados (P, G, E, M y C), mientras que en la Figura 9 se presentan los resultados del análisis de componentes principales realizados con los datos de la estructura de la comunidad (PLFA).

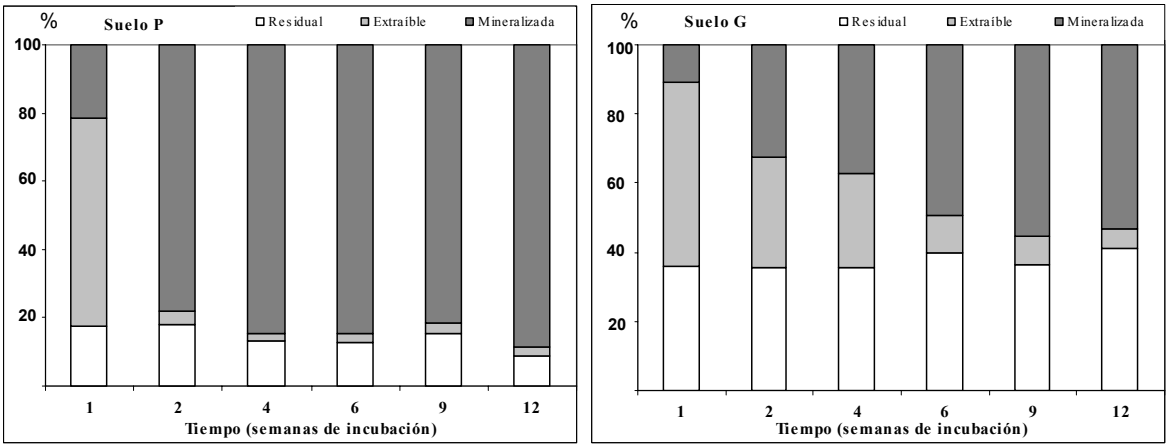

Figura 3. Distribución de la atrazina, expresada en porcentaje, en las fracciones no extraíble o "bound residues", extraíble y mineralizable, en dos suelos (P y G) a lo largo de un período de incubación de 12 semanas. (Datos tomados de Mahía y Díaz-Raviña, 2007).

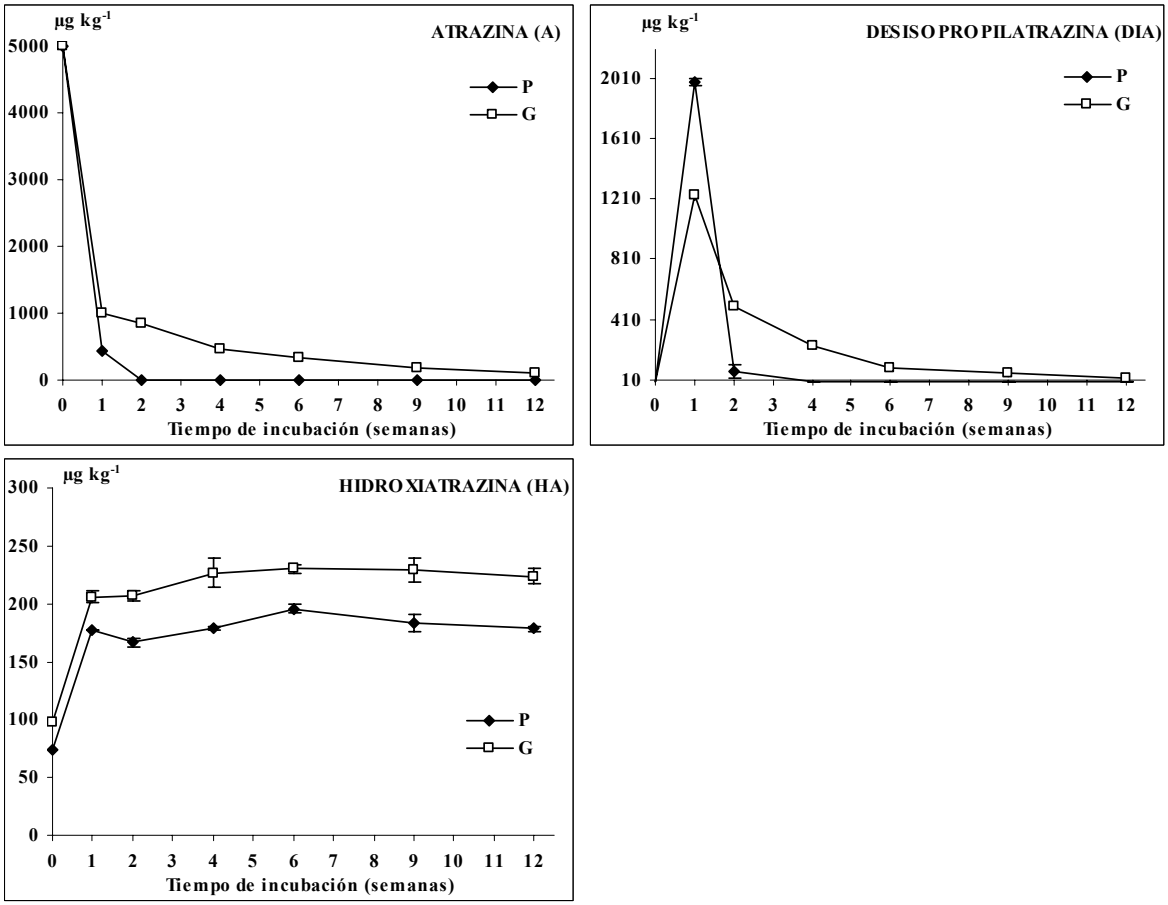

Figura 4. Evolución de la atrazina (A), desisopropilatrazina (DIA) e hidroxiatrazina (HA) en la fracción extraíble de dos suelos agrícolas (P y G) adicionados con 5 µ g kg^{-1} de atrazina e incubados durante 12 semanas (valor medio ± SE de las tres réplicas de incubación). La desetilatrazina (DEA) no fue detectada. (Datos tomados de Mahía y Díaz-Raviña, 2007).

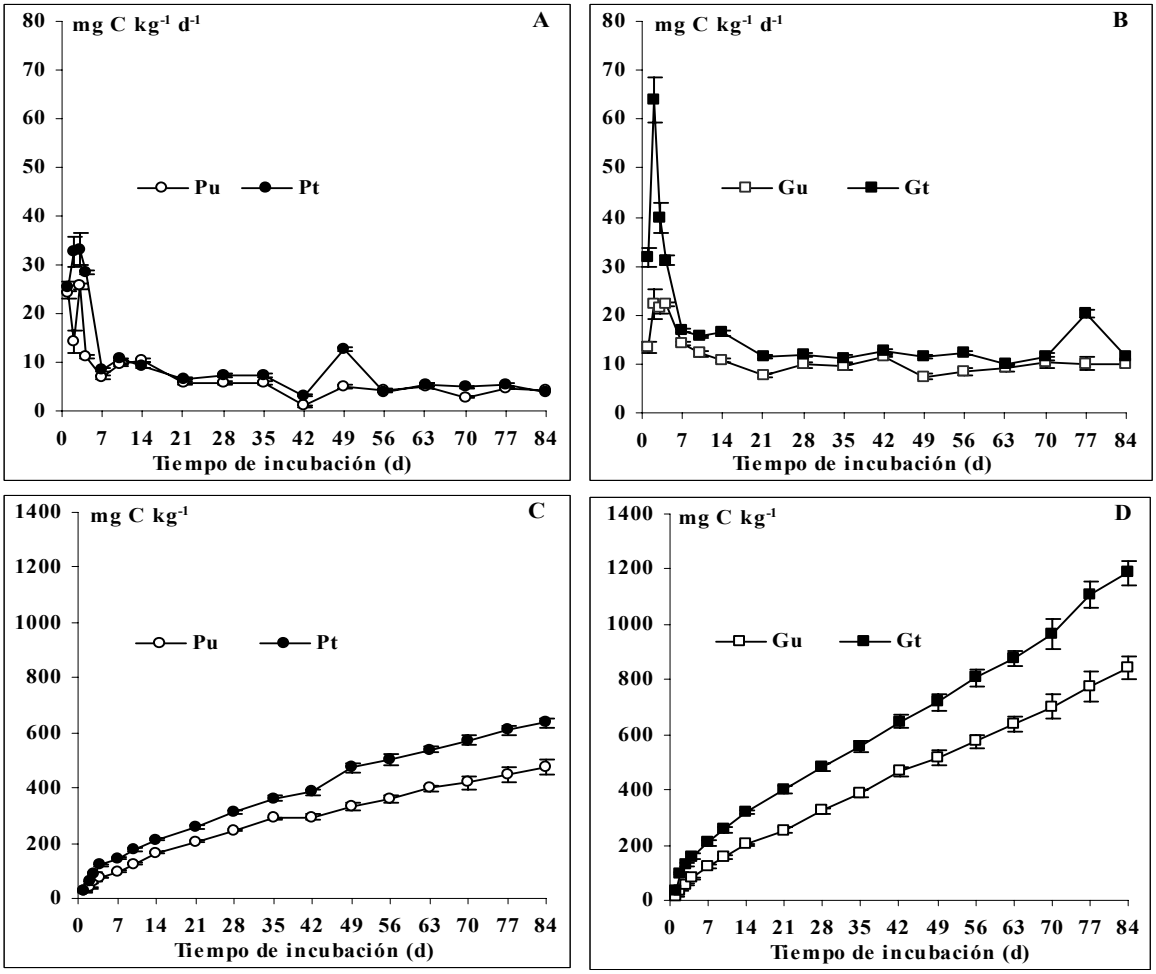

Figura 5. Cinética de mineralización del C en los dos suelos estudiados no adicionados y adicionados con atrazina a dosis de campo. Evolución del CO_2 desprendido diariamente (A, B) y acumulado (C, D) en las muestras no adicionadas (u) y adicionadas con atrazina (t) de los suelos P y G durante 12 semanas de incubación aeróbica (valor medio±SE). (Datos tomados de Mahía *et al.*, 2008a).

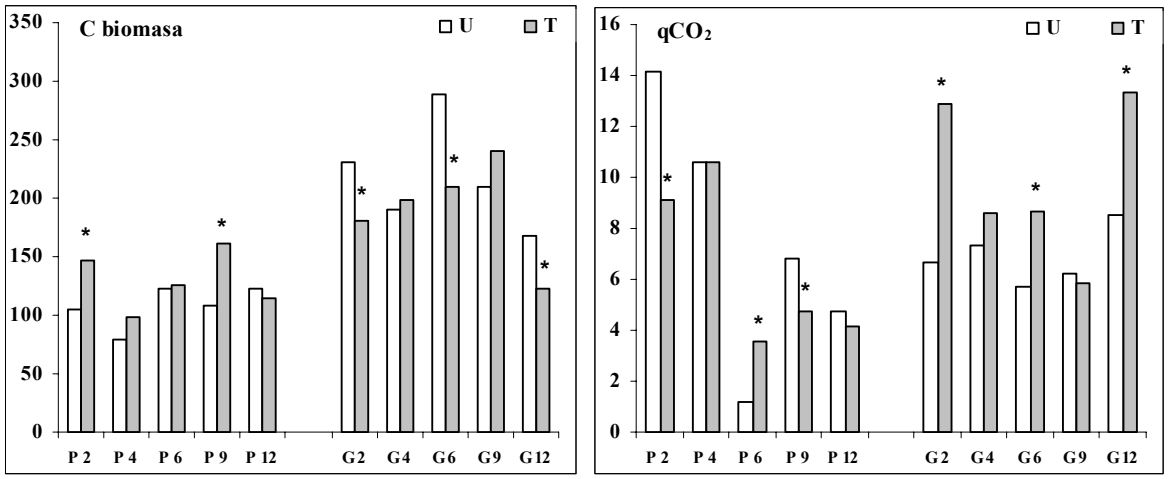

Figura 6. Biomasa microbiana (Cmic, mg C kg^{-1}) y coeficiente metabólico (qCO_2, µg CO_2 Cmic $día^{-1}$) en las muestras no adicionadas (U) y adicionadas con atrazina (T) de los suelos P y G después de 2, 4, 6, 9 y 12 semanas de incubación (valor medio±SE). *Valores medios estadísticamente diferentes del correspondiente suelo control. (Datos tomados de Mahía *et al.*, 2008a).

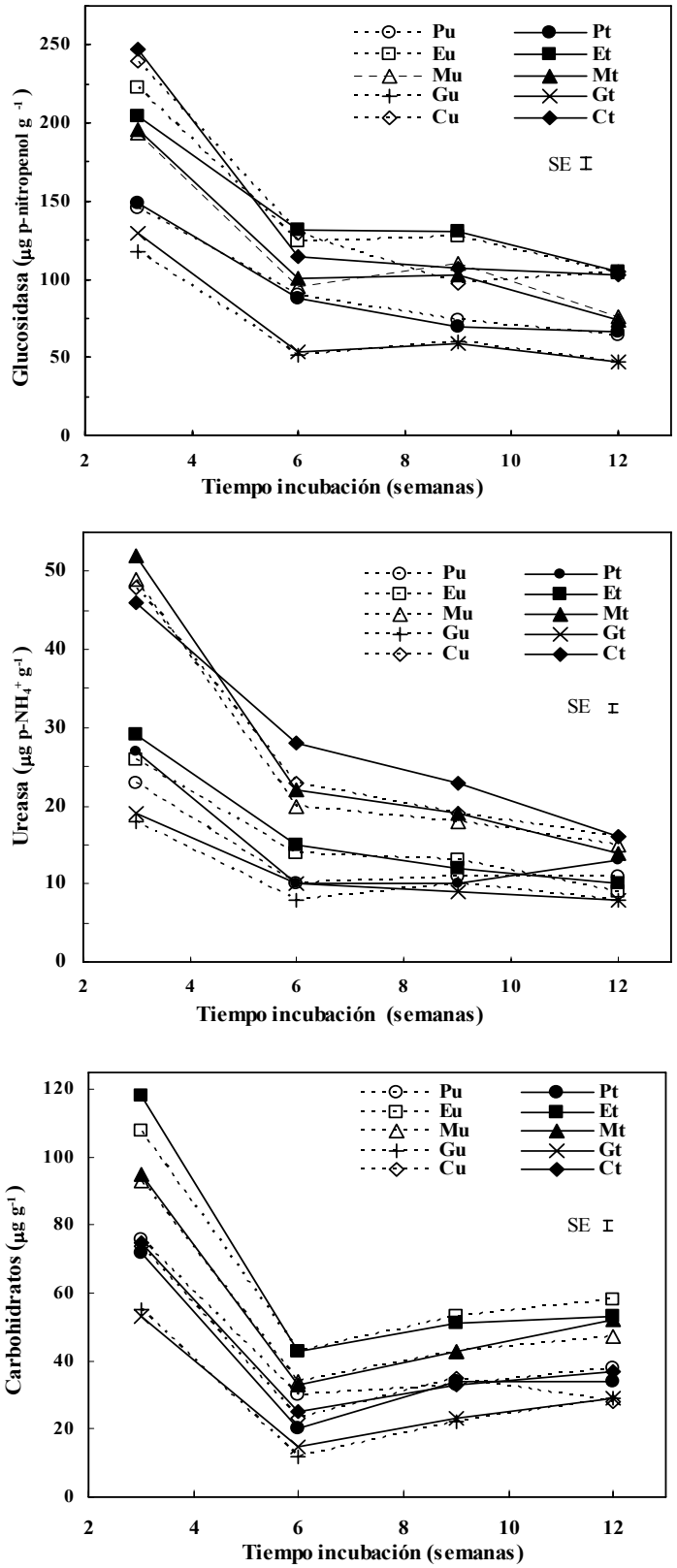

Figura 7. Propiedades bioquímicas de las muestras no tratadas (u) y tratadas con atrazina (t) en los suelos P, E, M, G y C, 3, 6, 9 y 12 semanas después de la incubación (valor medio de las 3 réplicas). (Datos tomados de Mahía *et al*., 2011).

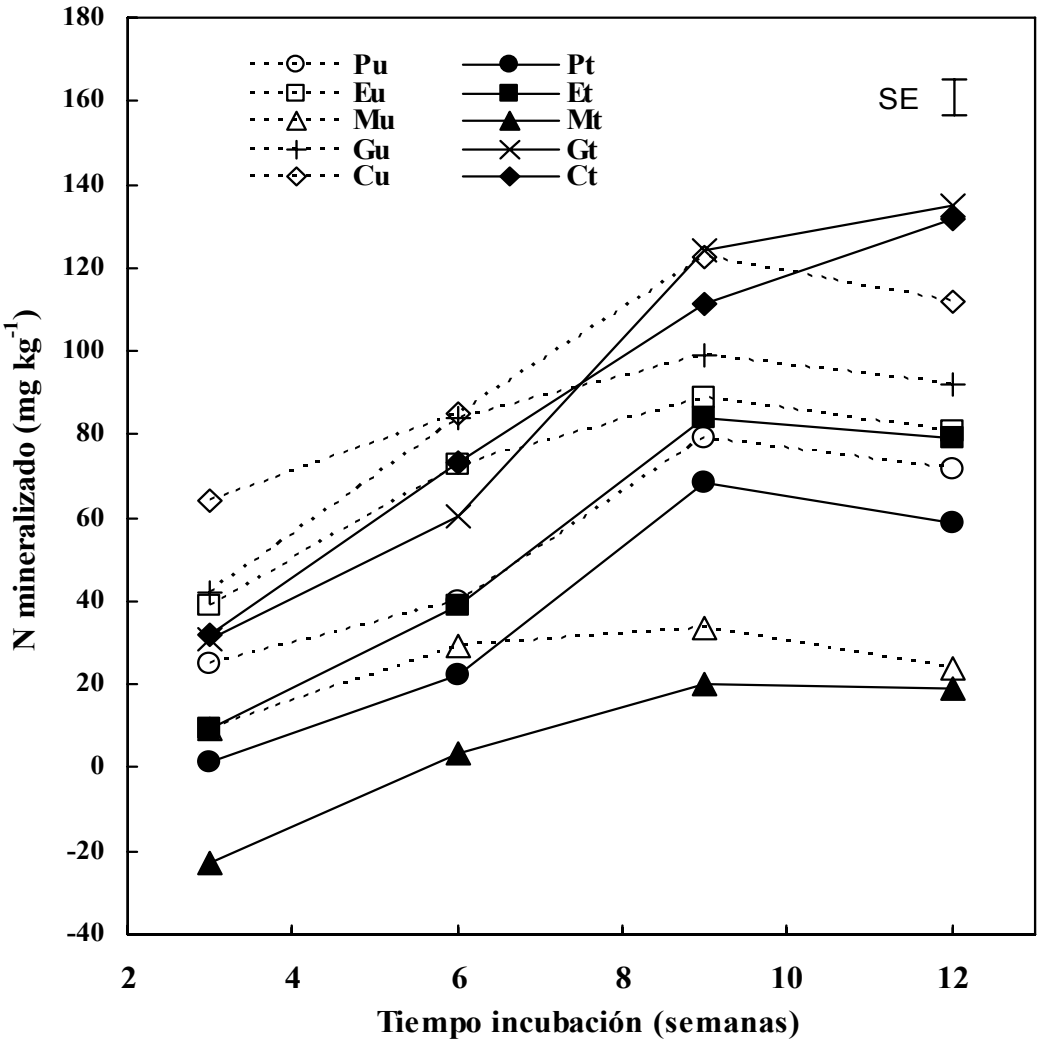

Figura 8. Mineralización neta del N (amonificación más nitrificación) en las muestras no tratadas (u) y tratadas con atrazina (t) en los cinco suelos (P, G, E, M y C) a las 3, 6, 9 y 12 semanas de incubación. (Datos tomados de Mahía *et al*., 2011).

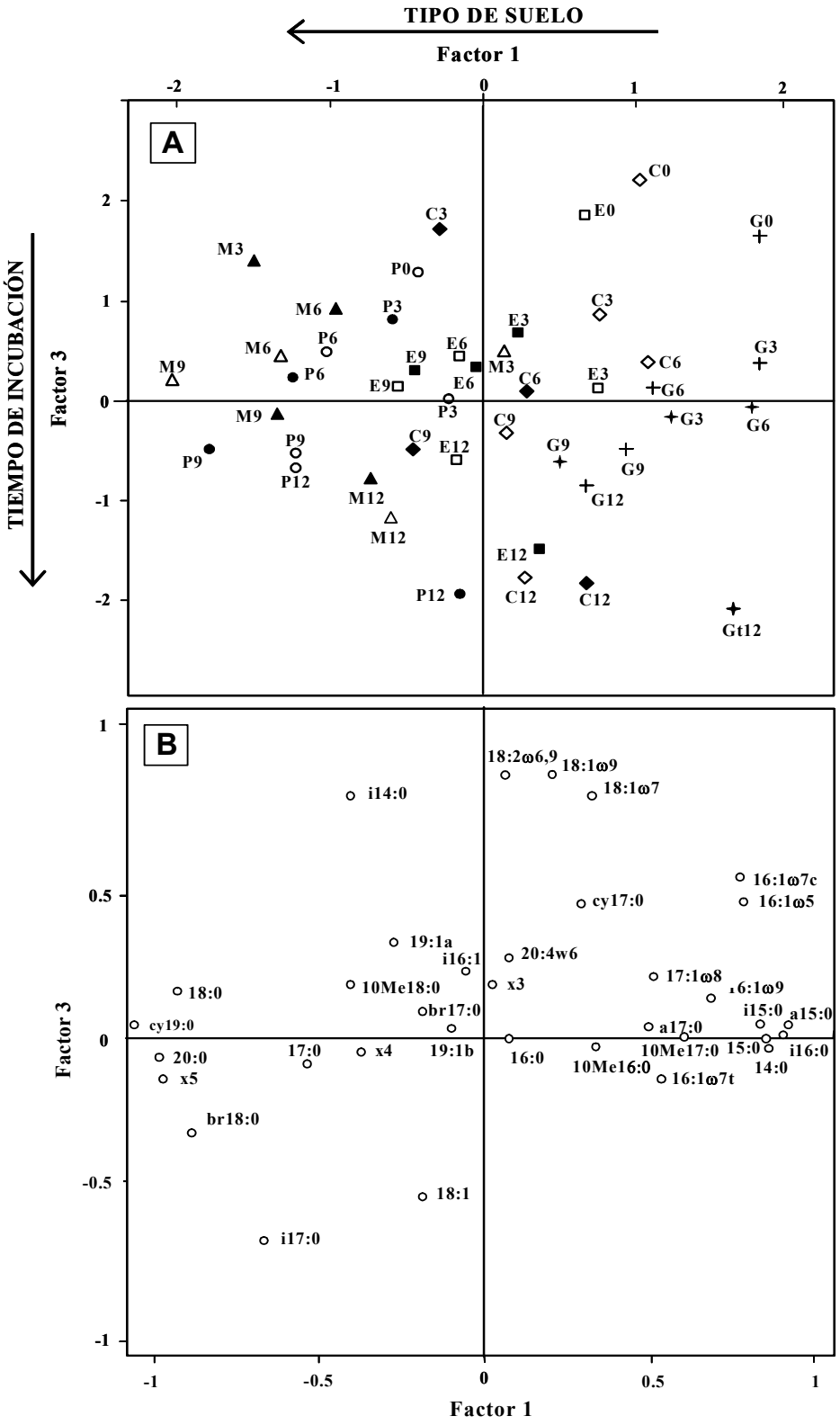

Figura 9. Análisis de componentes principales de las muestras no tratadas (u) y tratadas con atrazina (t) de los cinco suelos estudiados (P, G, E, M y C) a los diferentes tiempos de incubación (3, 6, 9 y 12 semanas). Los Factores 1 y 3 absorben el 34 y el 11% de la varianza, respectivamente. (Datos tomados de Mahía *et al*., 2011).

