UNIVERSIDAD DE GRANADA

PROGRAMA OFICIAL DE POSGRADO EN MICROBIOLOGIA (RD 56/2005)

DEPARTAMENTO DE MICROBIOLOGÍA

REPERCUSIÓN DE VERMICOMPOST AGROINDUSTRIALES EN LA BIOTA DEL SUELO Y EN LA BIODISPONIBILIDAD DE PLAGUICIDAS

TESIS DOCTORAL

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Memoria presentada por la Licenciado Microbiólogo Agrícola y Veterinaria por la Pontificia Universidad Javeriana de Bogotá, Colombia, Jean Manuel Castillo Díaz para optar al título de Doctor por la Universidad de Granada.

Granada, 26 de Octubre de 2015

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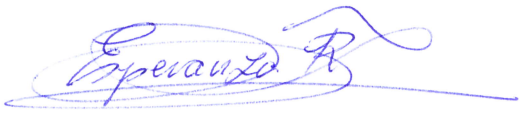

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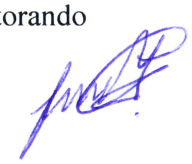

Fdo.: **Jean Manuel Castillo Diaz**

Para la realización del presente trabajo de Tesis Doctoral el Lcdo. Jean Manuel Castillo Díaz disfrutó de una ayuda predoctoral de Formación de Personal de Investigación (FPI) del Ministerio de Educación y Ciencia, referencia: BES-2007-15173.

Este trabajo de Tesis Doctoral ha sido realizado en el Departamento de Protección Ambiental (Grupo de investigación "Relaciones Planta-Suelo") de la Estación Experimental del Zaidín (EEZ-CSIC). El presente estudio se encuadró dentro de los objetivos científicos-técnicos propuestos en los proyectos de investigación subvencionados por la Comisión Interministerial de Ciencia y Tecnología dentro del programa Nacional de I+D de Ciencias y Tecnologías Ambientales y cofinanciado con fondos FEDER, CTM2006-12214/TECNO "Aprovechamiento de residuos agroindustriales como Enmiendas orgánicas del suelo para la reducción de la transferencia de plaguicidas a otros medios. Repercusión en la biodisponibilidad de estos xenobióticos, implicaciones de la microbiota en la descontaminación del suelo y validación de la tecnología mediante estudios de campo" y CTM2010-16807/TECNO "Utilización de residuos agroindustriales y de invernadero en el desarrollo de sistemas de biopurificación para la reducción de la contaminación puntual por plaguicidas". Algunas de las actividades científicas incluidas en este trabajo fueron realizadas en el Institut Nacional de la Recherche Agronomique, Unidad mixta de Investigación INRA/Universidad de Bourgogne-Dijon (Francia) en varias estancias breves realizadas en el extranjero por el Lcdo. Jean Manuel Castillo Díaz durante el periodo de disfrute de la Beca FPI concedida.

A Dios

A mi madre

A mi esposa

A la memoria de Nena

Yo te pido que seas fuerte y valiente, que no te desanimes ni tengas miedo, porque yo soy tu Dios, y te ayudaré por dondequiera que vayas.

Josué 1:9. (TLA)

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INDICE

RESUMEN

Esta memoria de Tesis doctoral se caracteriza por su carácter multidisciplinar, abordando diversos estudios a nivel físico-químico, bioquímico y microbiológico con el objetivo de estudiar el proceso de vermicompostaje de residuos agroindustriales provenientes de la producción de aceite de oliva y del vino, para posteriormente evaluar la repercusión del uso de estos nuevos vermicomposts como enmienda orgánica en la microbiota del suelo y en la biodisponibilidad de plaguicidas. Se cuantificaron varias actividades enzimáticas como son las involucradas en el ciclo del carbono (β-glucosidasa), nitrógeno (ureasa) y fósforo (fosfatasa ácida) y aquella que funciona como indicador de la actividad microbiana (deshidrogenasa). Se emplearon varias técnicas moleculares como QPCR, DGGE y ARISA, para analizar la abundancia y estructura microbiana. Por último, mediante herramientas estadísticas como son los diferentes análisis multivalentes, se obtuvieron relaciones ecológicas que ayudaron a comprender el efecto y la función de los vermicomposts como alternativa en el reciclado de residuos y como técnica de mitigación de plaguicidas en suelos.

Dos de los residuos más relevantes generados en la agroindustria en España son el alperujo en la producción de aceite de oliva, y el sarmiento en el cultivo de vid, ambos con una producción aproximada de 3-5x 10^6 toneladas por año. Estos residuos están constituidos por un material lignocelulósico altamente recalcitrante que requiere ser biotransformado. Por lo tanto, el desarrollo de estrategias de bajo coste que permitan revalorizar estos residuos son imprescindibles. En esta línea, en el capítulo 1 de esta tesis, se revalorizaron estos residuos agroindustriales mediante procesos de vermicompostaje a escala piloto. La estabilización de estos residuos se controló evaluando principalmente la dinámica microbiana durante el proceso de vermicompostaje y de maduración, haciendo énfasis en las interacciones con los cambios físico-químicos, el desarrollo de la lombriz *Eisenia fetida* y con los bioindicadores enzimáticos. Dentro de los resultados más relevantes se encontraron las correlaciones significativas entre la actividad β-glucosidasa y el desarrollo de la lombriz, así como también con la evolución de la estructura bacteriana y fúngica. Este resultado en ambos procesos condujo a proponer el seguimiento de la actividad β-glucosidasa como un nuevo indicador ecológico del estado y proceso del vermicompostaje. Otro resultado importante fue la evolución de las diferentes clases taxonómicas bacterianas durante cada proceso de vermicompostaje y maduración. En este caso los grupos funcionales Alfa y Gamma-proteobacteria disminuyeron durante el proceso a medida que Beta-proteobacteria y Actinobacteria aumentaban. Sin embargo, durante el proceso de maduración la clase Beta-proteobacteria disminuyó significativamente, mientras Actinobacteria solo decreció significativamente en el vermicompost de sarmiento (W).

Además, hay que destacar, que este último proceso de vermicompostaje (W) fue relevante por la explosiva abundancia fúngica observada en las primeras tres semanas del proceso o etapa de preacondicionamiento.

De la etapa de preacondicionamiento del vermicompostaje de W, se aislaron diferentes hongos con el fin de evaluar su potencial catabólico frente a xenobióticos persistentes en suelos. Este estudio se abordó en el capítulo 2 de la presente tesis, seleccionando como contaminante modelo la 3,4-dicloroanilina (3,4-DCA), considerado como el metabolito más toxico y recalcitrante en suelos tras la biodegradación del herbicida diuron. Se seleccionaron 3 hongos, dos cepas de *Fusarium oxysporum* (F1 y F2) y una de *Aspergillus niger* (F3) por su habilidad para crecer con 3,4-DCA como fuente de nitrógeno. Durante el proceso de crecimiento de los hongos se detectaron y cuantificaron por primera vez varios metabolitos. Los resultados obtenidos permitieron proponer una ruta metabólica de biodegradación de la 3,4-DCA indicando los procesos enzimáticos involucrados.

En función del potencial microbiológico que contiene el vermicompost y su alto contenido en carbono orgánico, los estudios realizados en los capítulos 3 y 4 se orientaron a conocer el papel que los vermicomposts de residuos agroindustriales pueden ejercer para mitigar los efectos adversos que Diuron e Imidacloprid pueden ocasionar cuando se aplican a suelos que presentan un bajo contenido en materia orgánica; como es el caso de la mayoría de los suelos de clima mediterráneo, donde además la aplicación de estos fitosanitarios es frecuente. El efecto de cada vermicompost sobre la capacidad adsorbente del suelo se investigó realizando isotermas de adsorción-desorción de cada plaguicida y de sus metabolitos principales en suelos previamente enmendados y envejecidos con esas enmiendas orgánicas. El efecto de la enmienda con los vermicompost sobre la disipación de los plaguicidas en el suelo se investigó empleando condiciones de microcosmos bajo dos condiciones diferentes de aplicación agronómica. La primera fue a partir del suelo previamente enmendado con dosis agronómicas de cada vermicompost y envejecidos mediante incubación durante varios meses. La segunda, a partir del suelo previamente fortificado con diuron o imidacloprid e incubado durante varios meses. En ambos microcosmos, tanto en suelos pre-enmendados como en suelos pre-tratados con cada plaguicida, se realizó a una aplicación con cada plaguicida y se evaluó su disipación a diferentes tiempos durante 90 días.