5.- Discusión

Degradación de la atrazina

Los estudios de una experiencia de laboratorio, realizados con los suelos P y G, mostraron que la hidroxiatrazina (HA) es el único producto intermedio detectado en la fracción extraíble de los suelos no tratados con atrazina durante el período de incubación de 3 meses (Figura 4). Dado que las muestras se recogieron en primavera antes de la siembra del maíz y de la aplicación anual de atrazina, los niveles de HA representan la acumulación de los residuos de atrazina de varias aplicaciones (1-40 años). Estos resultados indican que la persistencia de HA en los suelos es mayor que la de la atrazina o de los demás productos de su degradación (DIA, DEA). Resultados similares fueron observados por otros autores y atribuidos a su fuerte adsorpción y su relativamente lenta degradación (Winkelmand y Klaine, 1991; Assaf y Turco, 1994; Peixoto *et al.*, 2000). Después de la aplicación de la atrazina se observaron marcadas fluctuaciones en el contenido de la atrazina y de sus metabolitos (Figura 4). Los resultados de las experiencias de laboratorio mostraron que después de una semana, la atrazina representaba entre el 9 y el 20% de la concentración inicial y sus productos de degradación del 33 al 58%, siendo el contenido de DIA de 10 a 16 veces mayor que el de HA. Los datos indican que los primeros pasos en el metabolismo de la atrazina fueron la decloración (proceso principalmente químico) y la N-dealquilación y deaminación (procesos microbiológicos), que condujeron a la formación de HA e DIA, respectivamente. Estos resultados son concordantes con diversos estudios que indican que la degradación de atrazina ocurre a través de procesos bióticos y abióticos, pero que, sin embargo, la degradación microbiana es el proceso predominante en algunos suelos (Barriuso y Hout 1996; Gan *et al*. 1996; Kookana *et al*., 1998; Accinelli *et al.,* 2001). El contenido de atrazina y sus productos de degradación tendió a disminuir durante el período de incubación de 12 semanas; sin embargo, se observó un comportamiento diferente dependiendo del compuesto analizado en cada suelo. Después de 2

semanas de incubación, los residuos extraíbles representaron el 4 % del contenido inicial (0,2 % A, 2,5 % HA y 1,6 % DIA) en el suelo P y 32 % (17 % A, 2,7 % HA y 12 % DIA) en el suelo G. Más tarde, mientras que los valores de A y DIA disminuyeron ligeramente durante el período de 2 a 12 semanas en el suelo G, cantidades insignificantes eran detectadas en el suelo P. Al final de la incubación, todavía fue detectado un 2,5-5 % de atrazina residual, indicando un efecto residual 3 meses después de la aplicación. Resultados similares fueron observados en otro experimento de laboratorio, utilizando los cinco suelos agrícolas con diferentes características físico-químicas e historial de aplicación de atrazina, en el que se analizó la fracción extraíble (Mahía *et al*., 2008b).

La evolución de la mineralización de la atrazina en los suelos G y P a lo largo de la incubación se muestra en la Figura 3. En ambos suelos la mineralización se produjo rápidamente durante las primeras 2-6 semanas de incubación, con ausencia de la fase inicial de latencia, y luego se mantuvo constante (fase de degradación estacionaria) hasta el final de la incubación. Este comportamiento es concordante con otros estudios que demuestran la presencia de una comunidad microbiana adaptada, es decir, con la capacidad de mineralizar rápidamente el anillo de la atrazina en suelos con un historial previo de frecuente aplicación de atrazina (Barriuso y Houot, 1996; Ostrofsky *et al.*, 1997; Rousseaux *et al.*, 2001). Se observaron también diferencias en la cinética de mineralización de la atrazina entre los dos suelos estudiados, siendo el grado y la extensión de la mineralización menor en el suelo G, con 10 años de historial de aplicación, que en el suelo P con 40 años de historial. En el suelo P, el 78 % de la U-^{13}C-atrazina fue mineralizado en las dos primeras semanas de la incubación y luego este valor se incrementó ligeramente hasta alcanzar un 89% al final de la incubación. Por el contrario, en el suelo G, sólo un 33 % de la atrazina aplicada fue mineralizado durante las dos primeras semanas, el 49 % hasta la sexta semana y luego la mineralización

permaneció casi constante hasta el final de la incubación, en que se alcanzó un valor del 53 %. Estos valores de mineralización fueron mayores que los obtenidos en suelos no adaptados, es decir, sin aplicación de atrazina, y del mismo orden de magnitud que los obtenidos en suelos adaptados, con un historial similar de aplicación de atrazina (Barriuso y Houot, 1996; Ostrofsky *et al*., 1997; Yassir *et al*., 1999; Abdelhafid *et al.*, 2000a,b; Houot *et al*., 2000; Krutz *et al*., 2003).

La fracción no extraíble representó el 9-18 % y 35-41% en los suelos P y G, respectivamente (Figura 3). Las mayores concentraciones mostradas por el suelo G en comparación con el suelo P, se podrían explicar en base a determinadas propiedades de los suelos, tales como el contenido de materia orgánica y la textura, puesto que la formación de residuos no extraíbles se asocia con los contenidos de C orgánico (Peixoto *et al*., 2000; Moorman *et al.*, 2001; Huang *et al*., 2003) y con el tipo y contenido de arcilla (Koskinen y Clay, 1998; Houot *et al*., 2000). El contenido de materia orgánica puede también explicar la diferente evolución de la fracción no extraíble en los dos suelos estudiados. En el suelo G la fracción no extraíble apenas cambió o incluso se incrementó ligeramente durante la incubación, alcanzando el valor más elevado al final de la misma; por el contrario, en el suelo P se observaron cambios significativos con el tiempo mostrando, al final de la incubación, valores aproximadamente un 50 % menores que los presentados a la primera semana e indicando que la atrazina residual fue movilizada. Los datos son concordantes con los estudios de Huang *et al.* (2003), quienes encontraron una mayor desorpción al incrementarse el contenido de materia orgánica del suelo. El contenido de compuestos no extraíbles fue consistente con los datos de las fracciones extraíble y mineralizable, indicando un mayor potencial de contaminación de suelos y aguas para el suelo G que para el suelo P. Es un hecho conocido que concentraciones

elevadas de atrazina residual no extraíble representan un alto riesgo potencial de contaminación del ambiente cuando son movilizadas (Gan *et al.*, 1996). Los relativamente altos contenidos de la fracción residual de la atrazina en los suelos estudiados sugieren que se deberían hacer más investigaciones con el objetivo de estudiar los posibles efectos a largo plazo de estos residuos, así como la posibilidad de que, en condiciones naturales, estos residuos puedan ser fácilmente movilizados con el tiempo.

Los resultados de las experiencias de laboratorio se confirmaron bajo condiciones de campo. Durante el cultivo del maíz se observaron marcadas fluctuaciones en el contenido de atrazina y sus metabolitos en los 0-5 cm superficiales de la capa arable, particularmente en el tratamiento NT, lo cual es consistente con las mayores dosis aplicadas del herbicida (una dosis en pre-emergencia y, si es necesario, otra dosis en post-emergencia) (Figura 1). HA fue el principal metabolito detectado en los suelos antes de la siembra del maíz; así aunque se observó una significativa desaparición del metabolito entre los dos períodos consecutivos de crecimiento (los valores en el año 2002 fueron dos veces mayores que en el 2003), el rango de valores demostró la larga persistencia de este metabolito de la atrazina en este suelo. Tal como era de esperar, la aplicación de atrazina al suelo incrementó considerablemente el contenido de atrazina y sus metabolitos en la fracción extraíble, particularmente en el año 2003 al usar dosis mayores. La evolución de estos compuestos xenobióticos durante el cultivo de maíz mostró que inicialmente el contenido de atrazina tendía a disminuir y el de sus metabolitos, particularmente HA y DIA, tendían a aumentar, mientras que DEA sólo fue detectado en el año 2002. HA y DEA fueron también detectados, antes y 16 semanas después de la siembra del maíz, en la capa de 5-20 cm de parcelas bajo laboreo convencional y siembra directa, mostrando el efecto residual y alto riesgo potencial de contaminación de las

aguas superficiales y subterráneas (Mahía *et al*., 2007). Los resultados obtenidos también indicaron que la ruta de degradación de la atrazina y, por tanto, la proporción de metabolitos dealquilados, derivados de procesos biológicos (DEA y DIA), y el metabolito declorado, derivado principalmente de procesos no biológicos (HA), pueden variar entre años. Esto puede ser debido más a diferencias en el tipo y actividad de los microorganismos implicados en la degradación de la atrazina (por ejemplo fluctuaciones en las condiciones climáticas, disponibilidad de sustrato, etc.) que a diferencias en las propiedades físico-químicas. Es necesario señalar que sólo un 19-45 %, en 2002, y 10-51 %, en 2003, de la atrazina originalmente aplicada al suelo es extraíble (atrazina y metabolitos) en el período de 3-4 meses. Estos valores de compuestos extraíbles están dentro del rango dado para diferentes suelos con similar dosis de aplicación de atrazina (Gan *et al.*, 1996; Gaynor *et al.*, 1998; Chung y Alexander, 2002). Los resultados sugieren la existencia de una importante fracción residual de la atrazina y, por tanto, de una vía latente de contaminación, debido a varios factores que pueden causar su movilización y paso a la solución del suelo, lo que concuerda con los resultados obtenidos en las experiencias de laboratorio.

Resumiendo, los resultados de estos experimentos de laboratorio y campo muestran que, en estos suelos agrícolas de Galicia, la degradación microbiana a través de la ruptura del anillo *s*-triazínico (degradación completa o mineralización) es la principal ruta de descomposición de la atrazina y que la repetida aplicación anual de atrazina aumenta su degradación. HA y DIA son los principales metabolitos encontrados en la fracción extraíble, lo que parece demostrar que tanto procesos químicos como biológicos están implicados en la degradación de la atrazina. La formación de residuos no extraíbles también constituye un importante proceso abiótico para la atenuación del impacto de la aplicación de atrazina en estos suelos ácidos con relativamente alto contenido de materia orgánica. Además, combinando los resultados

obtenidos para los metabolitos analizados en la fracción extraíble y los porcentajes de U-^{13}C-atrazina (producción de CO_2, degradación total o mineralización) y etilamina-^{15}N-atrazina degradada (producción de NH_3, degradación parcial), la ruta de degradación de atrazina en estos suelos agrícolas pudo ser esclarecida (Mahía y Díaz-Raviña, 2007).

El estudio detallado de la degradación de la atrazina en suelos agrícolas de Galicia (metodología, resultados experimentales y discusión de resultados obtenidos) están recogidos con más detalle en los siguientes artículos:

Mahía J., A. Martín, T. Carballas y M. Díaz-Raviña. 2007. Atrazine degradation and enzyme activities in an agricultural soil under two tillage systems. *The Science of Total Environment*, 378: 187-194. (Véase Anexo I).

Mahía J., A. Martín y M. Díaz-Raviña. 2008. Extractable atrazine and its metabolites in agricultural soils from the temperate humid zone. *Environmental Geochemistry Health*, 30: 147-152. (Véase Anexo II).

Mahía J. y M. Díaz-Raviña 2007. Atrazine degradation and residues distribution in two acid soils from temperate humid zone. *Journal of Environmental Quality*, 36: 826-831. (Véase Anexo III).

Mahía J., S. J. González-Prieto, A. Martín, E. Bååth y M. Díaz-Raviña. 2011. Biochemical properties and microbial community structure of five different incubated soils untreated and treated with atrazine. *Biology and Fertility of Soils,* 47: 577-589. (Véase Anexo V).

Respuesta microbiana a la adición de atrazina

Aunque a lo largo de la incubación se observó, en algún caso, un efecto significativo positivo o negativo de la adición de atrazina sobre la microbiota edáfica, en general los valores del C de la biomasa fueron del mismo orden de magnitud en el suelo no adicionado y adicionado con atrazina, indicando que la adición del herbicida no modifica la biomasa microbiana (Figura 6). Los resultados obtenidos concuerdan con los de otros autores mostrando cambios inconsistentes o incluso ningún cambio en la biomasa microbiana después de la adición de atrazina a bajas dosis de aplicación (Ghani *et al.* 1996; Accinelli *et al.* 2002). La relación entre la respiración del suelo y la biomasa microbiana, conocida como respiración específica o coeficiente metabólico (qCO_2), refleja el estado fisiológico de la comunidad microbiana y puede usarse como un indicador de algún tipo de estrés en el ecosistema (Anderson y Domsch 1990); por tanto, las diferencias en los valores de qCO_2 pueden reflejar la magnitud de la respuesta inducida por el pesticida (Perucci *et al*., 2000; Jones y Ananyeva, 2001). En nuestro caso, los valores qCO_2 calculados para los suelos no tratados y tratados con atrazina, indicaron que el coeficiente metabólico no puede usarse para evaluar el impacto de la atrazina aplicada a dosis agronómicas sobre las comunidades microbianas (Figura 6). Resultados similares fueron observados usando los datos de los carbohidratos solubles y de las actividades enzimáticas del suelo (ureasa y β-glucosidasa), ya que generalmente los valores detectados en los dos diferentes tratamientos (suelo no tratado y suelo tratado con 5 mg atrazina kg^{-1} suelo) fueron del mismo orden de magnitud (Figura 7). Estos resultados están de acuerdo con los de otros estudios, que muestran efectos no consistentes de la atrazina sobre varias actividades enzimáticas (Voets *et al*., 1974; Gianfreda *et al.*, 1994; Sannino y Gianfreda, 2001), observándose también un efecto de la dosis (Kruglow *et al*., 1975; Davies y Greaves 1981; Perucci *et al.*, 2000; Moreno *et al*., 2007).

En concordancia con estos estudios, en condiciones de campo tampoco se observó ningún efecto o se detectaron incluso inconsistentes efectos positivos (estimulatorios) o negativos (inhibidores) sobre las actividades ureasa y β-glucosidasa como consecuencia de la aplicación de atrazina a dosis agronómica (Figura 2). También se manifestaron claramente marcadas fluctuaciones de las actividades enzimáticas con el tiempo de muestreo. Por ejemplo, los valores obtenidos para todas las actividades (excepto para la ureasa en 2003) fueron mayores en las muestras recogidas antes de la siembra y de la aplicación de la atrazina (tiempo 0) que en el resto de las muestras recogidas a distintos intervalos de tiempo después de la aplicación del herbicida. Estas variaciones con el tiempo se deben, al menos en parte, a la aplicación del herbicida, aunque la influencia de otros factores tales como el arado, la adición de otros agroquímicos, la disponibilidad del sustrato derivado de las raíces o de otro material incorporado al suelo, así como las condiciones climáticas (humedad y temperatura) tampoco puede ser descartada. Para el tratamiento CT se observó un inicial y significativo descenso del 30-45 % en los valores de las actividades una semana después de la aplicación de la atrazina y luego los valores apenas cambiaron con el tiempo, mientras que para el tratamiento NT el descenso fue de menor magnitud (10-20 %) y no fue significativo. Teniendo en cuenta que la dosis de atrazina era más alta en el tratamiento NT que en el tratamiento CT puesto que en 2003 se realizaron dos aplicaciones del herbicida en NT, una en pre-emergencia y otra en post-emergencia, los diferentes efectos observados bajo los dos sistemas de laboreo pueden explicarse por la historia de aplicación de la atrazina: los microorganismos del suelo sometido a no laboreo o siembra directa (NT) están más adaptados a la adición de atrazina y, por tanto, muestran una respuesta menor que la microbiota del suelo gestionado por laboreo convencional (CT). Esto está de acuerdo con los estudios de varios autores, quienes encontraron una diferente respuesta de la población microbiana a la adición de atrazina en suelos no adaptados y adaptados (Barriuso and Houot, 1996; Abdelhafid *et al.*, 2000b). En

contraste con estos efectos negativos, la actividad de la ureasa en 2003 mostró, una semana después de la aplicación de la atrazina, un incremento significativo del 60-69 % con respecto a los valores iniciales y luego disminuyó hasta alcanzar valores similares a los presentados por el suelo antes de la adición del herbicida. Los resultados obtenidos tanto bajo condiciones de laboratorio como de campo sugieren que los carbohidratos solubles y las dos actividades enzimáticas estudiadas no son índices adecuados para evaluar los efectos de la aplicación de atrazina en estos suelos, ya que, dependiendo de la propiedad considerada y del año de muestreo, reflejaron efectos positivos o negativos inconsistentes. Estos datos son concordantes con numerosos estudios de laboratorio y campo presentando resultados conflictivos y contradictorios debido a la dificultad de establecer la relación causa-efecto entre los pesticidas y las actividades enzimáticas (Davies y Greaves, 1981; Schäffer, 1993; Gianfreda *et al*., 1994; Sannino y Gianfreda, 2001; Accinelli *et al*., 2002; Haney *et al*., 2002).

Los resultados de la mineralización neta del N de las muestras no tratadas y tratadas con ^{15}N-etilamino-atrazina durante el período de 12 semanas de incubación se muestran en la Figura 8. Los valores de mineralización del N se incrementaron con el tiempo de incubación (P<0.005). Durante todo el proceso de incubación, el suelo M presentó los valores más bajos de mineralización del N, seguido de los suelos P y E y, finalmente, los suelos G y C mostraron los valores más altos de mineralización neta del N. Resultados similares fueron obtenidos tomando como base los porcentajes de mineralización neta del N, dado que los valores de este parámetro, que fueron calculados dividiendo la mineralización del N por el N total, siguieron el orden: M (-1,05-2,22 % del N total) < E = P (0,04-3,30 % del N total) < G (1,1-4,83 % del N total) < C (1,44-6 % del N total). Se observó una correlación negativa entre el N mineralizado en el suelo y la degradación de la ^{15}N-etilamino-atrazina (Mahía *et al*., 2011). De acuerdo con algunos autores, la mineralización de la atrazina disminuye cuando la

disponibilidad del N del suelo aumenta como consecuencia de la fertilización orgánica o inorgánica (Alvey y Crowley, 1995; Abbelhafid *et al*., 2000a,b; Haney *et al*., 2002). En nuestro estudio la mayor degradación de atrazina se observó en los suelos con un largo historial de aplicación del herbicida y baja disponibilidad de N, lo que apoya la hipótesis de que la atrazina puede ser utilizada como fuente de N por los microorganismos degradadores de la atrazina (Cook y Hunter, 1981; Entry *et al*., 1993; Mandelbaum *et al*., 1995; Radosevich *et al*., 1995; Bichat *et al*., 1999). Para todos los suelos y tiempos de incubación, las muestras tratadas con atrazina mostraron un rango de valores de amonificación y nitrificación ligeramente más bajo que las correspondientes muestras no tratadas, aunque en muchos casos no se observaron diferencias significativas (P<0.05). Así, la aplicación de 5 mg de atrazine kg^{-1} suelo dió lugar a una reducción de la mineralización del N de 1,6-34 mg N kg^{-1}, siendo detectados los valores más altos después de 3 o 6 semanas de incubación. También se observaron, sin embargo, incrementos significativos de la mineralización del N de 25 a 43 mg N kg^{-1} como consecuencia de la adición de atrazina a los suelos G and C después de 6-9 semanas de incubación. Resultados contradictorios se citan también en la literatura, indicando que los pesticidas pueden no afectar (Hart y Brookes, 1997) o afectar positiva o negativamente a la mineralización del N orgánico del suelo a N-NH_4^+ y luego a N-NO_3^- (Cheng *et al*., 2001; Haney *et al.* 2002). En nuestro caso observamos que a corto plazo (3-6 semanas) la atrazina modificó ligeramente la dinámica de mineralización del N, indicando que los microorganismos amonificantes y nitrificantes son sensibles a la adición de atrazina a dosis normales de aplicación en condiciones de campo. Asimismo, los resultados también indicaron que en estos suelos agrícolas, sometidos a la aplicación anual de atrazina, la medida de la mineralización neta del N (amonificación y nitrificación) es un indicador del impacto de la atrazina más sensible que la medida de determinadas actividades enzimáticas específicas de los ciclos del C (β-glucosidasa) y del N (ureasa).

Las curvas de evolución diaria del CO_2-C liberado de los suelos no tratados y tratados con atrazina durante el período de 12 semanas de incubación se muestran en la Figura 5. Se observó el mismo efecto de la atrazina independientemente del suelo considerado; así, durante todo el experimento el desprendimiento diario de CO_2-C fue mayor en los suelos adicionados con atrazina que en los correspondientes suelos control no adicionados, observándose únicamente un efecto más marcado en el suelo G que en el P. Para los dos suelos el incremento fue más acusado durante la primera semana de incubación, y luego el efecto disminuyó gradualmente aunque se mantuvo, en menor extensión, a lo largo de toda la incubación, indicando un efecto residual de la atrazina sobre la comunidad microbiana edáfica. Este efecto es bastante sorprendente dado que, como ya se mostró anteriormente, la mayoría de la atrazina recientemente adicionada al suelo se mineraliza rápidamente. Estos resultados muestran los efectos a largo plazo de este compuesto xenobiótico sobre los microorganismos del suelo, incluso después de que la mayor parte del compuesto original haya desaparecido, lo que está de acuerdo con las observaciones de Perucci *et al.* (2000). Al final de la incubación, los valores del C mineralizado acumulado fueron significativamente mayores en las muestras tratadas con atrazina que en las no tratadas, indicando que la adición del herbicida modificó la respiración de los dos suelos estudiados. El incremento neto del C mineralizado, provocado por la adición de atrazina, alcanzó, después de 12 semanas de incubación valores de 160 mg CO_2-C kg^{-1} suelo seco en el suelo P y de 344 mg CO_2-C kg^{-1} suelo seco en el suelo G, que representan el 33 % y 41 %, respectivamente, del C mineralizado por las correspondientes muestras no adicionadas con este herbicida (Fig. 6). El más pronunciado efecto de la atrazina observado en el suelo G, con un contenido más elevado de C orgánico y un menor historial de aplicación de atrazina, puede ser debido, en parte, al hecho de que los microorganismos de este suelo están menos adaptados a la adición del herbicida y, por tanto, muestran una mayor respuesta después de su aplicación (Rhine *et al.*,

2003). Un efecto estimulador de la aplicación de atrazina a dosis normales sobre la respiración del suelo también fue observado por otros investigadores (Ghani *et al.*, 1996; Accinelli *et al.*, 2002), aunque en estos estudios el aumento del CO_2-C no excedió de 20 mg CO_2-C kg^{-1} suelo seco y el efecto no duró más de 20 días. De acuerdo con De Nobili *et al.* (2001) un efecto "priming" puede ser inducido por concentraciones traza (por ejemplo, cantidades de μg g^{-1}) de adecuadas soluciones "trigger"; por lo tanto, una posible explicación para los resultados obtenidos podría ser que la atrazina y/o sus metabolitos de descomposición actuaron directamente como moléculas "trigger" o indujeron su progresiva liberación de la materia orgánica nativa del suelo durante toda la incubación. Una explicación más detallada de este positivo y marcado efecto de la adición de atrazina a dosis de campo sobre la mineralización de la materia orgánica del suelo puede encontrarse en el trabajo de Mahía *et al.* (2008a).