Dentro de los resultados obtenidos, se concluyó que los vermicomposts afectan la biodisponibilidad de imidacloprid, diuron y de sus metabolitos, observándose en ambos casos una mayor tasa de adsorción en el suelo enmendado con vermicompost W, mientras

que la mayor tasa de disipación ocurrió en el suelo enmendado con el vermicompost de alperujo (O). Además, la cinética de degradación fue significativamente más rápida en los suelos que fueron previamente fortificados con imidacloprid o diuron, correspondiendo la más alta tasa de degradación al diuron. En el caso del diuron, hay que destacar que la abundancia del gen *puh*B, cuantificado por QPCR, fue mayor en los suelos enmendados con respecto al suelo sin enmendar, aunque la abundancia relativa respecto al ADN total fue similar. No obstante, la abundancia de este gen *puh*B, que codifica la enzima hidrolasa fenilurea involucrada en la hidrólisis de diuron, fue correlacionada con la degradación de diuron y con los cambios en la estructura bacteriana del suelo obtenidos mediante el análisis automático del espacio intergénico ribosomal (ARISA). Estos resultados indicaron que la enmienda cambia significativamente la estructura bacteriana del suelo. Además, las diferencias estructurales inducidas por el diuron fueron contrarrestadas, observándose al final del periodo de incubación con este herbicida una estructura similar en los suelos enmendados y sin enmendar.

Estos resultados indicaron que la enmienda además de aportar una carga bacteriana capaz de modificar la estructura bacteriana del suelo, es también capaz de mantener las poblaciones que son dominantes y las que tienen capacidad de degradar diuron. Como conclusión, los resultados en estos dos capítulos, indicaron que estos vermicomposts de residuos agroindustriales pueden ser utilizados como herramientas útiles en las estrategias de mitigación de forma natural de estos plaguicidas, evitando su desplazamiento en el medio ambiente y promoviendo su biodegradación sin afectar la efectividad del plaguicida.

INTRODUCCIÓN

1. **Valoración y manejo de residuos en la agroindustria vitivinícola y oleícola**
2. **Utilización de los residuos orgánicos como enmiendas**
3. **Plaguicidas**
4. **Diuron**
5. **Imidacloprid**

1. VALORACIÓN Y MANEJO DE RESIDUOS EN LA AGROINDUSTRIA VITIVINÍCOLA Y OLEÍCOLA.

1.1. Residuos, clasificación y manejo.

La producción de residuos se encuentra en continuo aumento a nivel mundial. Por su envergadura y repercusión directa en la sostenibilidad del modelo económico y ambiental, es de vital importancia plantear continuamente estrategias de manejo que resuelvan tal problemática. En Europa, según la directiva 2008/98/CE del parlamento europeo y del consejo, se considera residuo cualquier sustancia u objeto del cual su poseedor se desprenda o tenga la intención o la obligación de desprenderse. Partiendo de este concepto, la Unión Europea generó en el año 2012 cerca de 2514 millones de toneladas de residuos, de los cuales 119 millones fueron generados por España, es decir, un 4.7% de la producción total de los 28 países de la Unión Europea (http://appsso.eurostat.ec.europa.eu/nui/show.do).

Debido al alto desarrollo industrial y tecnológico de la Unión Europea, acompañado de la generación de nuevos residuos, se ha venido desarrollando nuevos conceptos dentro del marco legislativo como son los residuos peligrosos, los aceites usados y los biorresiduos. Por otro lado se define el concepto de subproducto, el cual es un resultante de un proceso de producción que no es la finalidad primaria de dicho proceso. Al contrario de lo que sucede con los residuos, debe poder utilizarse ulteriormente.

Los residuos pueden clasificarse según su actividad productiva, estos pueden ser **(i)** los producidos en el sector primario, donde están involucrados aquellos residuos que provienen de la agricultura, ganadería, de actividad forestal y pesqueras. **(ii)** Los del sector secundario, como son los producidos por procesos industriales, así como también los derivados de la minería y la construcción. Por último, están **(iii)** los del sector terciario, que corresponden a los residuos urbanos, donde se incluyen residuos sólidos y aguas residuales.

En España, se ha realizado una transposición de la Directiva Europea 2008/98/CE al ordenamiento jurídico Español, bajo la ley 22/2011 sobre residuos y suelos contaminados. En esta normativa, se prevé la gestión de residuos teniendo en cuenta 4 puntos clave: **(i)** prevención en la generación de residuos, **(ii)** reciclaje, reutilización y valorización, **(iii)** recuperación energética de los residuos mediante tecnologías

respetuosas con el medio ambiente, **(iv)** manejo controlado y seguro de los residuos no reciclables.