El análisis de componentes principales, realizado con el conjunto de los datos de todas las muestras no tratadas y tratadas con atrazina de los cinco suelos estudiados mostró diferencias en el perfil de los ácidos grasos de los fosfolípidos (PLFA) dependiendo del historial de aplicación de la atrazina (Figura 9). El primer componente, que diferencia los suelos con bajo (suelos C y G, 8-10 años de historial de aplicación de atrazina), medio (suelo E, 30 años de historial de aplicación, los últimos 10 años sin aplicación) y alto (suelos M y P, 20-40 años de aplicación) historial de aplicación de atrazina, explicó el 34 % de la variación, mientras que el segundo componente, que diferencia las muestras de acuerdo con el tiempo de incubación, sólo explicó el 11 % de la variación. Los suelos con bajo historial de aplicación de atrazina y, por tanto, con bajo porcentaje de degradación (con valores positivos en el eje 1), se caracterizan por altas concentraciones de los ácidos grasos saturados de 14-16 átomos de C (i16:0, a15:0, i15:0, 14:0) y de los ácidos grasos monosaturados 16:1ω7c y 16:1ω5, mientras

que los suelos con un mayor historial de aplicación y mayores porcentajes de degradación (con valores negativos en el eje 1) están caracterizados por altas concentraciones de ácidos grasos saturados de 17- a -20- átomos de C. Los ácidos grasos que en las muestras de suelo no adicionadas y adicionadas con atrazina disminuyeron con la incubación (con valores positivos en el eje 2) son principalmente de origen fúngico (18:2ω6 y 18:1ω9) y bacteriano (18:1ω7, i14:0). La distribución de las muestras también mostró que, para el mismo tiempo de incubación, se observan pequeñas diferencias entre las muestras que recibieron la adición reciente de la atrazina y las correspondientes muestras no adicionadas, aunque no se observó un patrón de comportamiento constante para los 5 suelos estudiados; además, los cambios producidos son despreciables comparados con los provocados por la adición previa y repetida de atrazina (historial de aplicación) y el tiempo de incubación. Estos resultados coinciden con los encontrados en estudios de diversos autores, que muestran cambios en la estructura de la comunidad microbiana como consecuencia tanto de la incubación del suelo (Frostegård *et al*., 1996) como de la aplicación de atrazina (Chang *et al.*, 2001; Rhine *at al.* 2003; Seghers *et al.* 2003; Ross *et al*., 2006). Así, los resultados de Rhine *et al*. (2003) indican que la exposición reiterada del suelo a la atrazina proporciona una presión selectiva suficiente para provocar cambios en la composición de los ácidos grasos de los fosfolípidos de las comunidades microbianas del suelo; sin embargo, estos autores no pudieron identificar los grupos específicos de microorganismos que habían sido afectados. Además, Seghers *et al*. (2003) indicaron que el uso de atrazina a dosis normales de campo durante 20 años, altera la comunidad microbiana edáfica, en particular las bacterias metanotróficas, caracterizadas por amplificación por PCR con cebadores específicos y posterior análisis por electoforesis en geles con gradiente desnaturalizante (DGGE). Chang *et al.* (2001) y Ross *et al.* (2006) también encontraron diferencias en la estructura de la comunidad bacteriana, determinada por la técnica de PCR-DGGE, en experimentos de laboratorio a corto plazo realizados con

concentraciones excesivamente altas de atrazina. Debe señalarse, sin embargo, que los cambios observados bajo condiciones de campo fueron mucho menos marcados que los observados bajo condiciones de laboratorio, lo que puede deberse a las bajas dosis del herbicida, al tiempo transcurrido desde la aplicación y al efecto de otros factores (condiciones ambientales, propiedades del suelo, prácticas de manejo, efecto rizosférico, etc.) que enmascaran la influencia de la atrazina. En nuestro caso, sin embargo, a pesar de que se utilizaron dosis normales de aplicación del herbicida y de que la atrazina se degradó rápidamente durante las primeras semanas de aplicación, se observó un efecto residual de la aplicación previa y repetida de atrazina en base a los datos de diversidad microbiana (PLFA). Esto indica que la estructura de la comunidad microbiana determinada mediante el análisis del perfil de ácidos grasos de los fosfolípidos (PLFA) es una herramienta muy útil para detectar el efecto residual de la adición anual de atrazina en estos suelos agrícolas con 8-40 años de historial de aplicación de este herbicida.

Combinando la información proporcionada por las diferentes propiedades microbiológicas analizadas se puede concluir que la atrazina no sólo afecta a las malas hierbas sino también a las comunidades microbianas de estos suelos agrícolas con un historial de 8-40 años de aplicación anual del herbicida. Así, la adición reciente de atrazina a dosis normales de campo, aunque no altera substancialmente la mayoría de las propiedades bioquímicas analizadas (biomasa microbiana, actividad de β-glucosidasa y ureasa, carbohidratos solubles), incrementa notablemente la mineralización del C. Los resultados muestran claramente que la aplicación de atrazina a dosis normales de campo tiene importantes repercusiones en el ciclo del C y de los nutrientes y, por tanto, en la fertilidad de estos suelos ácidos con un historial previo de aplicación del herbicida. Actualmente, existe una preocupación constante acerca de

como el manejo de los suelos puede afectar a su calidad y/o a la velocidad de mineralización del C y, por consiguiente, al ciclo global del C; por tanto, deberían ser realizados más estudios referentes a la medida de la actividad y diversidad microbiana, con el fin de mejorar el conocimiento del impacto de la adición de los herbicidas sobre la dinámica de la materia orgánica del suelo y determinar si los resultados obtenidos en los suelos P y G son extrapolables a un amplio rango de suelos. Los resultados también indican que la mineralización del C, más bien que la medida del C de la biomasa y de las actividades enzimáticas, es un buen índice para detectar los cambios producidos en la calidad del suelo tras la aplicación del herbicida, mientras que la dinámica del N del suelo y el análisis de los ácidos grasos de los fosfolípidos son buenos indicadores del impacto de la adición frecuente y continua de atrazina durante varios años.

El estudio detallado de los efectos de la aplicación de atrazina sobre las propiedades bioquímicas de la comunidad microbiana en suelos agrícolas de Galicia (metodología, resultados experimentales y discusión de resultados obtenidos) están recogidos con más detalle en los siguientes artículos:

Mahía J., A. Martín, T. Carballas y M. Díaz-Raviña. 2007. Atrazine degradation and enzyme activities in an agricultural soil under two tillage systems. *The Science of Total Environment*, 378: 187-194. (Véase Anexo I).

Mahía J., A. Cabaneiro, T. Carballas y M. Díaz-Raviña 2008. Microbial biomass and C mineralization in agricultural soils as affected by atrazine addition. *Biology and Fertility of Soils*, 45: 99-105. (Véase Anexo IV).

Mahía J., S. J. González-Prieto, A. Martín, E. Bååth y M. Díaz-Raviña. 2011. Biochemical properties and microbial community structure of five different incubated soils untreated and treated with atrazine. *Biology and Fertility of Soils,* 47: 577-589. (Véase Anexo V).

6.- Resumen y conclusiones

El estudio sintetiza los resultados de varios experimentos que tienen como objetivo investigar el impacto y la dinámica de la atrazina (degradación y distribución de residuos, respuesta de la población microbiana) en suelos de cultivo ácidos del norte de España. Se estudió, bajo condiciones de laboratorio y campo, la mineralización de la atrazina y las fracciones extraíble (atrazina, hidroxiatrazina, desetilatrazina y desisopropilatrazina) y no extraíble, así como las propiedades bioquímicas (biomasa microbiana, respiración del suelo, carbohidratos solubles y actividad de las enzimas β-glucosidasa y ureasa), a diferentes intervalos de tiempo después de la adición del herbicida en dosis agronómica a suelos con diferentes características (contenido de carbono, textura e historial de aplicación de la atrazina).

Se observaron resultados similares en los experimentos de laboratorio y campo, mostrando que la degradación y el comportamiento de la atrazina variaba en función de las características del suelo.

La ruta principal de degradación de la atrazina fue la microbiana a través de la rápida ruptura del anillo s-triazínico, siendo la degradación mayor a medida que aumenta el historial de aplicación. Los principales metabolitos encontrados en la fracción extraíble fueron la hidroxiatrazina y la desisopropilatrazina, lo que parece indicar que tanto la degradación química como la biológica están implicadas en la ruta de descomposición de la atrazina. La formación de residuos no extraíbles también constituye un importante proceso abiótico de atenuación del impacto de la atrazina, particularmente en el suelo con un elevado contenido de materia orgánica y de arcilla.

Como consecuencia de la adición reciente de atrazina se observó un ligero descenso en la mineralización del N y un efecto variable o incluso un efecto nulo sobre la mayoría de las propiedades bioquímicas analizadas; por el contrario, la mineralización del C se incrementó considerablemente, indicando que la aplicación de atrazina a dosis normales de campo puede tener importantes repercusiones en el ciclo del C de estos suelos ácidos. Los resultados también indicaron que la dinámica del N del suelo y la medida del perfil de ácidos grasos de los fosfolípidos (PLFA) son útiles para detectar el efecto residual de la adición anual de atrazina en estos suelos agrícolas con 8-40 años de historial de aplicación.

En conclusión, nuestros resultados confirman que la degradación de la atrazina, así como los efectos de este herbicida sobre las comunidades microbianas, son aspectos importantes a la hora de evaluar el impacto ambiental de su aplicación. La aplicación de atrazina al suelo debe realizarse con precaución dado que, por una parte, la respiración del suelo y la dinámica de la atrazina (fracciones extraíble, mineralizable y no extraíble) muestran un efecto residual del herbicida incluso 3-12 meses después de su aplicación y, por otra, el perfil de ácidos grasos de los fosfolípidos muestra cambios en la estructura o diversidad de la comunidad microbiana varios años después de la aplicación de la atrazina.

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ANEXOS

ANEXO I

ANEXO I

Degradación de la atrazina y efectos de su aplicación sobre las actividades enzimáticas de un suelo agrícola bajo dos sistemas de laboreo

J. Mahía, A. Martín, T. Carballas y M. Díaz-Raviña

Resumen

Se estudió el contenido de atrazina y de sus metabolitos (hidroxiatrazina, desetilatrazina y desisopropilatrazina), así como la actividad de dos enzimas (ureasa y β-glucosidasa) en un suelo agrícola ácido, de la zona templado-húmeda (Galicia, NO. de España), con una rotación anual de centeno y maíz sometido a dos sistemas de cultivo diferentes, laboreo convencional (CT) y no laboreo (NT). Se recogieron muestras de suelo durante dos años consecutivos, a dos profundidades de la capa arable (0-5 cm y 5-20 cm), a diferentes intervalos de tiempo después de la aplicación de la atrazina. La hidroxiatrazina y deisopropilatrazina fueron los principales metabolitos formados como resultado de la degradación de la atrazina en el suelo ácido estudiado, detectándose los valores más elevados de estos compuestos en la zona más superficial (0-5 cm) del sistema de no laboreo (NT). Se observó un efecto residual de la aplicación de la atrazina en el suelo, ya que se detectó el metabolito hidroxiatrazina en la capa arable, a ambas profundidades (0-5 cm y 5-20 cm), incluso un año después de la aplicación del herbicida. Los valores de las actividades enzimáticas del suelo en la zona mas superficial (0-5 cm) bajo no laboreo (NT) fueron considerablemente más elevados que los encontrados en la misma capa bajo laboreo convencional (CT). Los valores de las actividades enzimáticas ureasa y β-glucosidasa descendieron a medida que aumentaba la profundidad en el suelo sometido a no laboreo (NT), pero no variaron con la profundidad en el suelo sometido a laboreo convencional (CT). En ambos sistemas de cultivo las actividades enzimáticas también reflejaron cambios temporales durante el cultivo del maíz; sin embargo, no se observó un efecto consistente de la aplicación del herbicida.

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Atrazine degradation and enzyme activities in an agricultural soil under two tillage systems

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Abstract

The content of atrazine and its metabolites (hydroxyatrazine, deethylatrazine and deisopropylatrazine) as well as the activities of two soil enzymes (urease and β-glucosidase) were evaluated in an acid agricultural soil, located in a temperate humid zone (Galicia, NW Spain), with an annual ryegrass–maize rotation under conventional tillage (CT) and no tillage (NT). Samples were collected during two consecutive years from the arable layer at two depths (0–5 cm and 5–20 cm) and different times after atrazine application. Hydroxyatrazine and deisopropylatrazine were the main metabolites resulting from atrazine degradation in the acid soil studied, the highest levels being detected in the surface layer of the NT treatment. A residual effect of atrazine was observed since hydroxyatrazine was detected in the arable layer (0–5 cm, 5–20 cm) even one year after the herbicide application. Soil enzyme activities in the upper 5 cm layer under NT were consistently higher than those in the same layer under CT. Urease and β-glucosidase activities decreased with depth in the profile under NT but they did not show any differences between the two depths for the plots under CT. For both tillage systems enzyme activities also reflected temporal changes during the maize cultivation; however, no consistent effect of the herbicide application was observed.

Keywords: Atrazine and its metabolites; Urease; β-glucosidase; Conventional tillage; No tillage

1. Introduction

For decades, the atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) has been widely used all over the world to control a variety of broadleaf and grass weeds in agriculture and forestry. The persistence of atrazine and its movement through soil are key factors influencing its potential to contaminate aquifers and groundwater. Despite its moderate water solubility, atrazine has a high mobility in soils, which results in the contamination of soil (Winkelmann and Klaine, 1991), surface and groundwater (Pick et al., 1992), rainwater (Buser, 1990) and tile drain water (Muir and Baker, 1976); thus, frequently, atrazine levels in water exceed the maximum level for drinking water which is 0.1 μg L^{-1} in Europe and 3 μg L^{-1} in USA. The presence of this chemical compound on the environment and its toxicological properties are being taken into account by regulatory bodies in EU countries, leading to increased restrictions on its use or to its banning.

Atrazine is considered a moderately persistent chemical in the environment with a half-life ranging from a few days to months (Khan and Saidak, 1981; Jones et al., 1982), depending on soil properties, prior application history and agricultural practices (Koskinen and Clay, 1998). However, residues of both the parent compound and its degradation products were detected in

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soils years after atrazine application (Schiavon, 1988). Biotic transformation is considered a major route whereby atrazine is decomposed in most soils (Kaufman and Kearney, 1970; Barriuso and Houot, 1996); however, the dechlorination of atrazine to hydroxyatrazine (chemical hydrolysis) has been reported as an important pathway of its dissipation in acid soils (Da Silva et al., 2000; Pimentel and Rosim, 2000). Therefore, atrazine degradation in soils occurs both via chemical and biological processes, resulting in the formation of metabolites such as hydroxyatrazine, deethylatrazine and deisopropylatrazine, which have a different mobility and toxicity than atrazine and hence also different contamination potential (Barrett, 1996). The presence of these xenobiotic compounds can modify drastically soil microbial communities thus altering the normal functioning of terrestrial ecosystems, which in turn has important implications for soil fertility and quality (Voets et al., 1974; Greaves, 1982; Schäffer, 1993).

In Galicia (NW Spain), there is a high potential risk of soil and aquifer contamination with xenobiotics of *s*-triazines group (aromatic heterocyclic ring with three N atoms in alternating positions), such as atrazine, due to the widespread use of these compounds in agricultural soils, the climatic conditions (abundant precipitation and low temperature) and soil characteristics (sandy texture, acid pH, high organic matter content, low microbial activity). However, despite its interest, there is no information concerning the impact of *s*-triazines on soil ecosystems. The aim of this work was to study the impact by the atrazine application in an agricultural soil from Galicia under two tillage systems, by determining the content of atrazine and its degradation products and by measuring functional parameters such as enzyme activities specific of N cycle (urease) or C cycle (β-glucosidase).

2. Materials and methods

The experimental soil is a Gleyic Cambisol with a sandy loam topsoil located in the Gayoso-Castro farm (43°06′N, 7°27′W, 420 m a.s.l.) at Castro de Ribeiras de Lea (Galicia, NW Spain). Since 1994, the same annual ryegrass–maize rotation has been cultivated under two tillage systems, conventional tillage (CT) and no tillage (NT), in a completely randomised block design with four replications and 2 m separation established around each plot (20 m × 5 m). Silage corn is being sown in rows 0.7 m apart in late May and harvested in September. Before sowing, plants established in the NT treatments were destroyed with glyphosate application at a dose of 5 L ha^{-1} whereas in the CT treatments they were discarded by ploughing at 25–30 cm. Further, pre-emergence agrochemicals applications were identical for both treatments (CT, NT): one mixture of herbicides (Harness, ai 33% acetachlor and 16.5% atrazine, at a rate of 4 L ha^{-1} in 2002 and Harness, ai 35% acetachlor and 20% atrazine, at rate of 7 L ha^{-1} in 2003), insecticide (Panda 48 LE, ai 48% clorpiriphos, 0.33 L ha^{-1}) and 12–12–24 NPK (700 kg ha^{-1}). In 2003, the NT treatment received an additional post-emergence application of the herbicides mixture (Harness, ai 35% acetachlor and 20% atrazine, at a rate of 7 L ha^{-1}) for controlling broadleaf and grassy weeds. Measurements of chemical and biochemical properties were carried out on all soil samples collected before sowing (0 time) and at different time intervals during the maize cropping (1, 2, 4, 12 and 16 weeks after sowing) in two consecutive years (2002, 2003). In each sampling plot, samples were taken at 0–5 cm depth from 16 points uniformly distributed in the central rows between the maize plants and mixed to obtain a composite sample per plot. In addition, samples were also collected in 2003 at 5–20 cm depth before sowing (0 time) and 16 weeks after sowing. After sieving at 4 mm, the homogenized soil samples were stored at 4 °C prior to further analyses of biochemical properties. The main characteristics of the soil studied are shown in Table 1.

Table 1
Main characteristics of a cultivated soil under conventional (CT) or no-tillage (NT) management

Sampling time year	Soil depth	Treatment	pH H_2O	Total C g kg^{-1}	Total N g kg^{-1}
2002	0–5 cm	NT	5.61±0.11	55.55±0.21	3.65±0.02
		CT	5.85±0.12	40.35±0.97	2.85±0.06
2003	0–5 cm	NT	5.16±0.05	57.05±0.91	3.70±0.05
		CT	5.47±0.08	41.97±1.24	2.91±0.10
	5 – 20 cm	NT	5.27±0.05	43.50±1.55	2.90±0.06
		CT	5.52±0.06	41.15±0.06	2.81±0.09

Mean values±SE of the values obtained at different sampling times.

The contents of atrazine and its metabolites in the soil samples were measured following the procedure described by Ghani et al. (1996) with some modifications. Selection of the extractant was made by mixing soil samples (5 g) with 10 mL of three different extractants, 10 mM $CaCl_2$, acetonitrile:water (9:1) and methanol:water (7:3) and shaking them for 24 h on an end-over-end shaker at the ambient temperature. Since the recovery of the added atrazine (73±3%, 88±3% and 91±3% for $CaCl_2$, acetonitrile:water and methanol: water, respectively) was the highest for the mixture methanol:water, this was the chosen extractant for the subsequent analyses. After centrifugation at 2500 rpm

for 11 min, the soil suspensions were filtered through 0.2 μm glass fibre filters and the extracts were stored at 4 °C until analysis. The quantification of atrazine and its metabolites (hydroxyatrazine, deethylatrazine and deisopropylatrazine) in the extracts was performed on an Agilent high-performance liquid chromatography (HPLC) system equipped with a C18 column of 5 μm and 150×4.6 mm and a C18 precolumn of 5 μm and 20×3.9 mm and a diode array detector set at 230 nm wave-length. The injected volume was 50 μL and the mobile phase was a two-solvent gradient of acetonitrile: water delivered at a flow rate of 1.5 mL min^{-1} as follows: from 0 to 2 min, 20% acetonitrile+80% water, from 2 to 6 min, 10% acetonitrile+90% water and from 6 to 23 min, 60% acetonitrile+40% water. These conditions allowed the detection of atrazine, hydroxyatrazine, deethylatrazine and deisopropylatrazine (retention times = 11.2, 1.4, 2.6 and 1.8 min, respectively). The content of atrazine and its metabolites in the soil samples was determined by the external standard method, using a standard mixture of atrazine, hydroxyatrazine, deethylatrazine and deisopropylatrazine in the concentration range of 0.1 to 5 μg mL^{-1} for each analyzed compound.

The activities of two hydrolases, urease and β-glucosidase, were measured as indicators of soil metabolic activity. The urease was chosen for its role in releasing inorganic N in the N cycle and β-glucosidase for its critical role in releasing low molecular weight sugars that are important sources of energy for microorganisms. Urease activity was estimated by incubating the soil samples with an aqueous urea solution and extracting the NH_4^+ with 1 M KCl and 0.01 M HCl followed by NH_4^+ colorimetric determination by a

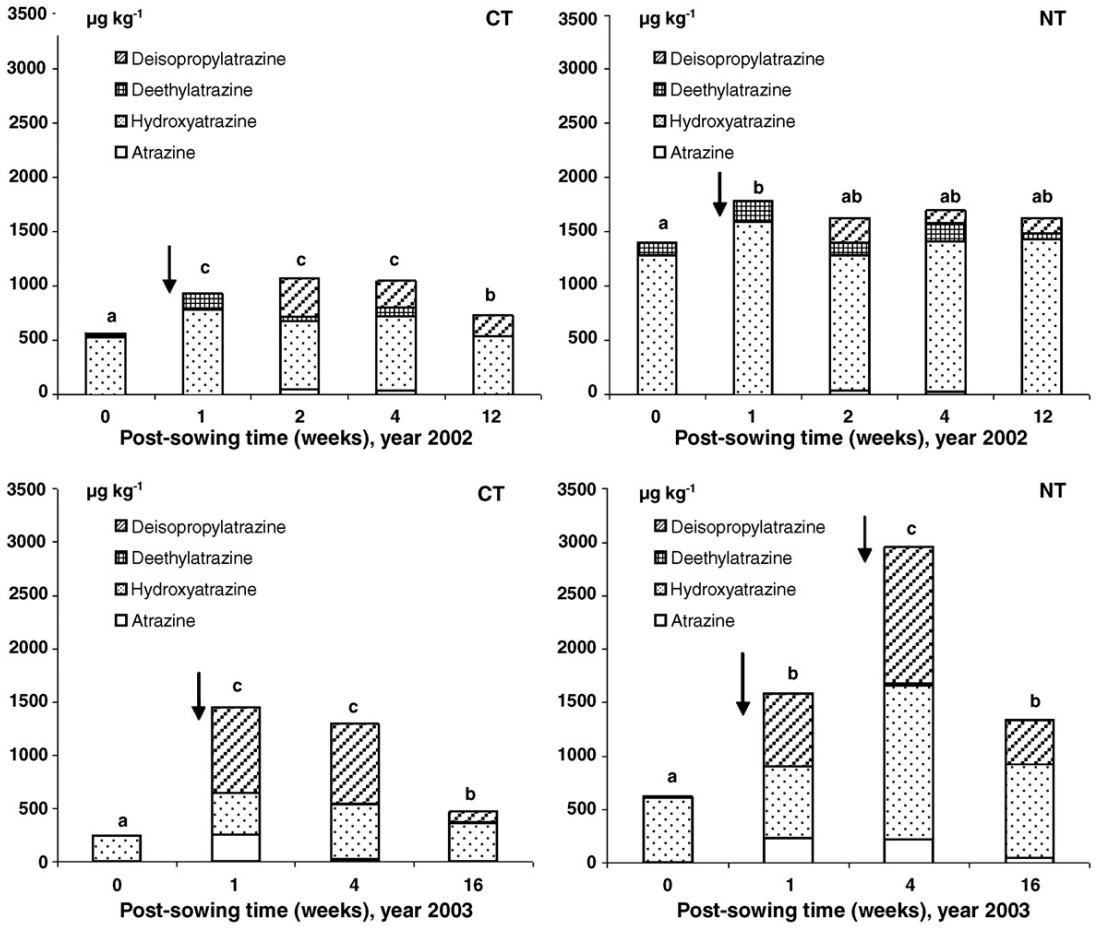

Fig. 1. Atrazine and its metabolite concentrations in the 0–5 cm layer of a soil under conventional tillage (CT) and no-tillage (NT) management at different sampling times (0, 1, 2, 4, 12 and 16 weeks) after maize sowing and atrazine application (↓). Different letters indicate significant differences ($P<0.05$) in the total content of the extractable compounds.

modified indophenol reaction (Kandeler and Gerber, 1988). The β-glucosidase activity was assessed following the procedure described by Eivazi and Tabatabai (1988), which determines the released *p*-nitrophenol after incubation of the soil with *p*-nitrophenyl-β-D-glucopyranoside solution for 3 h at 37 °C.

All results were obtained by triplicate determinations and were expressed on the basis of oven-dry (105 °C) weight of soil. Data obtained for the different field plots (4 replicates per treatment) were statistically treated by one-way analysis of variance (ANOVA 1) and, in cases of significant *F* statistic, Tukey's minimum significant difference test was used to separate the means ($P<0.05$). The percentage of data variation attributable to soil management and sampling time was calculated using two-way analysis of variance (ANOVA 2). All statistical analyses were performed using the SPSS program.

3. Results and discussion

The contents of atrazine and its metabolites in the 0–5 cm soil samples are shown in Fig. 1. Relatively high concentrations of these xenobiotic compounds were detected in the extractable fraction for all sampling times during the two growing seasons. The atrazine values ranged from 0 to 52 μg kg^{-1} (14±8 μg kg^{-1} and 18±12 μg kg^{-1}, mean values±SE of data obtained at different sampling times (the same below) in NT and CT treatments, respectively) in 2002 and from 0 to 253 μg kg^{-1} (130±60 μg kg^{-1} and 70±62 μg kg^{-1} for NT and CT treatments, respectively) in 2003. In 2002 the atrazine metabolites exhibited values between 525 and 1594 μg hydroxyatrazine kg^{-1} (1387±62 μg kg^{-1} in NT and 629±47 μg kg^{-1} in CT), between 0 and 192 μg deethylatrazine kg^{-1} (129±24 μg kg^{-1} in NT and 60±25 μg kg^{-1} in CT treatments) and between 0 and 344 μg deisopropylatrazine kg^{-1} (99±45 μg kg^{-1} in NT and 157±67 μg kg^{-1} in CT treatments). In 2003 higher contents of atrazine and deisopropylatrazine and lower concentrations of hydroxyatrazine and deethylatrazine were detected since the values varied from 237 to 1439 μg hydroxyatrazine kg^{-1} (894±191 μg kg^{-1} in NT and 375±56 μg kg^{-1} in CT), from 0 to 19 μg deethylatrazine kg^{-1} (8±4 μg kg^{-1} in NT and 2±2 μg kg^{-1} in CT) and from 1 to 1275 μg deisopropylatrazine kg^{-1} (594±265 μg kg^{-1} in NT and 415±212 μg kg^{-1} in CT).

During the maize cultivation marked fluctuations in the amounts of atrazine and degradation products were observed (Fig. 1). Hydroxyatrazine was the main metabolite detected in the soils before maize sowing; thus, although a significant dissipation of this metabolite occurred between the two consecutive growing seasons (the levels in 2002 were two times higher than those in 2003), the high range of values indicated a long persistence of this atrazine residue in soils. As expected, atrazine application to the soil increased notably the content of atrazine and its metabolites in the extractable fraction, particularly in 2003 when higher doses were used. Evolution of these xenobiotic compounds during the maize cultivation showed that the atrazine content tended to diminish and its metabolites, particularly hydroxyatrazine and deisopropylatrazine, tended to increase, whereas significant amounts of deethlyatrazine were only detected during maize cultivation in 2002. The results obtained also indicated that the route of atrazine degradation and hence the proportion of the dealkylated metabolites, derived from biotic processes (deethylatrazine and deisopropylatrazine), and the dechlorinated metabolite, mainly derived from abiotic processes (hydroxyatrazine), can differ between years. This may be due to differences in the type and activity of microorganisms involved in atrazine degradation (e.g. fluctuations in climatic conditions, substrate availability, etc.) rather than to differences in the soil physico-chemical properties (e.g. soil pH and organic matter content, Table 1). It is necessary to point out that only 19–45% in 2002 and 10–51% in 2003 of the atrazine originally applied to the soil became extractable (atrazine plus metabolites) over a 3–4 month period. These values of extractable compounds was in the reported range given for different soils with similar atrazine application dose (Gan et al., 1996; Gaynor et al., 1998; Chung and Alexander, 2002). The results suggested the existence of "bound" or non-extractable atrazine residues and hence a latent source of pollution due to various factors that can cause their remobilization in the soil solution. Quantification of the non-extractable fraction may be of great interest in this acid soil rich in organic matter with 8–9 years history of atrazine application since usually bound residues are closely related to the soil organic fraction and in addition they tended to increase with atrazine–soil reaction time (Ma and Selim, 1996).

In general, the content of atrazine and its metabolites was higher ($P<0.001$) in the NT than in the CT treatment (Fig. 1), which is consistent with the higher application concentrations of atrazine in the NT system (a pre-emergence and additionally a post-emergence dose when it was required) as well as with the surface herbicide application, organic residues accumulation and no mixing of the soil in these systems. Thus, for instance, hydroxyatrazine could be more strongly adsorbed on the 0–5 cm layer of the NT than in the CT treatment due to its higher organic matter content. The three degradation

Table 2
Distribution of atrazine and its metabolites and soil enzyme activities (mean values±SE of four field replicates) in the 0–5 cm and 5–20 cm depth layers of a cultivated soil under conventional tillage (CT) and no-tillage (NT) management at two sampling times in 2003 (before and 16 weeks after maize sowing and atrazine application)

	Sampling time weeks	CT		NT	
		0–5 cm	5–20 cm	0–5 cm	5–20 cm
Atrazine (μg kg^{-1})	0	0±0	9±4	7±4	1±1
	16	0±0	0±0	50±37	36±37
Hydroxyatrazine (μg kg^{-1})	0	237±13	279±44	601±90	269±23
	16	367±43	353±40	873±72	360±23
Deethylatrazine (μg kg^{-1})	0	0±0	0±0	3±3	0±0
	16	1±1	1±1	4±2	4±2
Deisopropylatrazine (μg kg^{-1})	0	1±1	61±43	14±6	8±3
	16	97±28	1±1	407±111	26±22
Urease (μg NH_4^+ g^{-1} h^{-1})	0	72±28	75±11	135±16	57±14
	16	67±1	65±10	108±14	58±16
Glucosidase (μg *p*-nitrophenol g^{-1} h^{-1})	0	325±33	322±11	759±95	242±14
	16	397±63	410±96	797±209	313±88

products studied were detected in this acid soil although hydroxyatrazine and deisopropylatrazine were the predominant metabolites. In agreement with the findings of Accinelli et al. (2002), results indicated that both biological and chemical degradation occurred, although the presence of high hydroxyatrazine concentrations confirmed that chemical hydrolysis is an important pathway of atrazine dissipation in acid soils (Da Silva et al., 2000; Pimentel and Rosim, 2000). In general, the atrazine was rapidly dissipated from the soil, which has important implications in its effectiveness to control weed grass, but its environmental impact persisted over

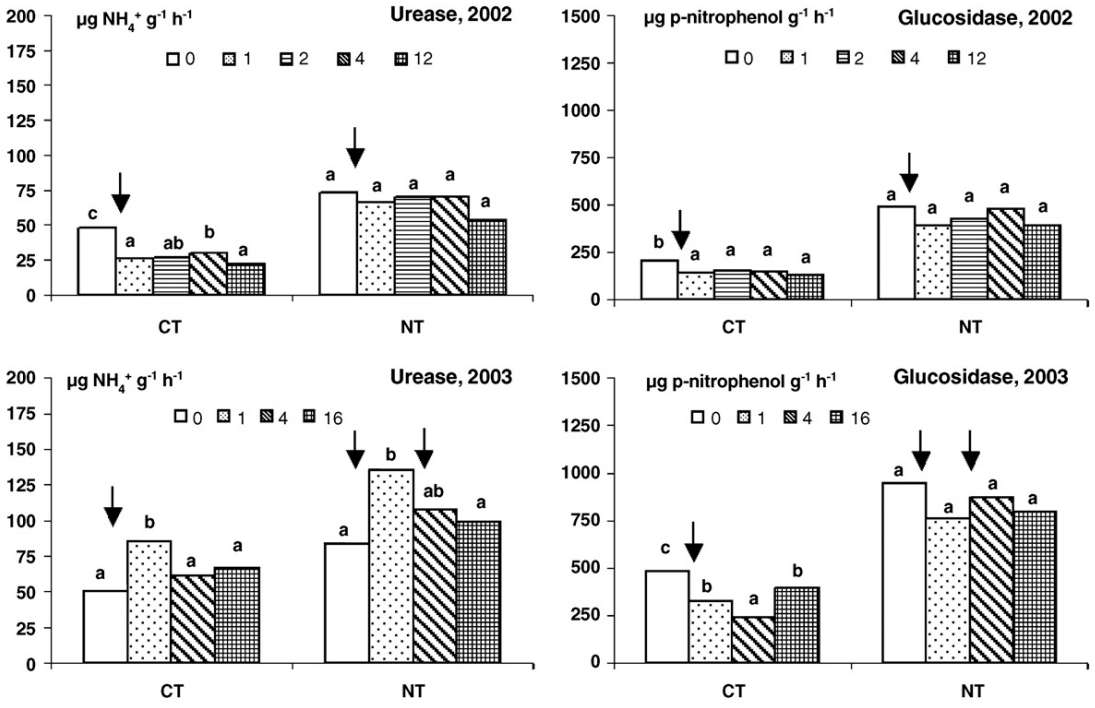

Fig. 2. Enzyme activities in the 0–5 cm layer of a cultivated soil under conventional tillage (CT) and no-tillage (NT) management at different sampling times (0, 1, 2, 4, 12 and 16 weeks) after maize sowing and atrazine application (↓). For each soil management different letters indicate significant differences ($P<0.05$).

the time since an increase of the degradation products (hydroxyatrazine and deisopropylatrazine) was detected 12–16 weeks after sowing when compared to their levels in the soil before sowing. Analysis of the degradation products rather than that of the parent compound is hence necessary to evaluate the atrazine environmental impact. Phytotoxicity is destroyed by hydroxylation but not by dealkylation (Kaufman and Blake, 1970); consequently, the results also indicated that the use of atrazine in this temperate humid acid soil under no tillage should be questioned since any successful approach to conservation tillage must be profitable and sustainable, protect our soil and water resources, and be environmentally sound (Blevins and Frye, 1993). Further studies performed with a wide range of soils, including the measurement of extractable and non-extractable fractions ("bound residues") of these compounds in soil surface and deeper layers and in groundwaters, are needed to evaluate the potential contamination problems.

The distribution of these xenobiotic compounds throughout the soil profile differed depending on the tillage system (Table 2). For the NT treatment, the content of hydroxyatrazine in the 0–5 cm layer was 2.2–2.4 times higher than that in the 5–20 cm layer whereas no depth effect was observed in the CT treatment. The content of deisopropylatrazine also varied with soil depth, although in this case a non-consistent trend was observed, particularly for the CT treatment. These results can be explained again on the basis of soil properties stratification in NT systems and no stratification or uniform distribution in CT treatments (Unger, 1990; Blevins and Frye, 1993) as well as on the different mobility of the xenobiotic compounds analyzed, the hydroxyatrazine being less mobile and the dealkylated metabolites more mobile than atrazine (Barrett, 1996). The presence of hydroxyatrazine into the soil profile is difficult to explain due to its low solubility and high adsorption to soil; therefore its penetration as atrazine in the deeper layers and further transformation *in situ* cannot be discarded (Muir and Baker, 1978; Ma and Selim, 1996). The detection of substantial concentrations of deethylatrazine (28–117 $\mu g\ kg^{-1}$) and hydroxyatrazine (250–1550 $\mu g\ kg^{-1}$) in the 0–5 and 5–20 cm layers of both NT and CT plots 1 year after atrazine application (Fig. 1 and Table 2) showed its residual effect and the high potential risk of surface and groundwater contamination.

The evolution of the enzyme activities in the 0–5 cm soil samples during the two consecutive years is shown in Fig. 2. In 2002 the urease activity values ranged from 21 to 70 $\mu g\ NH_4^+\ g^{-1}\ h^{-1}$ ($67 \pm 4\ \mu g\ NH_4^+\ g^{-1}\ h^{-1}$ and $34 \pm 4\ \mu g\ NH_4^+\ g^{-1}\ h^{-1}$, for NT and CT treatments, respectively) and those of the β-glucosidase activity from 130 to 491 μg *p*-nitrophenol $g^{-1}\ h^{-1}$ ($437 \pm 21\ \mu g$ *p*-nitrophenol $g^{-1}\ h^{-1}$ for NT and $153 \pm 14\ \mu g$ *p*-nitrophenol $g^{-1}\ h^{-1}$ for CT treatments). The two-way analysis of variance showed a significant effect of the two factors considered, tillage system and sampling time, which were independent as indicated by the non-significant effect of the interaction between them (data not shown). The tillage system explained most of the variance (72–85%), and the sampling time accounted only for a 4–12% of the variation. Values obtained in 2003 were 1.6–2.3 times higher than those found in 2002 with a range of 50–135 $\mu g\ NH_4^+\ g^{-1}\ h^{-1}$ ($106 \pm 10\ \mu g\ NH_4^+\ g^{-1}\ h^{-1}$ for NT and $66 \pm 8\ \mu g\ NH_4^+\ g^{-1}\ h^{-1}$ for CT treatments) for urease activity and 240–944 μg *p*-nitrophenol $g^{-1}\ h^{-1}$ ($842 \pm 41\ \mu g\ NH_4^+\ g^{-1}\ h^{-1}$ for NT and $360 \pm 51\ \mu g\ NH_4^+\ g^{-1}\ h^{-1}$ for CT treatments) for glucosidase activity. Again, variation in enzyme values was largely explained by the tillage system (52–72%), but the importance of the sampling time decreased for the glucosidase activity (not significant) and increased for the urease activity (32% of variance) while the interaction between these factors was again not significant. Differences in the results obtained for the two sampling years (2002 and 2003) were probably due to the different climatic conditions.

A significantly positive relationship between glucosidase and urease activities was observed ($r = 0.82$, $P < 0.001$, $n = 24$). The intercorrelation between these two enzyme activities indicated that they responded similarly to the management and land use systems and emphasized the interdependence of the activity associated with the biogeochemical cycles of C (β-glucosidase) and N (urease). Comparison of the enzyme activity values at all sampling dates showed significant differences ($P < 0.05$) between the two tillage systems, the values in the NT treatment being 1.5–2.5 and 2–3.6 times higher than those in the CT treatment for the urease and glucosidase, respectively (Fig. 2). This can be partly explained by differences derived from the influence of the tillage system used on soil temperature and organic matter content and hence in substrate supply and related soil physico-chemical properties (Doran, 1987; Biederbeck et al., 1994). The positive correlations between soil organic matter content and enzyme activities ($r = 0.68$ and 0.79 for urease and glucosidase, respectively, $P < 0.001$, $n = 20$) and the accumulation of crop residues in the soil surface (the total C and total N values in the 0–5 cm layer of the CT treatment being 70–80% of those in the NT system; Table 1), promoted by conservation tillage management, seem to support that assumption. Our results therefore indicated that

urease and glucosidase activities are able to discriminate between tillage systems and that the latter is more sensitive to detect soil disturbances. This is consistent with the results of Bandick and Dick (1999) who investigating the effects of field soil management on eleven enzyme activities found different sensitivities of enzymes to soil treatments, β-glucosidase being the better indicator of soil quality changes.

The glucosidase and urease activities showed similar patterns of behaviour related to depth in the soils under the different tillage treatment (Table 2). In the NT treatment, for both sampling times, the values in the 0–5 cm layer were always higher than those in the 5–20 cm layer, and although the effect of depth was more pronounced for glucosidase (decrease of 1.6–2.1 times) than for urease (decrease of 0.9–1.4 times), it was always significant ($P<0.001$) for the two parameters assayed. In contrast, in the CT treatment, urease and glucosidase activities did not show significant differences between the two soil layers (0–5 cm and 5–20 cm). This is in accordance with studies of diverse authors who also found a stratification of enzymes in soils under no tillage or reduced tillage and no stratification in soils under conventional tillage (Dick, 1992; Kandeler et al., 1999). Again, the content of soil organic matter and associated physico-chemical properties can partly explain this behaviour (Table 1).

Marked differences for the enzyme activities related with the time of sampling were evident (Fig. 2). For example, values for all activities (except for urease in 2003) were higher in samples collected before sowing and atrazine application (0 time) than in the rest of samples collected at different times after sowing and atrazine application. These variations found over time are partly due to atrazine application although other factors such as ploughing, addition of other agrochemicals (glyphosate, alachlor, acetachlor, clorpiriphos), availability of substrate derived from roots or from material incorporated to the soil and climatic conditions (temperature, moisture) cannot be discarded. For the CT treatment an initial and significant decrease of 30–45% ($P<0.001$) in the activity levels was observed 1 week after atrazine application and thereafter values hardly changed over time, whereas for the NT treatment the decrease was of lower magnitude (10–20%) and not significant. Atrazine dose was higher in NT treatment than in CT treatment since in some years (e.g. 2003) in addition to the first pre-emergence application a post-emergence application was added to control grass weeds. Therefore, the different effects observed in the soil under two tillage systems can be explained by the atrazine application history, the microorganisms of the soil under NT treatment being more adapted to atrazine and hence showing a lower response than those of the soil under CT treatment. This is in accordance with studies of several authors who found a different response of soil microorganisms to atrazine addition in non-adapted and adapted soils (Barriuso and Houot, 1996; Abdelhafid et al., 2000). In contrast to these negative atrazine effects, urease activity values in 2003 showed a significant increase of 60–69% with respect to the initial values 1 week after atrazine application and then values declined until reaching similar levels than those presented by the soil before atrazine application.

The obtained results suggest that the two enzymes studied are unsuitable means of assessing the effects of atrazine on these soil ecosystems since inconsistent adverse or stimulatory effects were observed depending on the enzyme activity measured and the sampling year. This is in accordance with numerous field and laboratory studies cited in the literature showing conflicting and contradictory results due to the difficulty of establishing the cause-and-effect relationships between pesticides and enzyme activities (Davies and Greaves, 1981; Schäffer, 1993; Gianfreda et al., 1994; Sannino and Gianfreda, 2001; Accinelli et al., 2002; Haney et al., 2002). Since atrazine residues can persist in this acid soil over time periods up to 1 year after the last herbicide application, it is probably that soil microorganisms could be damaged. Work is now in progress in our laboratory to evaluate the environmental impact of atrazine application examining, both under laboratory and field conditions, extractable and non-extractable (bound) atrazine residues in the soil profile as well as microbial response to the presence of these xenobiotic compounds measuring other parameters than enzyme activities such as microbial biomass, soil respiration and microbial community composition (PLFA pattern).

In conclusion, our results confirmed that the degradation of atrazine and the movement of parent compound and its degradation products in the soil profile are important aspects of their behaviour and indicated that atrazine application to acid soils from temperate humid zones should be performed with caution since atrazine residues can persist in the soil over time periods up to 1 year. The results also showed that urease and β-glucosidase activities are not good indices for assessing the environmental impact of atrazine application in these soils.