En relación a los residuos orgánicos, es quizás su valorización el resultado más importante en el manejo de este tipo de residuos, por tal razón la Directiva 2008/98/CE recomienda el uso de compostaje y otras técnicas medioambientales como alternativa para la gestión de estos residuos, utilizando luego el material estabilizado como enmienda orgánica en suelos. Teniendo en cuenta que los suelos mediterráneos y en especial los del sureste de España, tienen un factor K de erosionabilidad o vulnerabilidad a la erosión bastante alto (entre 0.038 - 0.045) atribuible al bajo contenido de materia orgánica (Zdruli et al., 2004; Panagos et al., 2014), en estos suelos el uso de enmiendas orgánicas estabilizadas es una alternativa para el mantenimiento y recuperación de las propiedades estructurales, biológicas y fisicoquímicas del suelo.

1.2. Residuos agroindustriales.

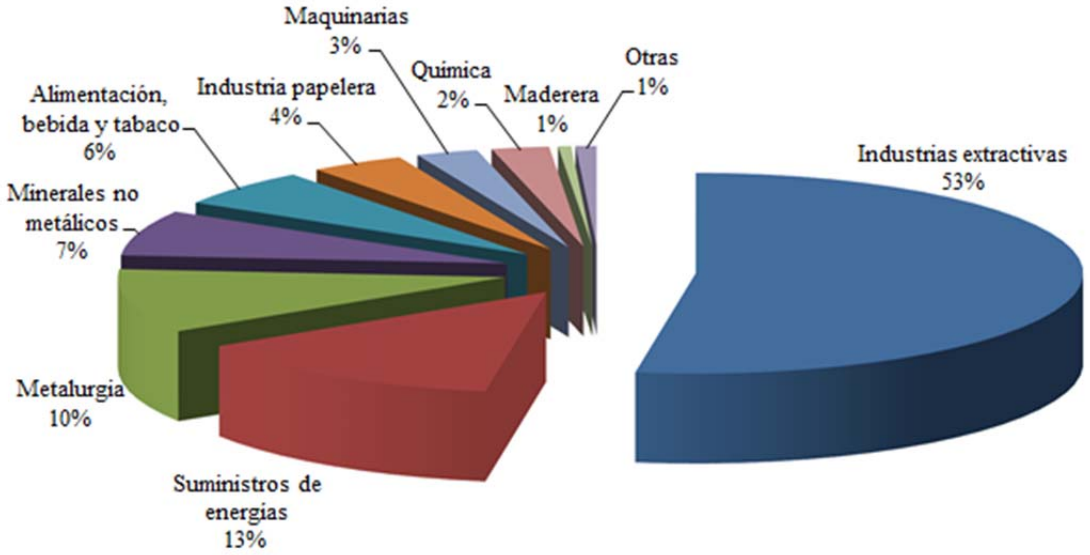


Figura 1. Residuos generados por sectores industriales. Año 2012. Nota de prensa del INE 7 octubre 2014.

Por su naturaleza lignocelulósica, estos residuos se consideran como materia orgánica, los cuales a través de tecnologías limpias, pueden ser reciclados ya sea por utilización del residuo integrado o retornándolo al sitio original. Según Eurostat, de los 119 millones de toneladas de residuos producidos en el año 2012 en España, 5.5 millones son producidos por actividades agrícolas, forestales y pesca. Por otro lado, la

industria generó 43 millones de toneladas de residuos, de los cuales el sector alimentación, bebidas y tabaco produjo 2.6 millones (Figura 1), categoría que engloba los residuos de las industrias vitivinícolas y oleícolas.

1.2.1. Industria vitivinícola y residuos generados.

El sector vitivinícola es uno de los más importantes en la agricultura mundial. Según el informe de la organización internacional de la viña y el vino para el año 2013 (OIV2013), la superficie vitícola mundial es de 7,5 millones de hectáreas con una producción de 69,1 millones de toneladas de uva. En cuanto a la producción de vino mundial este fue de 278 millones de hectolitros de los cuales 30 millones hl se produjeron en España convirtiéndose en el segundo productor de vino dentro de la Unión Europea sin contar el mosto, donde entonces España fue el primer productor mundial en 2013. Según Ruggieri et al. (2009) se estima que esta industria en España genera entre 2 y 3 millones de toneladas de residuos en el periodo de vendimia. La mayoría de estos residuos son orgánicos (Figura 2).