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ANEXO II

ANEXO II

Atrazina y metabolitos extraíbles en suelos agrícolas de la zona templado-húmeda

J. Mahía, A. Martín y M. Díaz-Raviña

Resumen

Se estudió, en condiciones de laboratorio, la fracción extraíble de la atrazina y de sus metabolitos (hidroxiatrazina, desetilatrazina y desisopropilatrazina) en cinco suelos agrícolas de la zona templado-húmeda (Galicia, NO. de España), con diferentes propiedades (carbono orgánico, textura e historial de aplicación de atrazina), adicionados o no adicionados con atrazina a dosis normales de aplicación en campo. La medida de los compuestos de descomposición de la atrazina se realizó a diferentes intervalos de tiempo (1, 3, 6, 9 y 12 semanas) durante un período de incubación de 3 meses. Los resultados mostraron que el único metabolito detectado en la fracción extraíble de los suelos no adicionados con atrazina fue la hidroxiatrazina, observándose valores prácticamente constantes a lo largo de todo el periodo de incubación. La adición de atrazina provocó un notable incremento de la concentración del herbicida y de los productos de su degradación en la fracción extraíble. Los principales metabolitos detectados en esta fracción fueron la desisopropilatrazina y la hidroxiatrazina, mientras que no se detectó desetilatrazina. Después de 7 días de incubación, el valor total de los residuos extraíbles, expresado en porcentaje de la atrazina añadida inicialmente, varió entre el 75 y el 86 %, encontrándose el 25-68 % de la atrazina inicial en forma de atrazina, el 7-11 % en forma de hidroxiatrazina y el 9-57 % como desisopropilatrazina. Este valor total descendió rápidamente durante las 3 primeras semanas de incubación, mostrando cifras del 2-8 % en suelos con un amplio historial de aplicación de atrazina y del 28-30 % en suelos con un bajo historial de aplicación. Al final de la incubación, todavía se detectó un 2-8 % de residuos extraíbles (0-4 % de atrazina, 2-3 % de hidroxiatrazina y 0-2 % de deisopropilatrazina), lo que indica un efecto residual de la adición de atrazina. Estas variaciones en la fracción extraíble indican que la mayor parte de la atrazina añadida fue degradada rápidamente, especialmente en suelos con un amplio historial de aplicación del herbicida.

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ORIGINAL PAPER

Extractable atrazine and its metabolites in agricultural soils from the temperate humid zone

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Abstract Extractable atrazine and its metabolites (hydroxyatrazine, deethylatrazine and deisopropylatrazine) were evaluated in agricultural soils from the temperate humid zone (Galicia, NW Spain) under laboratory conditions. The experiment was performed with five soils with different properties (organic C, soil texture and atrazine application history), both unamended and treated with atrazine at field application rate. Measurements of the atrazine compounds were made at different time intervals (1, 3, 6, 9 and 12 weeks) during a 3-month incubation period. Results showed that only hydroxyatrazine was detected in the extractable fraction of the unamended soils, with values remaining relatively constant throughout the incubation period. Atrazine addition notably increased the concentration of the parent compound and its degradation products; deisopropylatrazine and hydroxyatrazine were the main metabolites detected in the extractable fraction of the treated soils, whereas deethylatrazine was not detected. After 7 days incubation, values of total extractable residues, expressed as percentage of initially added atrazine, ranged from 75 to 86% (25–68% of atrazine, 7–11% of hydroxyatrazine and 9–57% of deisopropylatrazine). The values decreased rapidly during the first 3 weeks of incubation, showing values of 2–8% in soils with higher atrazine application and from 28 to 30% in soils with lower application history. At the end of the incubation, 2–8% of total extractable residues were still detected (0–4% of atrazine, 2–3% of hydroxyatrazine and 0–2% of deisopropylatrazine), indicating a residual effect of atrazine addition. These variations in the extractable fraction indicated that most added atrazine was rapidly degraded, especially in soils with higher application history.

Keywords Atrazine · Agricultural soils · Degradation products · Extractable residues

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Introduction

It is well known that atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine), one of the most widely used herbicides, can be chemically and microbially degraded (Kaufman and Kearney 1970; Erickson and Lee 1989; Blumhorst and Weber 1994; Barriuso and Houot 1996), and its chemical and microbial degradation products are frequently present in soils (Dao et al. 1979; Gaynor et al. 1998; Da Silva et al. 2000; Pimentel and Rosim 2000; Mahía et al. 2007). The presence of atrazine and its three metabolites, deethylatrazine (DEA, 2-amino-4-chloro-6-isopropylamino-*s*-triazine), deisopropylatrazine (DIA, 2-amino-4-chloro-6-ethylamino-*s*-triazine) and hydroxyatrazine (HA, 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine), have also been

shown in several field-monitoring studies of surface and ground waters (Poinke and Glotfeltly 1990; Thurman et al. 1994; Du Preez et al. 2005). Characterization and information relating to persistence of the parent compound and its metabolites are important in understanding the fate of atrazine in terrestrial ecosystems as well as for determining potential risks for soil and groundwater contamination, as some degradation products are nearly as toxic as atrazine (Kaufman and Kearney 1970).

Because of its widespread, repeated use in some areas of Galicia (NW Spain, temperate humid zone), soil and water contamination with atrazine has occurred, favoured by climatic conditions (abundant precipitation and low temperature) and soil properties (sandy texture, acid pH, high organic matter content, low microbial activity). However, there is no information concerning the atrazine fraction in soil that is available for degradation, leaching and surface runoff processes. The aim of the present study was to determine, under laboratory conditions, the content of extractable atrazine and its degradation products following atrazine application at normal field rates in different Galician agricultural soils.

Materials and methods

Five contrasting agricultural soils (MP, G, ME, MA and C), developed over different parent material (granite, sediments, granite, acid schists and amphibolite, respectively) with a history of 10–40 years of annual maize cropping and atrazine application (MP, 1964–2004; G, 1994–2004; ME, 1964–1994; MA, 1984–2004; C, 1996–2004), located in the temperate humid zone (Galicia, NW Spain) were used. Soil subsamples, collected randomly from each site in spring 2005 before maize planting, from the top 15 cm of the A horizon, were mixed, sieved (<2 mm) and thoroughly homogenized. Soil pH (H_2O) values ranged from 5.6 to 5.9, but other properties (organic matter, total N and soil texture) differed among soils (MP: 24.84 g C kg^{-1}, 2.39 g N kg^{-1}, 12% clay, 14% silt, 74% sand; G, 41.12 g C kg^{-1}, 2.79 g N kg^{-1}, 27% clay, 9% silt, 64% sand; ME: 41.91 g C kg^{-1}, 3.14 g N kg^{-1}, 13% clay, 12% silt, 75% sand; MA: 16.51 g C kg^{-1}, 1.51 g N kg^{-1}, 19% clay, 51% silt, 30% sand; C: 26.41 g C kg^{-1}, 2.20 g N kg^{-1}, 19% clay, 41% silt, 40% sand).

The incubation experiment was in temperature-controlled conditions (28°C in darkness) using replicates of 50 g oven-dried soil at a constant moisture level (85% of water holding capacity). The soils were treated with atrazine at the recommended application rate (8 ml of an atrazine solution of 30 mg l^{-1} to give a final concentration of 5 mg herbicide kg^{-1} soil). The same volume of distilled water was added to the unamended control soils that were then incubated under the same conditions. Measurements of atrazine and its metabolites were carried out at 1, 2, 4, 6, 9 and 12 weeks of incubation. Since samples were destructively treated and three replicates were used for each treatment–time combination, a total of 30 replicates of each soil (15 untreated and 15 treated with atrazine) were prepared. Concentrations of atrazine and its metabolites in the soil samples were measured in duplicate following the procedure described by Ghani et al. (1996) with some modifications. Soil samples (5 g) were extracted with 10 ml of a mixture of methanol and water (7:3) by shaking for 24 h on an orbital shaker at ambient temperature. After centrifugation at 2,500 rpm for 11 min, the soil suspensions were filtered through 0.2-μm glass fibre filters, and the extracts were stored at 4°C prior to analysis. Quantification of atrazine and its metabolites (hydroxyatrazine, deethylatrazine and deisopropylatrazine) in the extracts was performed on an Agilent high-performance liquid chromatography (HPLC) system equipped with a C18 column of 5 μm and 150 × 4.6 mm, a C18 precolumn of 5 μm and 20 × 3.9 mm and a diode array detector set at 230-nm wave-length. The injected volume was 50 μl, and the mobile phase was a two-solvent gradient of acetonitrile:water delivered at a flow rate of 1.5 ml min^{-1} as follows: from 0 to 2 min, 20% acetonitrile + 80% water, from 2 to 6 min, 10% acetonitrile + 90% water and from 6 to 23 min, 60% acetonitrile + 40% water. These conditions allowed the detection of atrazine, hydroxyatrazine, deethylatrazine and deisopropylatrazine (retention times = 11.2, 1.4, 2.6 and 1.8 min, respectively). The content of atrazine and its metabolites in the soil samples was determined by the external standard method, using a standard mixture of atrazine, hydroxyatrazine, deethylatrazine and deisopropylatrazine in a concentration range from 0.1 to 5 μg ml^{-1} for each analysed compound. The values were expressed as absolute values in both untreated and atrazine-treated soils (μg kg^{-1} dry soil). The

evolution of extractable atrazine residues following addition to soils was studied by subtracting values obtained in unamended soils (only hydroxyatrazine was detected) from the corresponding treated soils and expressing these as percentages of the amounts added (5 mg atrazine kg^{-1} soil).

Results and discussion

The content of atrazine and its metabolites obtained from untreated and atrazine-treated soils at different incubation times is showed in Table 1. Hydroxyatrazine was the only intermediate atrazine product detected in the extractable fraction from untreated soils during the 3-month incubation period; values were quite similar among soils and varied from 111 to 194 μg kg^{-1} (atrazine, deethylatrazine and deisopropylatrazine levels were below detectable limits). Incubation hardly changed the hydroxyatrazine values; thus, the hydroxyatrazine content tended to initially increase, showing the highest concentrations after 3 or 6 weeks' incubation. Thereafter it decreased until at the end of the incubation period, values were similar or slightly higher than those observed after 7 days' incubation. Since soil samples were collected in spring before maize sowing and annual atrazine application, hydroxyatrazine levels represent residue accumulations from previous applications (1–40 years). The data indicated that the persistence of hydroxyatrazine is longer in soils than either atrazine or its chlorinated degradation products. Similar results were reported by others and were attributed to its strong adsorption to soil and relatively slow degradation (Winkelmann and Klaine 1991; Assaf and Turco 1994; Peixoto et al. 2000; Mahía et al. 2007).

Marked fluctuations in the content of the parent compound and its intermediate products were observed in the treated soils following atrazine application (Table 1, Fig. 1). The data showed that 75–86% of added atrazine (21–61% atrazine, 6–11% hydroxyatrazine and 9–52% of deisopropylatrazine)

Table 1 Atrazine (A), hydroxyatrazine (HA) and deisopropylatrazine (DIA) content (μg kg^{-1} dry soil) in five different unamended control soils and atrazine-treated soils (5 mg atrazine kg^{-1}) during the incubation period (mean values ± SD)

Soil			Incubation time (weeks)				
			1	3	6	9	12
MP	Control	HA	129 ± 9	137 ± 7	147 ± 13	114 ± 11	116 ± 7
	Treated	A	1,260 ± 25	1 ± 2	bld	bld	bld
		HA	460 ± 10	230 ± 11	253 ± 3	212 ± 5	233 ± 5
		DIA	2,099 ± 15	bld	bld	bld	bld
G	Control	HA	146 ± 10	182 ± 16	156 ± 7	120 ± 6	127 ± 10
	Treated	A	2,046 ± 105	591 ± 70	445 ± 47	213 ± 181	145 ± 16
		HA	492 ± 7	251 ± 7	250 ± 4	207 ± 4	225 ± 7
		DIA	1,047 ± 55	590 ± 81	166 ± 21	114 ± 54	87 ± 23
ME	Control	HA	150 ± 8	175 ± 7	181 ± 12	121 ± 4	122 ± 8
	Treated	A	1,064 ± 4	119 ± 6	39 ± 31	bld	bld
		HA	579 ± 10	261 ± 6	285 ± 6	212 ± 3	236 ± 7
		DIA	1,891 ± 21	148 ± 10	10 ± 17	bld	bld
MA	Control	HA	114 ± 7	118 ± 16	103 ± 7	123 ± 3	111 ± 6
	Treated	A	3,395 ± 72	28 ± 9	bld	bld	bld
		HA	381 ± 3	208 ± 7	209 ± 2	222 ± 3	228 ± 20
		DIA	467 ± 21	Bld	bld	bld	bld
C	Control	HA	150 ± 7	194 ± 4	169 ± 7	120 ± 9	117 ± 8
	Treated	A	3,292 ± 30	1,018 ± 83	716 ± 38	316 ± 12	179 ± 1
		HA	530 ± 7	256 ± 2	244 ± 4	203 ± 4	215 ± 6
		DIA	377 ± 19	340 ± 82	192 ± 22	95 ± 7	60 ± 18

Deethylatrazine was not detected; bld, below detectable limits

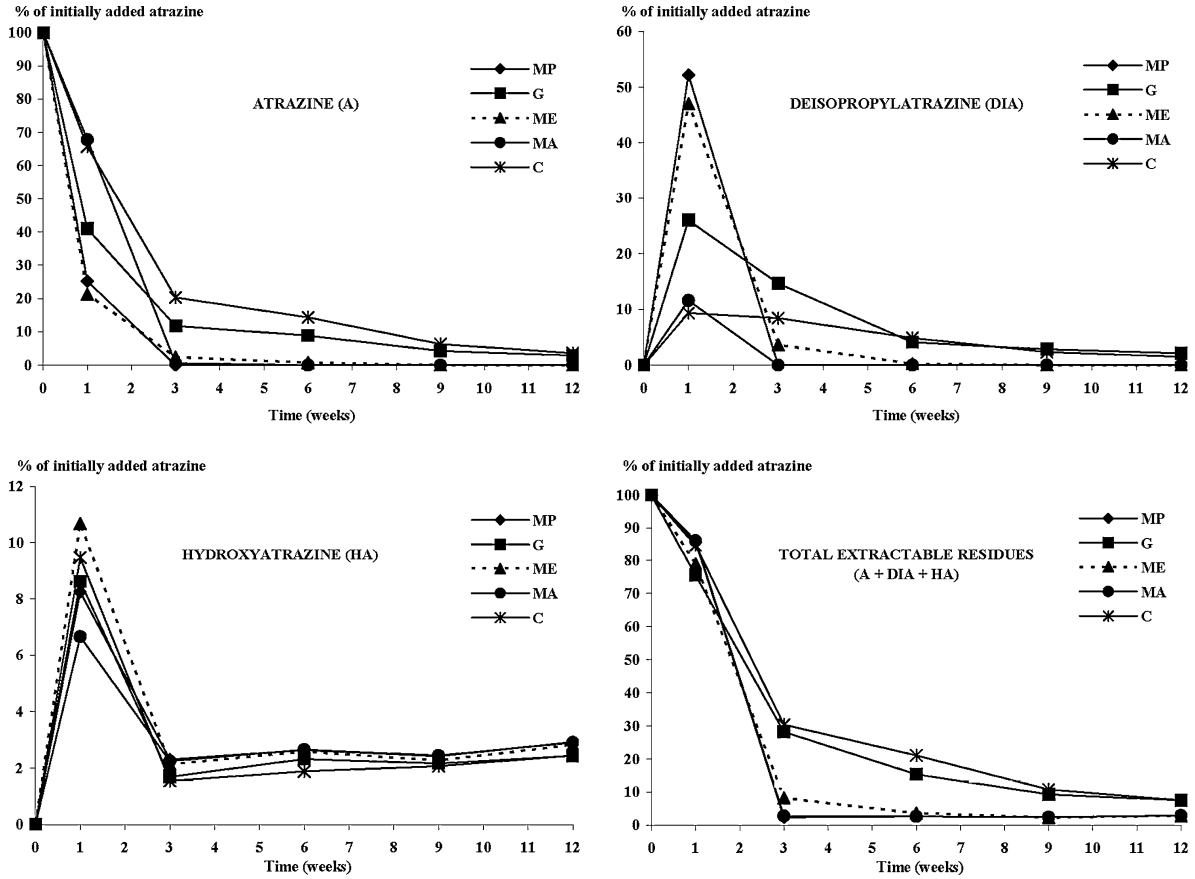

Fig. 1 Atrazine (A), hydroxyatrazine (HA), deisopropylatrazine (DIA) and total extractable residue evolution during the 12-week incubation period in the different studied soils (deethylatrazine was not detected)

was recovered in the extractable fraction after 7 days incubation. Atrazine values ranged from 1,260 to 3,395 μg kg^{-1}, representing between 66–67%, 41% and 21–25% of initially added atrazine for MA and C soils, G soil and for ME and MP soils, respectively. The atrazine metabolites exhibited values between 381 and 578 μg hydroxyatrazine kg^{-1} (7–11% of initially added atrazine) and between 467 and 2,099 μg deisopropylatrazine kg^{-1} (47–52% of initially added atrazine for MP and ME soils, 26% for G soil and 9–11% for MA and C soils), whereas levels of deethylatrazine were below detectable limits. These results indicated that the first steps of atrazine metabolism were dechlorination (chemical process), N-dealkylation and deamination (microbial process), leading to formation of hydroxyatrazine and desisopropylatrazine, respectively. This is consistent with observations that biotic and abiotic atrazine degradation occurred, but that microbial breakdown is dominant in some soils (Barriuso and Houot 1996; Gan et al. 1996; Accinelli et al. 2001).

The content of atrazine and its degradation products tended to decrease during the 12-week incubation period, but a different pattern was observed depending on which compound was analysed in which soil (Table 1, Fig. 1). After 3 weeks' incubation, atrazine concentrations were low or even undetectable in the MP, M and ME soils (0–119 μg kg^{-1}, 0.1–2% of added atrazine) and relatively high in the C and G soils (591–1,018 μg kg^{-1}, 12–20% of added atrazine). Hydroxyatrazine content was quite similar among soils after 3 weeks, varying between 207 and 261 μg kg^{-1} (1.3–2.3% of added atrazine); these values hardly changed over the remainder of the incubation period. In MP and MA soils, deisopropylatrazine was not detectable after 3 weeks and values ranged from 148 to 590 μg kg^{-1} for the other soils (4–15% of added atrazine). Most dissipation occurred during the

first 3 weeks of incubation since only 2–5% of the added atrazine in MA, ME and MP soils and 28–30% of atrazine residues were recovered in the extractable fraction. Accelerated atrazine dissipation, although to a lesser degree, has also been reported in soils with a long history of continuous maize planting and atrazine application (Barriuso and Houot 1996; Ostrofsky et al. 1997; Vanderheyder et al. 1997). No appreciable variations with incubation time were observed between 3 and 6 weeks in MA, MP and ME soils, whereas values decreased slightly with incubation time in G and C soils. At the end of the incubation period, atrazine values ranged from 0 to 179 μg kg^{-1} (undetectable values in MA, ME and MP soils and 144–179 μg kg^{-1} for G and C soils, 3–4% of added atrazine), whilst atrazine metabolites were present between 215–236 μg hydroxyatrazine kg^{-1} (2.5–3% of added atrazine) and 0–87 μg deisopropylatrazine kg^{-1} (0 values for MA, ME and MP soils and 60–87 μg kg^{-1} for G and C soils, 1.5–2% of added atrazine).

Soils could be divided into two different groups with respect to atrazine metabolism: one group formed by MP, ME and MA soils with rapid changes in the extractable fraction (20–40 years atrazine application, 1.6–4.1% C, loamy silt–silt loam texture) and a second group formed by G and C soils with more gradual variations (8–10 years application history, 2.6–4.1% C, sandy clay loam–loam texture). At the end of the incubation, 2–8% of total extractable residues were still detected, indicating a residual effect of atrazine 3 months after application. Despite the differences between the two groups, all results show that most atrazine in the extractable fraction disappeared rapidly (1–3 weeks of incubation), which can have important implications for the environment. There is only short-term persistence in the soil solution and hence low potential risk for soil and water contamination; in contrast, plant productivity can be negatively affected due to low herbicide efficiency, particularly if atrazine is lost before controlling the growth of grasses in the maize crop. Caution should be taken when laboratory results are extrapolated to the field where the activity of degrader microorganisms and hence the atrazine mineralization process can be retarded or reduced by unfavourable environmental conditions (moisture, temperature, etc.). Atrazine can be dissipated via mineralization (biological process) or via immobilization due to physico-chemical processes with formation of bound or non-extractable residues, which are not detected under standard extraction and analytical procedures. The presence of sequestrated residues, revealed in field and laboratory experiments conducted with labelled atrazine, was found to be associated with organic C content as well as the prolonged persistence of the herbicide in soil (Chung and Alexander 2002; Barraclough et al. 2005; Mordaunt et al. 2005). Further studies are now being conducted in our laboratory with either ring-labelled (U–^{13}C-atrazine) or chain-labelled herbicide (ethyl-amino-^{15}N-atrazine) in order to determine the relative importance of the mineralization and immobilization processes in the atrazine degradation pathway of these acid agricultural soils with relatively high content of organic matter and medium- to long-term histories of herbicide application.

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ANEXO III

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Degradación de la atrazina y distribución de sus metabolitos en dos suelos ácidos de la zona templado-húmeda

J. Mahía y M. Díaz-Raviña

Resumen

Se estudió, en condiciones de laboratorio, la mineralización de la atrazina y la formación de residuos extraíbles y no extraíbles en dos suelos con características diferentes (C orgánico, textura e historial de aplicación de atrazina) del N de España. Los suelos, un Cambisol Húmico (MP) y un Cambisol Gleico (G), fueron incubados, después de añadirles atrazina marcada en el anillo con ^{13}C a dosis normales de aplicación en el campo, y se realizaron medidas a diferentes intervalos de tiempo durante 3 meses. Tanto la degradación como el comportamiento de la atrazina durante el período de incubación fue muy diferente en los dos suelos. Así, el tiempo necesario para la degradación del 50 % de la atrazina añadida (DT50) fue de 9 días para el suelo MP y de 44 días para el suelo G. En el suelo MP, con 40 años de historial de aplicación de atrazina y bajo contenido en C orgánico y arcilla, más del 89 % de la ^{13}C-atrazina añadida fue mineralizada después de 12 semanas de incubación, produciéndose la mayor parte de la mineralización durante las 2 primeras semanas. Por el contrario, el suelo G, con 10 años de historial de aplicación de atrazina, mostró una mineralización de la ^{13}C-atrazina más progresiva, produciéndose después de 12 semanas únicamente la mineralización del 54 % de la ^{13}C-atrazina añadida. La hidroxiatrazina y la desisopropilatrazina fueron los únicos metabolitos detectados en la fracción extraíble, lo que demuestra que en la degradación de la atrazina intervienen tanto procesos químicos como biológicos. Durante toda la incubación los valores de la fracción residual extraíble en el suelo G fueron mayores que en el suelo MP, lo que indica un alto riesgo potencial de contaminación de suelos y aguas. El estudio reveló que la rápida degradación microbiana de la atrazina a través de la ruptura del anillo triazínico es la principal vía de descomposición de la atrazina en los dos suelos estudiados. La fracción residual no extraíble también fue muy diferente en los dos suelos, observándose valores del 9 y 41 % de la atrazina añadida, correspondiendo el valor más alto al suelo con un mayor contenido en materia orgánica y arcilla (suelo G).

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Atrazine Degradation and Residues Distribution in Two Acid Soils from Temperate Humid Zone

J. Mahía and M. Díaz-Raviña*

ABSTRACT

Mineralization of atrazine and formation of extractable and non-extractable "bound" residues were followed under laboratory conditions in two contrasting soils (organic C, texture, and atrazine application history) from northern Spain. The soils, a Humic Cambisol (MP) and a Gleyic Cambisol (G) were incubated with labeled atrazine (ring-^{13}C atrazine) at field application dose and measurements were made at different time intervals during 3 mo. Fate and behavior of atrazine along the incubation showed different patterns between the two soils, the time taken for degradation of 50% (DT50) being 9 and 44 d for MP and G soils, respectively. In MP soil, with 40 yr of atrazine application and lower organic C and clay content, more than 89% of U-^{13}C-atrazine added was mineralized after 12 wk, with most mineralization occurring within the first 2 wk. G soil, with 10 yr of atrazine application, exhibited a more progressive U-^{13}C-atrazine mineralization, reaching 54% of initially added atrazine at 12 wk. Hydroxyatrazine and deisopropylatrazine were the metabolites founded in the extractable fraction, demonstrating that both chemical and biological processes are involved in atrazine degradation. Soil G showed during all the incubation times an extractable residues fraction greater than that in MP soil, indicating a high potential risk of soil and water contamination. Rapid microbial degradation through *s*-triazine ring cleavage was proposed to be the main decomposition pathway of atrazine for the two soils studied. Bound residues pool also differed notably between soils accounting for 9 and 41% of initially added atrazine, the higher values shown by soil with higher organic matter and clay content (G soil).

ATRAZINE [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], the most widely applied herbicide for pre- and post-emergency control of broad-leaf and grassy weeds, is highly toxic and frequently appears in surface and ground water at concentrations that exceed the maximum permitted levels of 0.1 μg L^{-1} in Europe and 3 μg L^{-1} in the USA (Capriel et al., 1985; Herwig et al., 2001). The persistence of this herbicide in the environment depends on soil characteristics with a half-life ranging from a few days to several months (Erickson and Lee, 1989; Assaf and Turco, 1994; Koskinen and Clay, 1998). The persistence and toxicity of the transformation products of atrazine in natural environments is not as well understood, but is also of concern since many of the dechlorinated and dealkylated atrazine metabolites have been detected in soils and ground water at concentrations that are greater than that of the residual parent compound (Schiavon, 1988; Kookana et al., 1998; Battaglin et al., 2000). Because of these findings, several European countries have restricted or banned the use of this *s*-triazine herbicide.

Atrazine degradation in soils occurs both via chemical and biological processes, resulting in the formation of metabolites such as hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine), deethylatrazine (2-amino-4-chloro-6-isopropylamino-*s*-triazine), and deisopropylatrazine (2-amino-4-chloro-6-ethylamino-*s*-triazine), which have a different mobility and toxicity than atrazine and hence also different contaminant potential (Barrett, 1996). Chemical dechloration (hydroxyatrazine) dominates in some soils, particularly those showing low pH (Peixoto et al., 2000; Queiroz and Monteiro, 2000), whereas in other soils microbial breakdown is the main decomposition pathway with formation of dealkylated metabolites (deethylatrazine and deisopropylatrazine) and ring cleavage (Kaufman and Kearney, 1970; Barriuso and Houot, 1996; Abdelhafid et al., 2000a, 2000b). Formation of non-extractable or bound residues also constitutes a very important mechanism for the attenuation of the atrazine, which is associated with the soil organic matter content (Capriel et al., 1985; Peixoto et al., 2000). There is, therefore, a need to thoroughly understand the fate of atrazine after it enters the environment, including its binding to components of soil, and biotic and abiotic degradation.

Degradation and movement of herbicides in soils are important aspects of their behavior in relation to effectiveness and potential for environmental contamination. In Galicia (NW Spain), there is a widespread use of *s*-triazine compounds such as atrazine in agricultural soils and a high potential risk of surface and ground water contamination due to abundant precipitations and sandy textured soils. However, information concerning the degradation of *s*-triazine compounds in these acidic soils with relatively high organic matter content is scarce. A previous field study performed with an agricultural soil under two tillage systems showed that hydroxyatrazine was the main atrazine metabolite in the extractable fraction over a 5- to 12-mo period after the herbicide application, suggesting that the dechlorination is an important pathway associated with the residual activity of atrazine in soils (Mahía et al., 2006). In the present work the various forms of the atrazine (mineralized, extractable atrazine and its metabolites and non-extractable or "bound" residues) were monitored under laboratory conditions to study the fate and behavior of this *s*-triazine herbicide in two Galician contrasting soils.

MATERIALS AND METHODS

Soils

Two contrasting galician agricultural soils, a loamy sand textured soil (MP) classified as Humic Cambisol with a 40 yr

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atrazine history and a sandy clay loam textured soil (G) classified as Gleyic Cambisol with a 10 yr atrazine history, were sampled from the 0 to 15 cm top layer and used for the incubation experiment. The MP soil, with a pH of 5.6, contained 24.85 g total C kg^{-1} and 2.39 g total N kg^{-1}, whereas the G soil, with a similar pH (5.88), showed values of 41.12 and 2.79 g kg^{-1} for total C and N, respectively. Both soils were partially air-dried, homogenized, and sieved at 4 mm.

Chemicals

Analytical standards of atrazine (A, purity 99.2%) and its metabolites [atrazine-2-hydroxy (HA, purity 96%), atrazine-deethyl (DEA, purity 99.9%), atrazine-deisopropyl (DIA, purity 96.1%)] were purchased from Riedel-de Häen (Seelze, Germany). Uniformly ring-labeled-^{13}C-atrazine (purity 99%) was purchased from Cambridge Isotopes Laboratories Inc. (USA) and ethylamino-chain-labeled-^{15}N-atrazine (purity > 98.5%) from Isotec (USA). U-^{13}C-atrazine and ethylamino-^{15}N-atrazine solutions were prepared in water at 30 mg L^{-1}.

Incubation Experiment

The incubation experiment was run in a water bath using 50 g soil replicates at 28°C and constant moisture level (85% of water holding capacity) added with a U-^{13}C-ring-labeled atrazine solution (30 mg L^{-1}) at recommended agronomic dose (5 mg atrazine kg^{-1} soil). Distilled water was added to the control unamended soils which were then incubated under the same conditions. Measurements of different forms of atrazine residues (mineralized, extractable, and non-extractable or "bound" residues) were performed at 0.6, 2, 4, 6, 9, and 12 wk of incubation. Since samples were destructively treated, three replicates were prepared for each treatment-time combination. Three replicates of each soil were also added with ethylamino-^{15}N-atrazine, and measurements of ethylamino side-chain degradation were made after 12 wk of incubation.

Analysis of Atrazine Fractions

The contents of atrazine and its metabolites in the extractable fraction were measured following the procedure described by Ghani et al. (1996) with some modifications. Soil samples (5 g) were extracted with 10 mL of a mixture of methanol and water (7:3) by shaking for 24 h on an orbital shaker at the ambient temperature. Extracts were centrifuged at 2500 rpm for 11 min and filtered through a 0.2 μm glass fiber filter for direct analysis by high-performance liquid chromatography (HPLC). Extraction efficiency ranged from 89 to 93% of the total atrazine. Atrazine and its metabolites (HA, DEA, and DIA) were analyzed on a Novapak C18 column (Waters, 5 μm, 150 mm × 4.6 mm) with an Agilent Instrument equipped with a UV detector diode array at 230 nm. The injected volume was 50 μL and the mobile phase was a two-solvent gradient of acetonitrile/water delivered at a flow rate of acetonitrile/water 1.5 mL min^{-1} as follows: from 0 to 2 min, 20% acetonitrile + 80% water, from 2 to 6 min, 10% acetonitrile + 90% water, and from 6 to 23 min, 60% acetonitrile + 40% water. Under these conditions the retention time for A, HA, DEA, and DIA was 11.2, 1.4, 1.8, and 2.6 min, respectively. The atrazine and metabolites content in soil samples was determined by the external standard method, using a standard mixture of A, HA, DEA, and DIA in the concentration range of 0.1 to 5 μg mL^{-1} for each analyzed compound.

The δ ^{13}C and δ ^{15}N values were measured on finely ground treated and untreated samples (<100 μm) with an elemental analyzer (Carlo Erba CHN 1108) coupled 'in line' with an isotopic mass spectrometer (Finnigan Mat, delta C, Bremen, Germany). Atrazine residues were calculated from the equation: ^{13}C or ^{15}N remained atrazine = ^{13}C or ^{15}N in soil samples added with atrazine − ^{13}C in soil samples without added atrazine. A mass balance, expressed as percentage of initially added ring or side chain labeled, was used to determine atrazine dissipated as follows: % atrazine dissipated = 100 − % atrazine remaining in soil. Comparison of degradation rates among soils was accomplished by calculating the time taken for degradation of 50% of active substance originally applied (DT50), determined from the slope of the decreasing linear part of the plot of percentage of mineralization vs. incubation time and used as "half-life" of atrazine (approximate value). The non-extractable or "bound" atrazine residues were determined subtracting the extractable fraction from the remained ^{13}C-atrazine residues.

RESULTS AND DISCUSSION

Evolution of extractable A and the dechlorated (HA) and dealkylated (DIA and DEA) metabolites obtained for soil samples studied during the incubation experiment is shown in Fig. 1. The results showed that initially only HA was found in non-incubated samples, whereas that A, DIA, and HA were detected after atrazine application and further soil incubation. Deethylatrazine was found in amounts lower than the detection limits throughout the whole incubation period. This is in accordance with other short-term laboratory studies indicating that both biological and chemical processes are involved in atrazine degradation (Gan et al., 1996; Abdelhafid et al., 2000a, 2000b; Houot et al., 2000; Accinelli et al., 2001) as well as a previous field experiment performed with an acid soil from the same temperate humid region under two tillage systems showing that HA was the main long-term metabolite found in soil (Mahía et al., 2006).

Shortly after the application, the atrazine content tended to diminish rapidly and atrazine degradation products initially increased and then decreased with time, the pattern being more accentuated in MP soil than that in G soil. After 4 d of incubation atrazine accounted for 9 to 20% of initial concentration and degradation products for 33 to 58%, the DIA content being 10 to 16 times higher than that of HA. This is consistent with observations of several authors suggesting that microbial transformation was the first and predominant process in the atrazine degradation pathway (Barriuso and Houot, 1996; Kruger et al., 1997; Gaynor et al., 1998). Others have found HA to be a major degradation product of atrazine (Mandelbaum et al., 1995; Radosevich et al., 1995; Peixoto et al., 2000). After day 4, HA levels remained constant until the end of incubation, the values being always greater in G soil than in MP soil due probably to the greater organic matter that catalyzes the chemical hydroxylation in acid environments (Vanderheyden et al., 1997; Peixoto et al., 2000). After 2 wk, extractable residues accounted for 4% of initial content (0.2% A, 2.5% HA, and 1.6% DIA) in MP soil and 32% (17% A, 2.7% HA, and 12% DIA) in G soil. Then, while A and DIA values slightly decreased during the following 2 to 12 wk of incubation in G soil, undetectable amounts were found in MP soil.

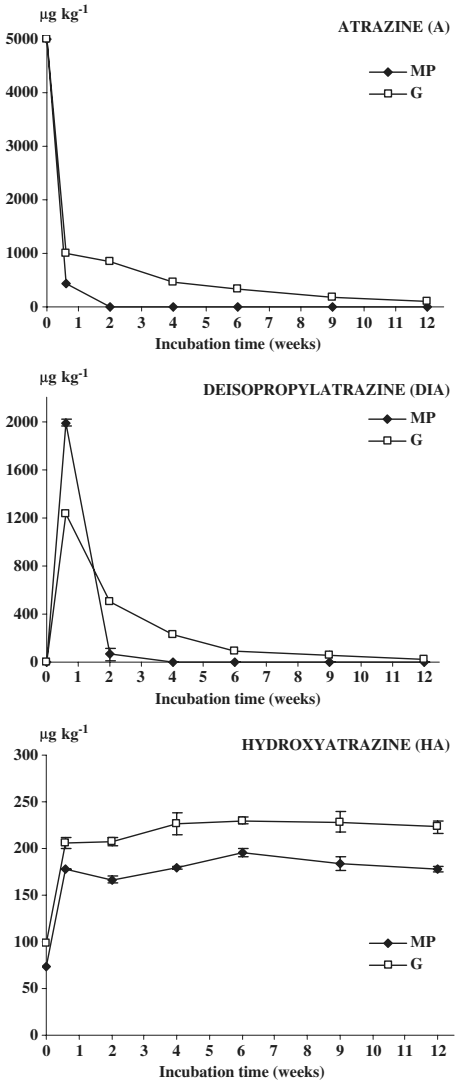

Fig. 1. Evolution of atrazine (A), deisopropylatrazine (DIA), and hydroxyatrazine (HA) in the extractable fraction of two contrasting agricultural soils (MP and G) added with 5 µg kg^{-1} atrazine and incubated during 12 wk (mean values ± SE of three incubation replicates). Deethylatrazine was not detected.

At the end of incubation, a residual atrazine fraction accounting for 2.5 to 5.5% of initial content (2.5% HA in MP soil, 2% A, 3% HA, and 0.5% DIA in G soil) was still detected. This pattern of extractable fraction showing pronounced peaks of atrazine and its metabolites within the first weeks is characteristic of soils with a long period of atrazine application (Barriuso and Houot, 1996; Abdelhafid et al., 2000a, 2000b). As it was expected, MP soil (40 yr of atrazine application) exhibited variations in the extractable fraction that were more accentuated than G soil (10 yr of application history); thus G soil showed throughout the whole experiment a greater extractable fraction, which is available for leaching and surface runoff processes and causes a higher potential risk of ground and surface water contamination.

Pathways of atrazine degradation consist of a series of hydrolytic cleavages of chloro-, amino-, and alkylamino-groups from the *s*-triazine ring that converge to cyanuric acid, which is subject to hydrolytic ring cleavage to CO_2 and NH_4^+ via hydrolysis of biuret and urea (Cook, 1987). Since U-^{13}C-atrazine was labeled on the *s*-triazine ring, any production of $^{13}CO_2$ can be attributed to complete degradation or mineralization via ring cleavage (Radosevich et al., 1995; Gan et al., 1996). The atrazine mineralization as a function of time is shown in Fig. 2. In both soils mineralization rapidly occurred during the first 2 to 6 wk of incubation with no initial lag phase and then remained constant (stationary degradation phase) until the end of incubation. This is consistent with other studies showing the presence of an adapted microbial community with the capacity to rapidly mineralize the triazine ring in soils which have frequently received atrazine (Barriuso and Houot, 1996; Ostrofsky et al., 1997; Rousseaux et al., 2001). Differences in the kinetics of atrazine ring mineralization and therefore in extrapolated half-lives were however observed between the two studied soils, with both the rate and extent of mineralization being lower in G soil, with a 10-yr history of atrazine treatment, compared with the MP soil, with 40-yr history of atrazine treatment. A half-life of 9 d in MP soil was significantly lower than 44 d in G soil. In MP soil 78% of the applied U-^{13}C-atrazine was mineralized by week 2 and then values increased slightly reaching a value of 89% at the end of incubation. In contrast, in G soil 33% of the applied atrazine was mineralized by week 2 and 49% by week 6, and then values remained fairly constant until the end of the experiment when a value of 53% was observed. These atrazine mineralization values were much higher than those obtained in soils with no atrazine application but were in the reported

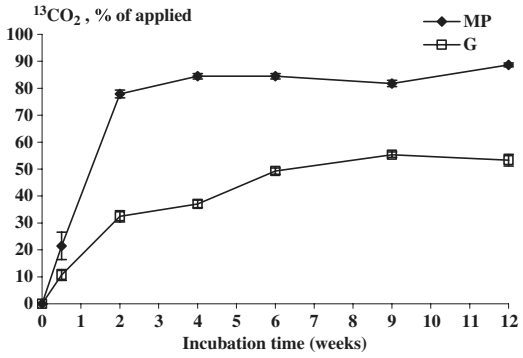

Fig. 2. Mineralization of (U-^{13}C-ring) atrazine on a percentage basis in two contrasting agricultural soils (MP and G soils) added with 5 µg kg^{-1} atrazine and incubated during 12 wk (mean values ± SE of three incubation replicates).

range given for soils with similar atrazine application history (Barriuso and Houot, 1996; Ostrofsky et al., 1997; Yassir et al., 1999; Abdelhafid et al., 2000a, 2000b; Houot et al., 2000; Krutz et al., 2003).

Variations in the number and activity of microorganisms growing at the expenses of atrazine and hence using the chemical as a source of carbon and energy (Ralebitso et al., 2002) are responsible for the kinetics of the herbicide dissipation in the two soils with previous annual herbicide treatment of 10 to 40 yr. The enhanced microbial degradation of atrazine found shortly after the herbicide addition with no lag phase can be explained by selection and proliferation of a very active atrazine mineralizing community already present in these MP and G soils even long after the herbicide has been metabolized. The data also indicated that initially the number of atrazine-degrading microorganisms in soil with a 10-yr history of atrazine application (G soil) was not as high as it was in soil with 40-yr history of application (MP soil), suggesting that the proportion of microorganisms capable of degrading atrazine was dose-dependent and therefore increased with continued annual application of the same herbicide (Barriuso and Houot, 1996; Ghani et al., 1996; Sparling et al., 1998; Abdelhafid et al., 2000a, 2000b). The low activity of atrazine degraders during the 2 to 12 (MP soil) or 6 to 12 (G soil) weeks of incubation (stationary phase) can be attributed to the lack of nutrients and/or herbicide in the available fraction (Queiroz and Monteiro, 2000).

By combining the results of analyzed metabolites in the extractable fraction and percentages of U-^{13}C-atrazine (CO_2 production, total degradation) and ethylamine-^{15}N-atrazine degraded (NH_3 production, partial degradation) after 12 wk of incubation, somehow atrazine routes in these agricultural acid soils from a temperate humid zone can be elucidated (Fig. 3). Shortly after the atrazine application (day 4), both HA and DIA were detected in the extractable fraction of the two studied soils, suggesting that atrazine degradation could be initiated both via hydroxylation or via dealkylation. However, the concentration of DIA metabolite was 10 to 16 times greater than that of HA indicating that dealkylation was the predominant process. The data of U-^{13}C-atrazine added soils revealed that 89 and 54% of atrazine had been mineralized after 12 wk in MP and G soils, respectively. In contrast, the data of ethylamine-^{15}N-atrazine added soils showed that the percentages of atrazine dissipated via formation of ammelide were 34% in MP soil and 22% in G soil, which pointed to the conclusion that about 38 to 40% of U-^{13}C-atrazine mineralized was degraded via formation of this intermediate metabolite. On the other hand, since DEA was not detected and DIA was the predominant primary metabolite, the data also suggest that deisopropylhydroxyatrazine, 2,4 dihydroxy 6(N-ethyl)amino 1,3,5 triazine, and, to a lesser extent, deisopropyldeethylatrazine, are important secondary metabolites in the atrazine degradation route. In principle, the presence of all of these xenobiotic compounds can have important implications for soil fertility and quality; however, since it is well known that phytotoxicity is destroyed by hydroxylation but not by

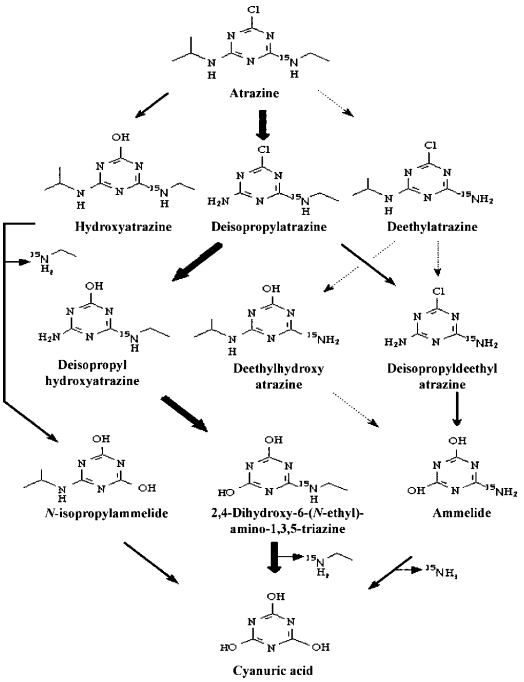

Fig. 3. Putative pathway of atrazine degradation in two contrasting agricultural soils (MP and G) from temperate humid zone (NW Spain). Dominant (thick arrow), probable (thin arrow) and non-probable (dotted arrow) routes are indicated.

dealkylation (Kaufman and Kearney, 1970), a higher potential environmental impact should be expected when DIA and deisopropyldeethylatrazine are accumulated. In addition, the environmental impact of the HA should be not discarded due to the persistence of relatively high amounts of this compound in the extractable fraction over the 3-mo incubation period.

Bound residues have been widely reported to be the major portion of atrazine residues in soil and its formation is considered a natural detoxification process (Capriel et al., 1985; Gan et al., 1996). In this study bound residues accounted for 9 to 18% and 35 to 41% in MP and G soil, respectively (Fig. 4). It may be point out, however, that since only main degradation products were quantified in the extractable fraction—HA, DEA and DIA—bound residues can be slightly overestimated when secondary metabolites were formed. The higher levels exhibited by G soil as compared with MP soil can be explained on the basis of soil characteristics such as organic matter content and soil texture since the formation of bound residues has been found to be associated with organic C content (Peixoto et al., 2000; Moorman et al., 2001; Huang et al., 2003) and clay content in soils (Koskinen and Clay, 1998; Houot et al., 2000). Organic matter levels can also partly explain the different evolution of bound residues in the two studied soils. In the G soil the bound residues content hardly

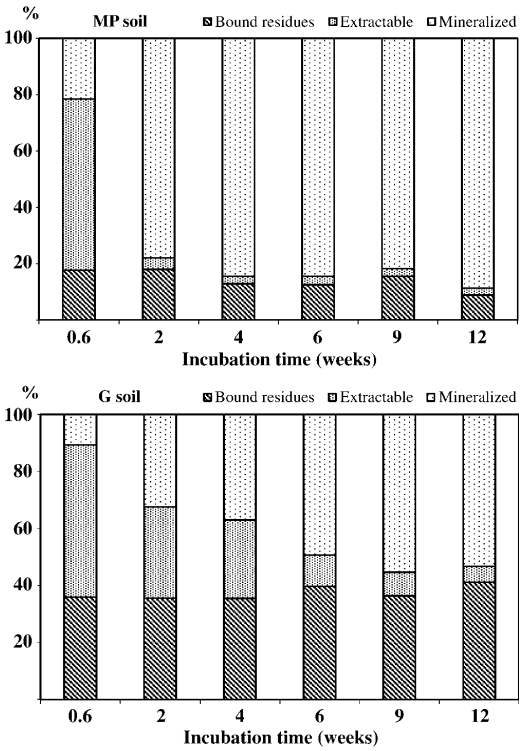

Fig. 4. Distribution of atrazine in mineralized, extractable, and "bound residues" fractions on a percentage basis in two contrasting agricultural soils (MP and G soils) over the 12-wk incubation period.

changed or even slightly increased with the incubation time, reaching the highest value at the end of the incubation; in contrast, significant changes over time were observed in the MP soil with values at the end of incubation that were 50% lower than those at week 1 and indicated than bound residues were re-released. The data are consistent with studies of Huang et al. (2003) who found higher desorption with increasing organic matter content. The amount of non-extractable compounds are coincident with data of both the extractable fraction and half-lives of the herbicide indicating that the potential contamination of soil and water is higher for G soil than for MP soil. However, since in this laboratory incubation the vertical movement of herbicide is not taken into account, complementary studies of leaching must be performed to confirm this hypothesis. It is well known that bound residues at high concentrations represent a high potential risk for environmental contamination when they are re-mobilized (Gan et al., 1996). The relatively high levels of bound atrazine residues found in the studied soils suggested that further investigations should be addressed to examine the possible long-term effects of atrazine-bound residues as well as whether these residues can be re-released over time under natural conditions.