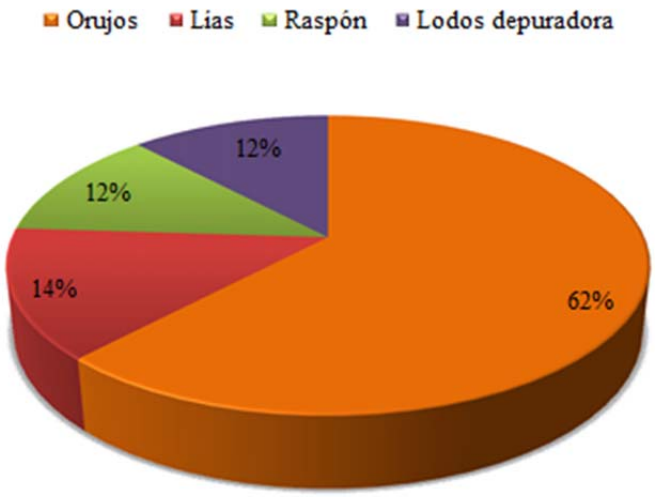


Figura 2. Distribución aproximada de los residuos orgánicos producidos en la industria del vino.

Para conocer los distintos tipos de residuos generados en la industria vitivinícola y donde se producen, es necesario conocer la cadena de producción (Figura 3). Durante el proceso se generan 6 residuos o subproductos principalmente:

1. Sarmientos de vid (vine shoot). En estado natural la vid es una rama trepadora que puede alcanzar hasta 30 metros. La producción de la uva no es proporcional al desarrollo frondoso de la vid, por lo tanto es necesario podar para reducir la longitud de los sarmientos y obtener menos racimos pero con más grosor y mayor calidad. Según la FAO, 2004, la producción de sarmientos en España puede alcanzar los 5.4 millones de toneladas por año. Este residuo ha sido valorizado en la alimentación animal, en la producción de alquitranes, como fertilizante mediante procesos de compostaje y en la producción biomasa como combustible.