CONCLUSIONS

The results therefore showed that, in these adapted agricultural galician soils with 10 to 40 yr of atrazine history, rapid microbial degradation through *s*-triazine ring cleavage (complete degradation or mineralization) is the main decomposition pathway of atrazine and that repeated annual application of herbicide enhanced the degradation capacity. Formation of bound residues, however, also constituted an important abiotic process for attenuation of the atrazine in these acid soils with relatively high organic matter content. The half-lives of the herbicide and the amounts of extractable and non-extractable residues indicated that potential contamination of soil and water is different for two contrasting soils. Considering that atrazine breakdown is largely mediated by microorganisms, factors which stimulate or inhibit microbial components likely exert similar effects on the degradation of atrazine. The response of microbial communities to stress and disturbance induced by land management practices are therefore of great interest in these soils since a decrease in the size and activity of relevant biodegraders would diminish the dissipation of atrazine increasing the potential risk of soil and water contamination.

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ANEXO IV

ANEXO IV

Efectos de la aplicación de atrazina sobre la biomasa microbiana y la mineralización del C en suelos agrícolas

J. Mahía, A. Cabaneiro, T. Carballas y M. Díaz-Raviña

Resumen

En este estudio se estiman los efectos de la atrazina sobre el carbono de la biomasa microbiana y la dinámica de mineralización del C en dos suelos agrícolas con diferentes características (C orgánico, textura e historial de aplicación de atrazina) ubicados en Galicia (NO. de España). Se añadió atrazina a los dos suelos, un Cambisol Húmico (H) y un Cambisol Gleico (G), a la dosis agronómica recomendada, y se determinaron el C mineralizado (CO_2 emitido) y la biomasa microbiana en muestras adicionadas y no adicionadas con atrazina, a diferentes intervalos de tiempo durante un periodo de incubación aeróbica de 12 semanas. La curva que representa la cinética de mineralización del C (CO_2-C desprendido a lo largo del tiempo, acumulado), se ajustó a un modelo cinético de primer orden [$Ct=Co\ (1-e^{-kt})$], cuyos parámetros cinéticos fueron cuantificados, observándose diferencias entre los parámetros de los dos suelos estudiados. El suelo G, con un mayor contenido en materia orgánica y en carbono de la biomasa microbiana y un menor historial de aplicación de atrazina, mostró valores más elevados de C total mineralizado (Ct) y de C lábil potencialmente mineralizable (Co) que los mostrados por el suelo H. La adición de atrazina modificó los parámetros cinéticos y provocó un incremento considerable del C mineralizado; al final de la incubación los valores de CO_2-C acumulado en los suelos adicionados con atrazina, fueron entre un 33 y un 41 % más elevados que los valores de los correspondientes suelos no adicionados con atrazina. Por el contrario, la adición de atrazina mostró un efecto variable, o incluso ningún efecto, sobre la biomasa microbiana de los suelos. Los resultados demostraron claramente que la aplicación de atrazina a dosis normales de campo puede tener importantes implicaciones en el ciclo del C de estos dos suelos ácidos con diferentes características.

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SHORT COMMUNICATION

Microbial biomass and C mineralization in agricultural soils as affected by atrazine addition

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Abstract This study examines the effects of atrazine on both microbial biomass C and C mineralization dynamics in two contrasting agricultural soils (organic C, texture, and atrazine application history) located at Galicia (NW Spain). Atrazine was added to soils, a Humic Cambisol (H) and a Gleyic Cambisol (G), at a recommended agronomic dose and C mineralization (CO_2 evolved), and microbial biomass measurements were made in non-treated and atrazine-treated samples at different time intervals during a 12-week aerobic incubation. The cumulative curves of CO_2–C evolved over time fit the simple first-order kinetic model [$Ct=Co\ (1-e^{-kt})$], whose kinetic parameters were quantified. Differences in these parameters were observed between the two soils studied; the G soil, with a higher content in organic matter and microbial biomass C and lower atrazine application history, exhibited higher values of the total C mineralization and the potentially mineralizable labile C pool than those for the H soil. The addition of atrazine modified the kinetic parameters and increased notably the C mineralized; by the end of the incubation the cumulative CO_2–C values were 33–41% higher than those in the corresponding non-added soils. In contrast, a variable effect or even no effect was observed on the soil microbial biomass following atrazine addition. The data clearly showed that atrazine application at normal agricultural rates may have important implications in the C cycling of these two contrasting acid soils.

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Keywords Atrazine · Microbial biomass · Carbon mineralization · Agricultural soils

Introduction

Soil microorganisms and the processes that they govern are essential for long-term sustainability of agricultural systems since they control the breakdown of organic matter and, hence, the net fluxes and amounts of soil carbon and nutrients through decomposition, mineralization, and immobilization processes (Jenkinson 1988; Pankhurst et al. 1997); there is, therefore, concern about the effects of herbicides on soil microbial biomass and activity in order to preserve soil health (Perucci et al. 2000; Accinelli et al. 2002). Measurements of soil respiration and microbial biomass have been used as useful tools for evaluating the side-effects of herbicides on soil microbial communities and, hence, the environmental impact of their application to agricultural ecosystems (Hart and Brookes 1996; Lin and Brookes 1999; Perucci et al. 2000; Jones and Ananyeva 2001; Haney et al. 2002). The ratio between soil respiration and microbial biomass, known as specific respiratory rate or metabolic quotient (qCO_2), reflects the physiological status of the microbial community and may be used as an indicator of ecosystem stress (Anderson and Domsch 1990); therefore, differences in the qCO_2 values may represent the magnitude of pesticide-induced response (Jones and Ananyeva 2001; Perucci et al. 2002).

In recent years many European countries have been restricted or banned the use of atrazine as herbicide due to its persistence on the environment and its toxicological properties. The influence of *s*-triazines on soil microorganisms has been intensively investigated. Several studies have demonstrated that atrazine may influence the population

dynamics of certain microbial groups (Voets et al. 1974; Ostrofsky et al. 1997; Rhine et al. 2003; Ross et al. 2006), although the response of total microbial biomass and soil respiration is less well understood (Wardle and Parkinson 1990; Ghani et al. 1996; Lin and Brookes 1999; Accinelli et al. 2002). The application of herbicides to soils induces either inhibitory (e.g., through effects on soil microorganisms) or stimulatory effects (e.g., through resistant microbes using herbicides as resources) on these microbial parameters. A variable effect was also observed depending on soil properties, dose of herbicide, and time passed after their application (Haney et al. 2002; Moreno et al. 2007). Most these investigations have involved short-term laboratory effects following a single herbicide application at large concentrations to non-adapted soils. However, in the field, the herbicide is often repeatedly applied at low dose to the same soil for many years (adapted soils). Therefore, the impact of atrazine on microbial biomass and activity is still poorly understood since it is difficult to extrapolate the results of these laboratory experiments to field conditions.

In Galicia (NW Spain), atrazine is still the most widely herbicide used to control broad-leaf and grassy weeds in agricultural soils. However, despite its interest, there is no information concerning the herbicide effect on both microbial biomass and soil respiration and therefore on carbon and nutrient cycling, which in turn can have important implications for long-term fertility of soils. The aim of the present study was to evaluate the changes induced in soil microbial biomass C and C mineralization by atrazine addition and thus to assess the herbicide impact on soil organic matter dynamics.

Material and methods

A Humic Cambisol (H) and a Gleyic Cambisol (G) developed on different parent materials, granite and sediments, respectively, were sampled from the 0–15 cm Ap horizon of experimental fields located in Galicia (NW Spain). The H soil (pH 5.6, organic C 24.84 g kg^{-1}, total N 2.39 g kg^{-1}, microbial biomass 142 mg C kg^{-1}, sand 74%, silt 14%, clay 12%) was continuously cultivated with maize since 1964 and treated with atrazine (47.5%) and linuron (52.5%) at an annual rate of 5 L ha^{-1}. The G soil (pH 5.9, organic C 41.12 g kg^{-1}, total N 2.79 g kg^{-1}, microbial biomass 253 mg C kg^{-1}, sand 64%, silt 9%, clay 27%) has been cultivated under annual ryegrass–maize rotation since 1994 and treated with acetachlor (35%) and atrazine (20%) at an annual rate of 7 L ha^{-1}. Representative samples (composite of 16 soil subsamples) from both soils were collected before maize planting and agrochemicals application, partially air-dried, sieved at 4 mm and homogenized.

Incubation experiment The incubation experiment was run in a water bath using 50-g soil replicates placed in 500-ml Erlenmeyer flasks and maintained at 28°C and 85% of field capacity for 12 weeks. The soils were treated with an atrazine solution at the recommended agronomic dose (5 mg herbicide kg^{-1} dry soil). The untreated control soils were added with a water solution and incubated under the same conditions. Measurements of microbial biomass and activity were carried out at different time intervals during the incubation period. Since samples were destructively treated for microbial biomass estimates and three replicates were used for each treatment–time combination, a total of 30 replicates of each soil (15 untreated and 15 treated with atrazine) were prepared.

Microbial activity In order to determine the metabolic activity of soil microorganisms, the CO_2 release from the nonadded and atrazine-added soil samples was monitored throughout 84 days of incubation, performing measurements at 1, 2, 3, 4, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 days. The incubation method was that of Carballas et al. (1979), who used an intermittent air flow for aeration. The atmosphere of the 500-ml Erlenmeyer flask was renewed periodically with humidified and CO_2-free air and the CO_2 evolved was trapped by bubbling for 2 h through a 2 N NaOH solution of known strength, which was subsequently titrated against 0.2 N HCl. The CO_2–C was then quantified by subtraction, using two empty flasks incubated under the same conditions as the controls. The potential C mineralization, expressed as grams of CO_2–C evolved per kilogram of dry soil and the percentage of the total organic C that was mineralized (C mineralization coefficient) were calculated. The cumulative curves of the CO_2–C released over time were fitted to the simple first-order kinetic model [$Ct=Co(1-e^{-kt})$] proposed by Stanford and Smith (1972) to quantify the kinetics of the C released from the soils H and G. In this model, which considers a unique C pool with a unique mineralization rate, Ct is the cumulative C released after time t (day), Co is the potentially mineralizable C in the labile pool, and k (day^{-1}) is the instantaneous mineralization rate. The nonlinear parameter estimation procedures in SPSS were used to fit the experimental data to the model. To avoid errors in the parameter estimation, the convergence criteria indicated by Updegraff et al. (1995) were used.

Microbial biomass The microbial biomass C was measured at 2, 4, 6, 9, and 12 weeks of incubation as described by Vance et al. (1987) with some modifications (Basanta et at. 2002). After soil fumigation with $CHCl_3$ for 24 h, the organic C was extracted from the unfumigated and fumigated samples with a 0.05 M K_2SO_4 solution using a 1:4 soil/extract ratio. The extracts were freeze-dried for their further C analysis by direct combustion in an

elemental analyzer (Carlo Erba CHN 1108). The soil microbial biomass C values were calculated from the equation: Cmic (mg C kg^{-1} dry soil)=2.64 E_C, where E_C is the extractable C flush (difference between the extractable organic C from the fumigated and unfumigated samples). The specific respiration rate or metabolic quotient (qCO_2) was calculated from the data for soil respiration rate and microbial biomass C (μg CO_2–C mg^{-1} Cmic day^{-1}) (Anderson and Domsch 1990).

Results and discussion

Microbial biomass C (Cmic) values obtained at different times during the incubation period, for the untreated and atrazine-treated samples from the H and G soils, are shown in Fig. 1. In the untreated H samples, Cmic ranged from 79 to 122 mg C kg^{-1} dry soil and represented between 0.31% and 0.49% of the organic C (average values ± SD of data obtained at different incubation times, 107±17 mg C kg^{-1} dry soil and 0.43±0.07%), whereas, in the untreated G samples, the values ranged from 167 to 231 mg C kg^{-1} dry soil and represented between 0.41% and 0.70% of the organic C (average values, 217±46 mg C kg^{-1} dry soil and 0.53±0.11%). Therefore, the Cmic values in the soil under crop rotation, with 4.1% of C and sandy clay loam texture (G soil), were about twice higher than those exhibited by the soil under monoculture with 2.5% of C and loamy sand texture (H soil). These values were much lower than those reported for a wide range of Galician undisturbed soils (Díaz-Raviña et al. 1988, 1993; Leirós et al. 2000), which can be partly explained by the lower organic C concentrations found in these agricultural soils. In the H atrazine-treated soil, the values ranged from 98 to 161 mg C kg^{-1} dry soil and represented between 0.39 and 0.65% of the organic C (average values, 129±25 mg C kg^{-1} dry soil and 0.52±0.10%); and in the G-treated soil, they ranged between 122 and 241 mg C kg^{-1} dry soil and represented between 0.30 and 0.59% of the organic C (average values, 190±44 mg C kg^{-1} dry soil and 0.46±0.10%). Although a significant positive or negative effect ($p<0.05$) could be observed at some incubation time, in general, the Cmic values were of the same magnitude order in both untreated and atrazine-treated soils, indicating that soil microbial biomass was not substantially modified by the atrazine addition. The results obtained are in agreement with those previously reported, showing inconsistent changes or even no changes in soil microbial biomass following addition of low atrazine concentrations to soils (Ghani et al. 1996; Accinelli et al. 2002).

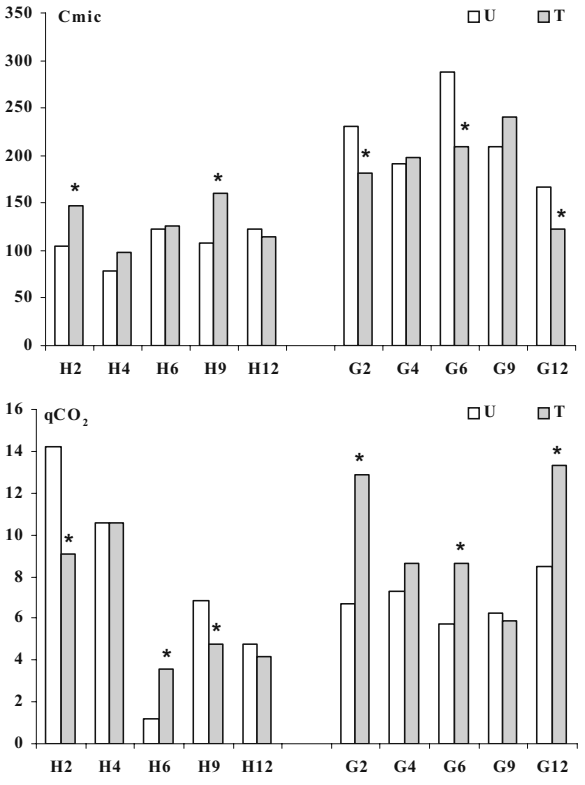

Fig. 1 Microbial biomass (*Cmic*, mg kg^{-1}) and metabolic quotient (qCO_2, μg CO_2 mg Cmic day^{-1}) for the untreated (*U*) and atrazine-treated (*T*) samples from the H and G soils, 2, 4, 6, 9 and 12 weeks after incubation. *Asterisks*, mean values significantly different from the corresponding untreated control

The daily and cumulative curves for the CO_2–C released from the untreated and atrazine-treated soils during the 12 weeks of incubation are shown in Fig. 2, whereas their C mineralization kinetic parameters are shown in Table 1. Approximately 1.7–2 times more CO_2–C was evolved overall from the untreated G soil samples compared to that from the untreated H soil samples, presumably reflecting the larger microbial biomass and readily decomposable organic matter fraction, as clearly indicated by the values of the rapidly potentially mineralizable C (Co), which pointed to the existence of an about threefold higher easily degradable fraction in the G soil than in the H soil. This is in accordance with the results of previous studies showing that soils with the highest organic matter content exhibited the highest microbial biomass C level and released most CO_2 (Díaz-Raviña et al. 1988, 1993; Leiros et al. 2000). In spite of their different C concentrations, both soils had similar C mineralization coefficients with values of 1.92% of the total C in soil H and 2.05% of the total C in soil G (Table 1). These values indicate a low potential mineralization activity, as also reported by other authors for undisturbed soils of the same zone (Díaz-Raviña et al. 1988; Fernández et al. 1997, 1999). However, the mineralization rate constant value (*k*) was three times

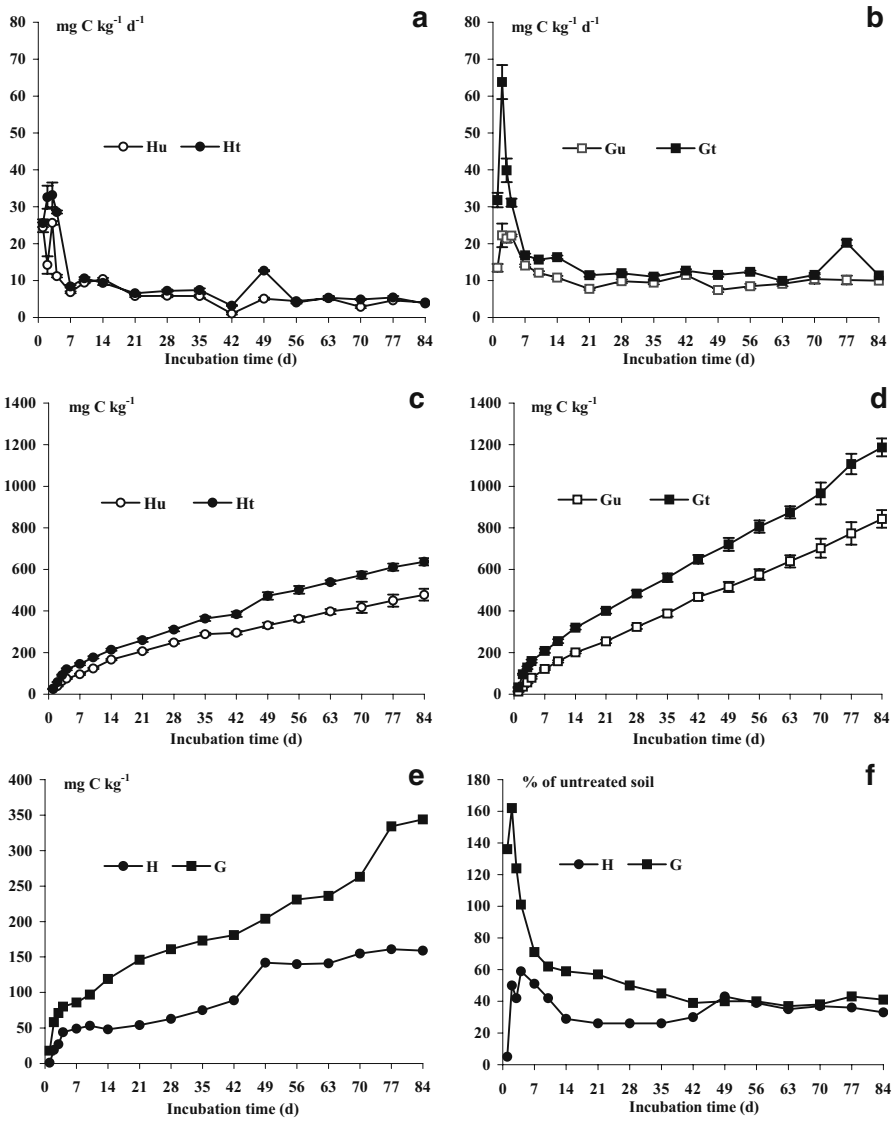

Fig. 2 C mineralization in two studied soils as affected by atrazine addition at recommended agronomic dose. Daily (**a**, **b**) and cumulative (**c**, **d**) C released as CO_2 by the untreated (*u*) and atrazine treated (*t*) samples from the H and G soils during 12 weeks of aerobic incubation (mean values±SE). Cumulative extra CO_2-C evolution from the H and G soil samples treated with atrazine (herbicide effect) expressed as absolute (**e**) and relative values (**f**)

Table 1 Total C mineralization, C mineralization coefficient, and kinetic parameter values for the simple first-order model $Ct=Co\ (1-e^{-kt})$ applied to the cumulative C released as CO_2 by the untreated (u) and atrazine treated (t) samples from the H and G soils during 12 weeks of aerobic incubation

Sample	C mineralization (g C kg^{-1})	C mineralization coefficient (g C 100 g^{-1} TC)	Kinetic parameters		
			Co (g kg^{-1})	*k* (day^{-1})	R^2
Hu	0.478±0.028	1.927±0.113	0.530±0.032	0.023±0.003	0.986
Ht	0.638±0.035	2.566±0.141	0.745±0.059	0.021±0.003	0.981
Gu	0.843±0.082	2.051±0.199	1.839±0.288	0.007±0.001	0.995
Gt	1.187±0.043	2.887±0.104	1.976±0.379	0.010±0.003	0.982

TC total carbon, *Ct* cumulative C released after time *t* (day), *Co* potentially mineralizable C in the labile pool, *k* instantaneous mineralization rate

higher in the untreated H soil, pointing to a $t_{(1/2)}$=22 and 71 days to mineralize one-half of the labile C pools, Co, in the H and G soils, respectively. The values for Co and k were close to those obtained for the C mineralization of different undisturbed forest ecosystems located in the same temperate humid zone (Fernández et al. 1997; 1999).

The C mineralization and C mineralization coefficient values, as well as the values of the kinetic parameters obtained for the atrazine-treated soils, differed considerably from those in the corresponding untreated soil (Table 1). The herbicide increased the potentially mineralizable C of the labile pool (Co) of the H and G soils, which were about 1.1–1.4-fold higher in the atrazine-treated samples than in the corresponding untreated ones. The mineralization rate constants were also slightly modified by the herbicide treatment, although an opposite effect was observed for the two soils (increase for the G soil and decrease for the H soil). A similar atrazine effect was observed independently of the soil considered; thus, during the experiment, the daily release of CO_2–C was generally larger for the soil added with atrazine compared with that of the corresponding untreated soil, this effect being more accentuated in the treated G soil than in the treated H soil (Fig. 2). For both soils, the increase was more marked during the first week, then the effect decreased gradually but it was maintained in a lower extent over the entire incubation period, indicating a long-lasting atrazine residual effect on soil microbial communities. Such an effect was quite surprising since the time taken under laboratory conditions for 50% of the active substance originally added being degraded is reported to be approximately 9 days for the soil with 40 years of atrazine application (H) and 44 days for the soil with 10 years of atrazine application (G) (Mahía and Díaz Raviña 2007). Thus, the results highlight the possible long-term effects of xenobiotic compounds on soil microorganisms even after most of the original molecules have disappeared, which is in agreement with the findings of Perucci et al. (2000). At the end of the incubation, the cumulative mineralized C values were significantly higher ($p<0.05$) in the soil samples treated with atrazine (638 mg CO_2 kg^{-1} dry soil and 1187 mg CO_2 kg^{-1} dry soil in the H and G soils, respectively) compared to those in the corresponding untreated samples (478 mg CO_2 kg^{-1} dry soil and 843 mg CO_2 kg^{-1} dry soil in the H and G soils, respectively), indicating that respiration was markedly affected by atrazine addition in both soils (Fig. 2). The net increase of C mineralized, provoked by the atrazine addition, reached, after 12 weeks of incubation, values of 160 mg CO_2–C kg^{-1} dry soil in the H soil and 344 mg CO_2–C kg^{-1} dry soil in the G soil, which represented 33% and 41%, respectively, of the C mineralized by the corresponding untreated samples (Fig. 2). The more marked effect of the atrazine in the G soil showing higher organic C content and shorter atrazine history may be partly due to the fact that microorganisms in this soil environment are less adapted to the herbicide and, hence, exhibited a greater response to the herbicide application (Rhine et al. 2003). A stimulatory effect of atrazine application at normal agricultural rates on soil respiration was also reported by other authors (Ghani et al. 1996; Accinelli et al. 2002), although in these studies, the enhancement of CO_2–C did not exceed 20 mg CO_2–C kg^{-1} dry soil and the effect did not last for more than 20 days.

Priming effects in soils are described as positive or negative changes in the rate of mineralization of soil organic matter following the addition of substrates to the soil (Kuzyakov et al. 2000). In our work, it should be noticed that the increase of CO_2–C evolved from the soils treated with atrazine was considerably higher than the amount of C added as atrazine, being 72 and 155 times higher for the H and G soils, respectively; therefore, it is likely that atrazine induced an accelerated mineralization of native organic matter, resulting in a "priming effect" (Ghani et al. 1996). This positive effect remained during the entire incubation period and was much higher than the priming effects reported by other authors in soils of the same area (Carballas et al. 1979; Díaz-Raviña et al. 1988; González-Prieto et al. 1991) after application of high, easily degradable substrates and organic residue doses. De Nobili et al. (2001) also observed that trace concentrations (i.e., μg g^{-1} quantities) of appropriate "trigger solutions" of glucose, amino acids, and root exudates caused the biomass to evolve about two- to five-times more C as CO_2 than that contained in the original trigger solution, which was explained by the shift from a dormant to an active state to maintain the cell in a state of "metabolic alertness" to take immediate advantage of a food event. Likewise, similar data showing an immediate and significant activation of the soil microorganisms following addition of trace amounts of different simple and complex substrates (glutamic acid, aminoacids mixture, glucose, protein hydrolysates, carbohydrates, compost extract) were reported recently by Mondini et al (2006), which is consistent with an effective survival strategy in a nutrient-poor soil environment. Taking into account all these studies, a possible explanation for data here obtained is that the atrazine and/or their decomposition metabolites either act directly as trigger molecules or induce their progressive liberation from the native soil organic matter over the entire incubation period. Results of a previous study on U-^{13}C-atrazine degradation showed that, in the H soil, more than 89% of the atrazine was mineralized after 12 weeks of incubation, with most mineralization occurring within the first 2 weeks, whereas the G soil exhibited a more progressive atrazine mineralization, reaching 54% of the initially added atrazine at 12 weeks of the incubation (Mahía and Díaz-Raviña 2007).

Therefore, important residual atrazine fractions persisted in the soils over the entire incubation time, particularly in the G soil, which showed a more marked priming effect (Fig. 2). The probable sources of the priming effect were an enhanced turnover of the native soil microbial biomass and/or an enhanced mineralization of the nonbiomass soil labile organic matter pool. The magnitude of the priming effect and the size and evolution of the soil microbial biomass pool over the incubation period (Fig. 1), as well as the size of the potentially mineralizable labile pool (Table 1), seem to indicate that both facts could be responsible for this extra CO_2-C evolved from the atrazine-added soils (Fig. 2).

Specific respiration values in the H and G untreated samples ranged from 1.22 to 14.18 μg CO_2 mg Cmic day^{-1} (average 7.44±4.8) and from 5.76 to 8.40 μg CO_2 mg Cmic day^{-1} (average 6.96±0.96), respectively (Fig. 1). In the atrazine-treated samples, the qCO_2 ranged from 3.54 to 10.58 μg CO_2 mg Cmic day^{-1} (average 6.48±3.12) in the MP soil and from 5.87 to 13.35 μg CO_2 mg Cmic day^{-1} (average 9.84±3.12) in the G soil. An increase in qCO_2 can be considered as indicative of a detrimental effect of the herbicide treatments, which can force microbes to utilize a large part of their energy budget for maintenance in order to support the stress provoked by xenobiotic compounds, thus reducing the efficiency for substrate utilization (Anderson and Domsch 1990; Perucci et al. 2000; Jones and Ananyeva, 2001). However, according to our qCO_2 results, the detrimental effect was only evident for week 6 for the H soil and at most incubation times for the G soil. It is interesting to point out that, in the G soil, by the end of the incubation, the atrazine seemed to exert a detectable toxic effect similar to that observed at the beginning of the experiment (2 weeks). These results are quite surprising since the most serious effect of the herbicides should be exerted at the beginning of the experiment due to the fact that the half-life of this herbicide was reported to be approximately 9 and 44 days for the H and G soils, respectively (Mahía and Díaz-Raviña 2007). Changes in microbial composition could partly explain this behaviour since bacterial/fungi ratios can drastically affect the qCO_2 values (Nannipieri et al. 2003). The qCO_2 undoubtedly indicates microbial efficiency for substrate utilization, but this quotient may not reflect disturbance and ecosystem development (Wardle and Ghani 1995). Our data clearly support this hypothesis, indicating that the qCO_2 cannot be used for evaluating the effect of atrazine at normal application doses on soil microbial communities. It should be noticed, however, that reported studies concerning the usefulness of qCO_2 measurements to examine the herbicides' impact on soil microorganisms were made using a much higher concentration than the recommended agronomic dose.

Combining the information derived from microbial biomass and respiration measurements in both untreated and atrazine-treated samples, somehow it is possible to determine if the size of the microbial biomass is important in regulating the C mineralization. The data showed that the soils recently added with atrazine at agronomic dose evolved much more CO_2 than the corresponding untreated soils; however, the microbial biomass estimates, particularly in the G soil, were similar or even smaller than those in the corresponding untreated soils, showing that the carbon mineralization rates are not proportional to the microbial biomass size. This seems to support the hypothesis of Kemmitt et al. (2008), who propose that mineralization of humified organic matter is a two-stage process, firstly an abiological destabilizing step that transforms nonbioavailable substrates to bioavailable substrates, and secondly, a biologically mediated step. According to this hypothesis, our data can also indicate that this regulatory mechanism is changed as a consequence of the atrazine addition due either to the herbicide effects on soil microbial communities (size, activity, or composition) or to the interactions of the atrazine and its metabolites with humified soil organic matter affecting possible mechanisms involved in the conversion of nonbiologically available to biologically available soil organic matter (chemical oxidation or hydrolysis, diffusion from inaccessible soil pores or aggregates, desorption from solid phase, or action of extracellular-stabilized enzymes). Taking into account the magnitude of the extra CO_2 evolved derived from the recent atrazine addition and the small reported changes induced in several soil biochemical properties (Mahía, personal communication), the latter seem to be the most probable hypothesis.

In conclusion, the data clearly showed that the application of atrazine at normal field doses may have important implications in the carbon and nutrient cycling and hence in the fertility of these two contrasting acid soils with a 10–40 yr of atrazine history. Nowadays, there is considerable awareness of how soil management affects soil quality and interest in the effects of changes in C mineralization rates on feedbacks within the global carbon cycle; therefore, more studies concerning the measurement of several microbial activity and diversity parameters should be performed in order to improve the understanding of herbicide impacts on soil organic matter mineralization and to determine if the results here obtained can be extrapolated to a wide range of soils.

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ANEXO V

ANEXO V

Propiedades bioquímicas y estructura de la comunidad microbiana en cinco suelos diferentes después de la aplicación de atrazina

J. Mahía, S. J. González-Prieto, A. Martín, E. Bååth y M. Díaz-Raviña

Resumen

La atrazina es uno de los herbicidas más usados en el mundo; sin embargo, las consecuencias de su amplio y prolongado uso en la agricultura son todavía desconocidas. Se realizó un estudio de laboratorio con el fin de determinar los cambios producidos en las propiedades microbianas de cinco suelos ácidos de Galicia (NO. de España) con diferentes propiedades físico-químicas e historial de aplicación de atrazina, tras la adición a los mismos de etilamin-^{15}N-atrazina, a la dosis agronómica recomendada, e incubación aerobia durante 12 semanas. Se observó mineralización neta del N en todos los suelos, predominando los nitratos entre las sustancias formadas; los valores más elevados se detectaron en los suelos con un menor historial de aplicación de atrazina. Entre un 2 % y un 23 % de la ^{15}N-atrazina se encontró en el "pool" de N inorgánico, siendo detectados los mayores valores después de 9 semanas de incubación en los suelos con un mayor historial de aplicación de atrazina y una menor mineralización del N nativo del suelo. En el corto plazo, la adición de atrazina redujo ligeramente el contenido de N mineralizado y de biomasa microbiana. El contenido en carbohidratos solubles y la actividad de la β-glucosidasa y la ureasa descendieron con el tiempo de incubación, pero no se vieron afectados significativamente por la adición de atrazina. La estructura de la comunidad microbiana resultó afectada tanto por el tipo de suelo como por el tiempo de incubación, pero no se detectaron cambios en el patrón de los ácidos grasos de los fosfolípidos (PLFA) debidos a la reciente adición de atrazina a dosis normales de campo. Se observó una mayor abundancia relativa de los ácidos grasos saturados 17-C y 20-C en los suelos con un mayor historial de aplicación de atrazina y un descenso de la biomasa fúngica, tal como indica el PLFA 18:2ω6,9, con el tiempo de incubación. Los resultados sugieren que el patrón de los ácidos grasos de los fosfolípidos (PLFA) y la dinámica del N del suelo pueden detectar el impacto a largo plazo de la aplicación repetida de atrazina en suelos agrícolas.

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ORIGINAL PAPER

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-^{15}N-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-^{15}N 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 18:2ω6,9, 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

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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.

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 (NH_4^+ and NO_3^-) was analyzed with an extraction–diffusion method at room temperature, the evolved NH_3 being trapped in 10 ml of 0.005 M H_2SO_4 and measured by back titration with 0.01 M NaOH (Couto-Vázquez and González-Prieto 2006). The resulting $(NH_4)_2SO_4$ solutions were then acidified with 1 ml 0.005 M H_2SO_4 and evaporated to obtain $(NH_4)_2SO_4$ salts, whose $^{15}N/^{14}N$ 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, NH_4^+-N, NO_3^--N) during the incubation subtracting the initial N content, and (b) the soil inorganic N content and its atom% ^{15}N 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 ^{15}N-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 NH_4^+ with 1 M KCl and 0.01 M HCl followed by colorimetric NH_4^+ 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

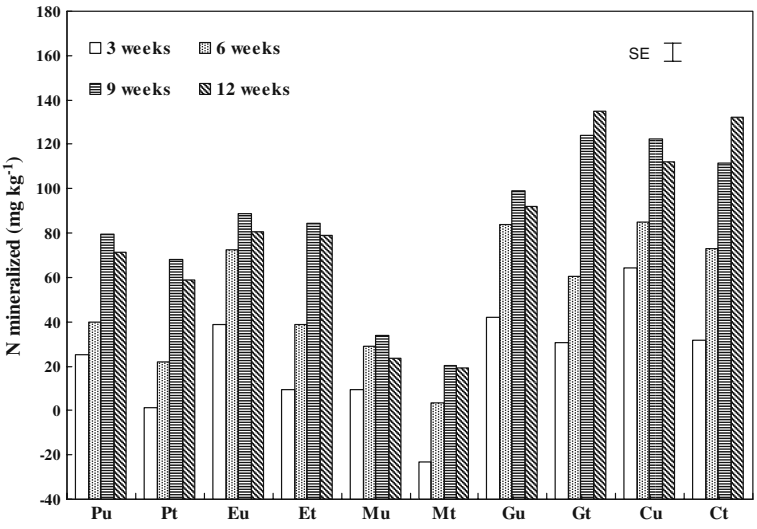

Fig. 1 Net soil N mineralization (ammonification plus nitrification) in samples non-added (*u*) and added (*t*) with ^{15}N-ethylamino-atrazine in the soils studied (*P*, *E*, *M*, *G*, and *C*) at different incubation times. Mean values of three incubation replicates

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-ethylamine-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. $^{15}NHCH_2CH_3$ and $^{15}NH_3$ are released in the intermediate processes of the ^{15}N-ethylamino-atrazine degradation pathway; thus the presence of NH_4^+-^{15}N and NO_3^--^{15}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-ethylamine-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-NH_4^+ 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).

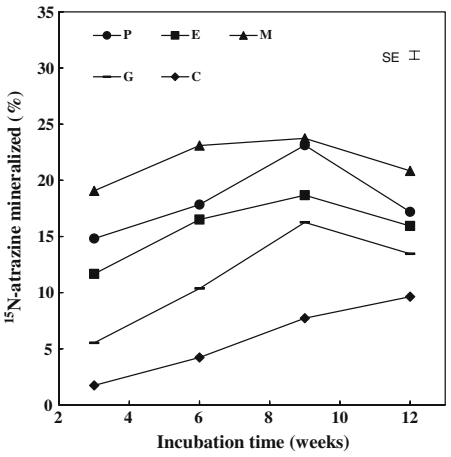

Fig. 2 Atrazine-derived inorganic-N (NH_4^+ + NO_3^-) expressed as percentage of added ^{15}N-ethylamine-atrazine in the soils studied (*P*, *E*, *M*, *G*, and *C*) at different incubation times. Mean values of three incubation replicates

The microbial biomass, estimated as totPLFAs, ranged from 1.3 to 3.6 μmol 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 significative 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 16:1ω7c and 16:1ω5, 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 (18:2ω6,9, 18:1ω9) and bacterial (18:1ω7, 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.

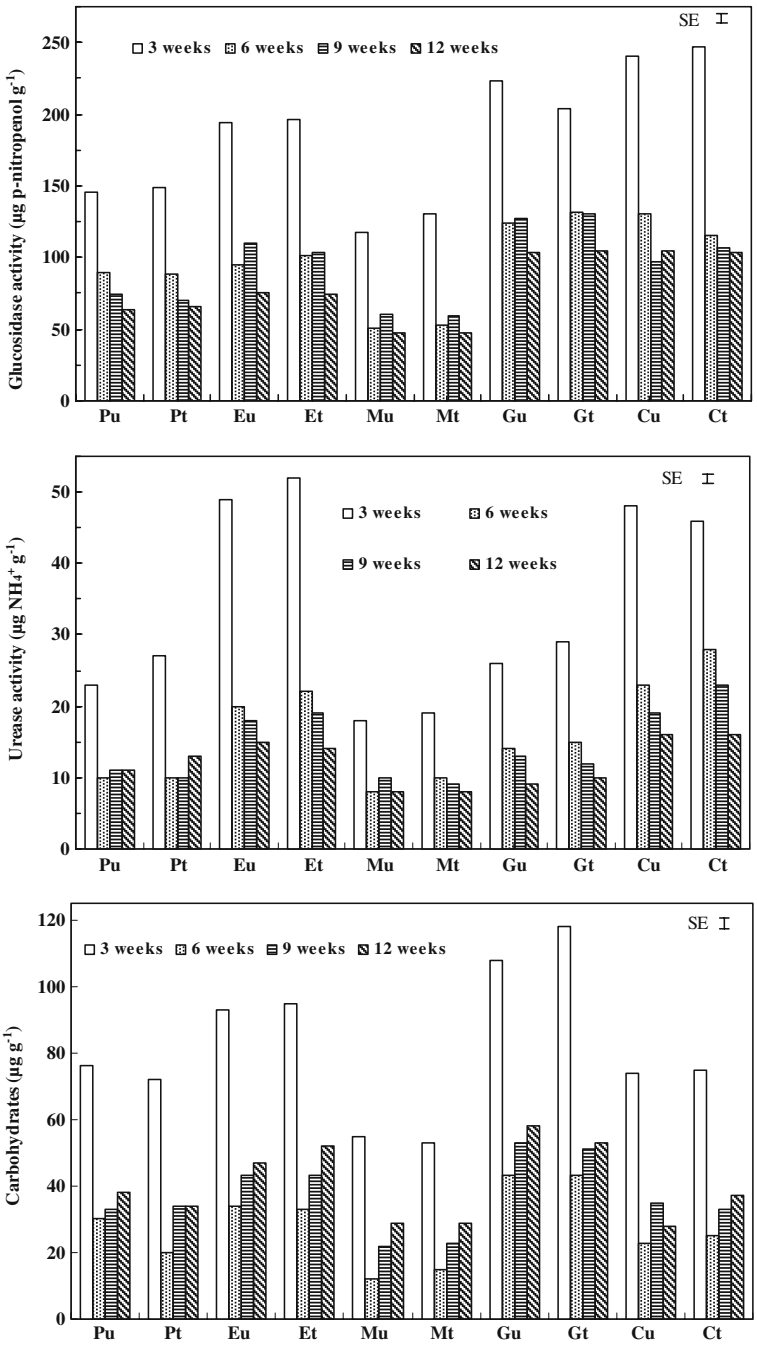

Fig. 3 Soil biochemical properties in the studied soils (*P*, *G*, *E*, *M*, and *C*) untreated (*u*) and treated (*t*) with atrazine at different incubation times. Mean values of three incubation replicates

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 (Schaëfer 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

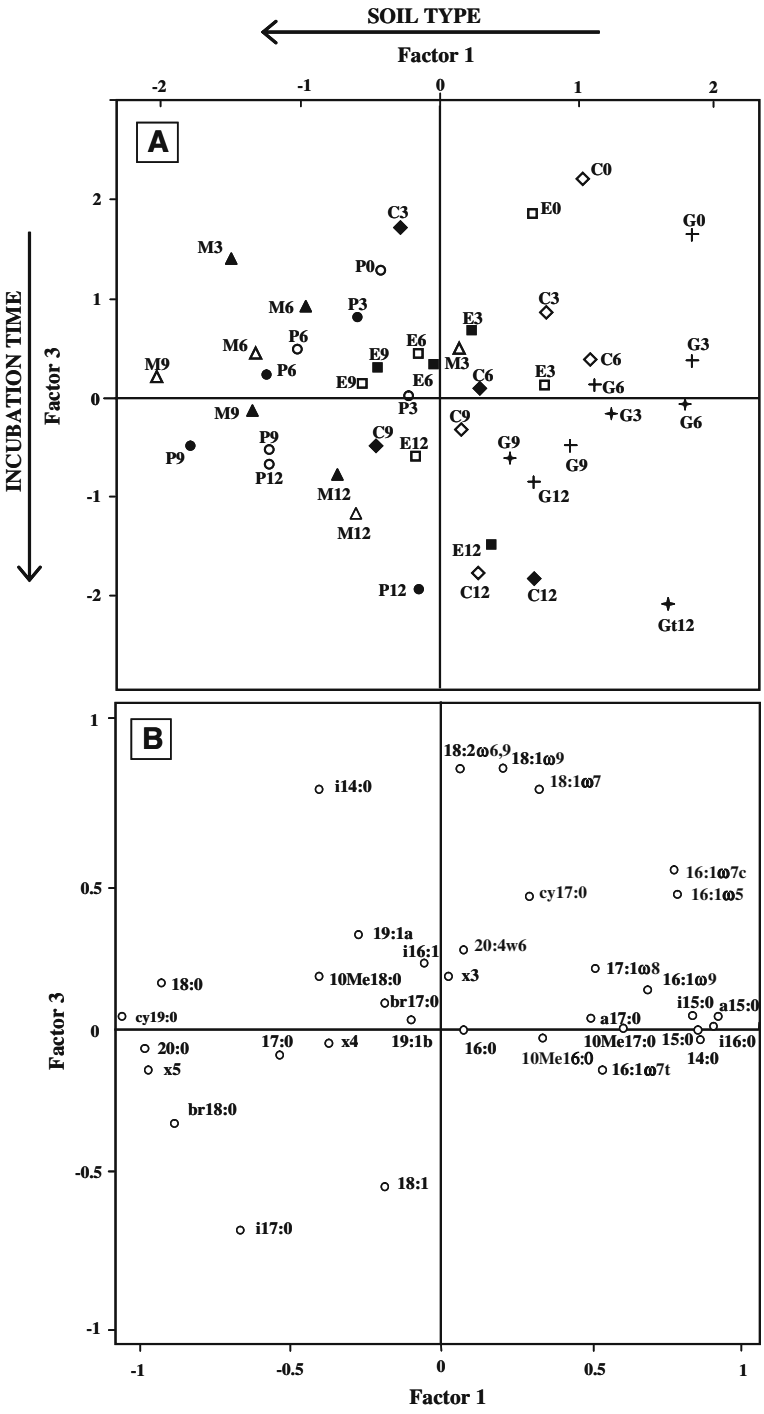

Fig. 4 Score and loading plots from principal component analysis performed on the PLFAs of the samples untreated (*open symbols*) and treated (*filled symbols*) with atrazine from the studied soils (*P*, *E*, *M*, *G*, and *C*) at different incubation times. Factors 1 and 3 accounted for 34% and 11% 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 18:2ω6,9 and 18:1ω9, characteristic of fungi, and 18:1ω7, 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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