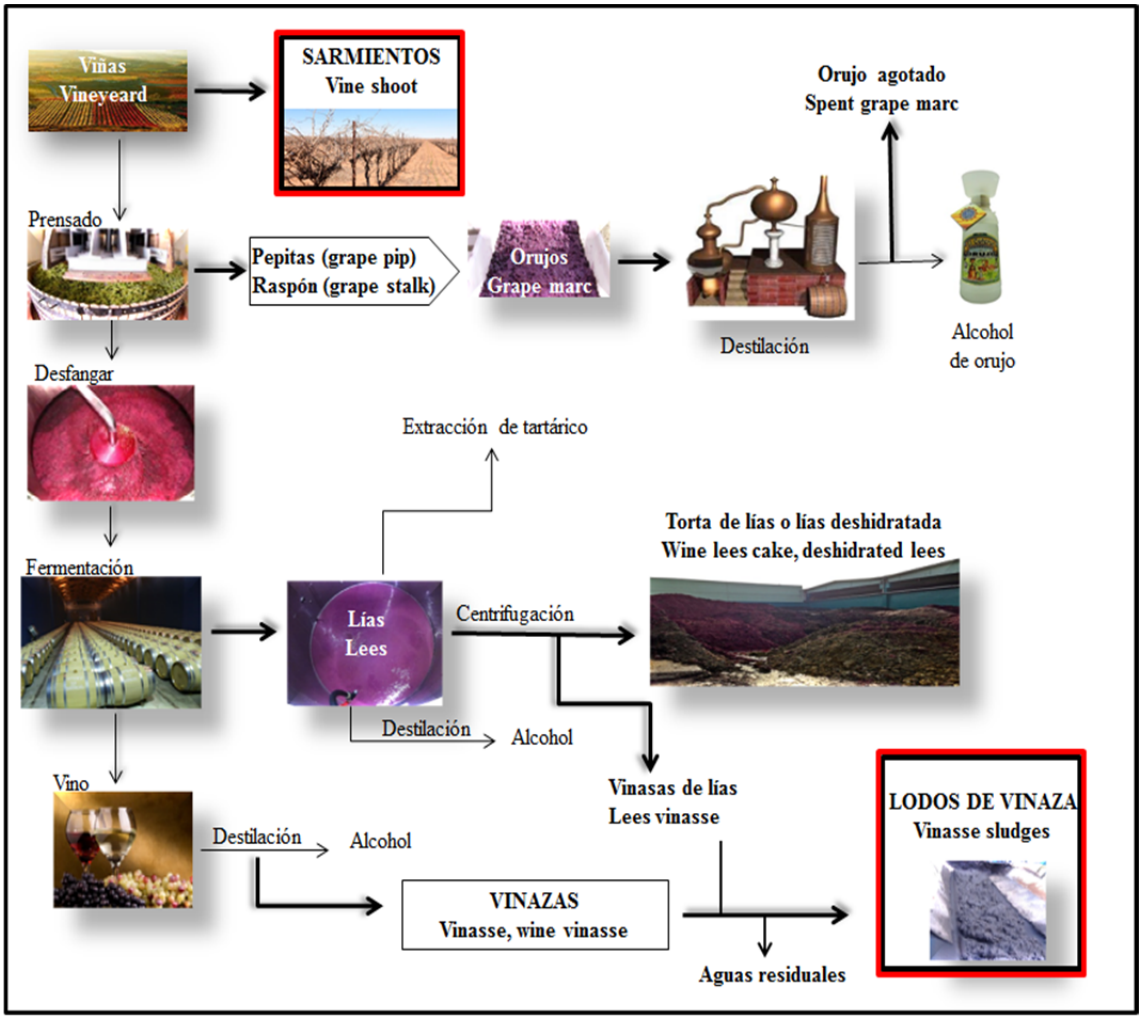


Figura 3. Proceso de elaboración del vino, residuos y subproductos generados.

2. Orujos dulces (grape marc). Es el principal subproducto del proceso de elaboración del vino. Están compuesto principalmente por piel u hollejos, semillas y restos de pulpa. Si los racimos no han sido despalillados entonces los raspones serán parte de este subproducto. Del peso total de la uva prensada, este subproducto supone entre un 10% a

un 25%. Se estima que la producción de orujos frescos en España alcanza el millón de toneladas anuales (Red Nacional de Compostaje (2015).

3. Orujos agotados (Spent grape marc). A partir de la desalcoholización y destartarización de los orujos frescos que tiene lugar en las alcoholeras, se genera este subproducto. Estos son materiales lignocelulósicos de carácter ácido con alto contenido de polifenoles y escaso en nutrientes.

4. Lías (Lees). Subproducto generado en los procesos de fermentación, estos decantan y son de color amarillo o violeta según el tipo vino. Estos sedimentos contienen sales tartáricas, levaduras muertas, restos de vino, entre otras sustancias.

5. Orujos y lías agotados (Wine lees cake). Generado a partir de la mezcla de orujos frescos con lías donde se hace una destilación del alcohol y obtención de sales tartáricas utilizadas en la remoción de restos de proteínas en suspensión después del proceso de fermentación.

6. Vinazas (Vinasses). Se genera en las alcoholeras a partir de la destilación del vino de baja calidad, orujos frescos, lías y de las aguas residuales generadas en los procesos de limpieza de los tanques de fermentación. Son residuos muy contaminantes, con mucha acidez y de baja viscosidad. Al tener elevadas concentraciones de sólidos en suspensión, DQO y DBO, es imprescindible su tratamiento.

7. Lodos de vinaza (Biosolid vinasse or vinasse sludge). Son los lodos residuales generados en las estaciones de depuración de vinazas.

1.2.2. Industria oleícola y residuos generados.

La producción de aceite de oliva es una de las industrias más importantes en España. La superficie de olivar ocupa una superficie de 2.5 millones de hectáreas, de las cuales 2.3 millones son de aceituna para almazara (Agencia para el aceite de oliva). La comunidad autónoma que mayor superficie de cultivos tiene es Andalucía con 1.5 millones ha con 174 millones de olivos cultivados.

El proceso de elaboración del aceite de oliva ha venido evolucionando con los años (Figura 4). Desde el sistema tradicional de presión hasta el sistema de centrifugación de masas en 3 fases y de 2 fases, diferenciándose por las tecnologías empleadas y las características de los subproductos durante el proceso de extracción del aceite. En la actualidad el sistema mayoritario en España es el centrifugado en 2 fases.

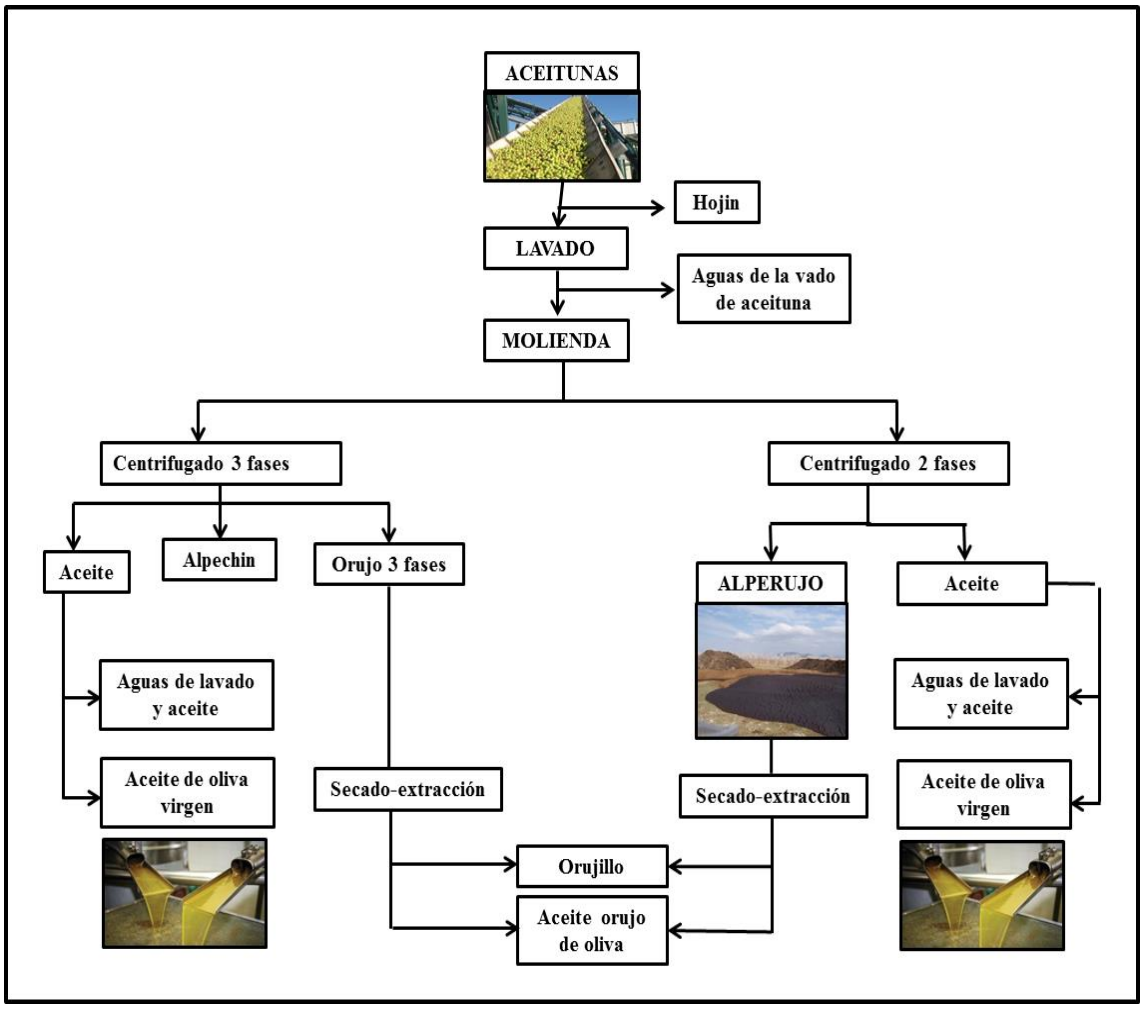


Figura 4. Elaboración del aceite de oliva, subproductos y residuos generados.

Aproximadamente a partir de una tonelada de aceituna se obtiene un 27% de aceite y un 73% de alperujo del cual 11.5% es hueso, 27% orujillo, 1,5% aceite de orujo y 60% agua (Mateo, 2011). La presencia de un residuo u otro dependerá del método de producción del aceite. Entre los más importantes están:

1. Alpechín (Olive mil wastewater): Se entiende como el residuo acuoso proveniente principalmente del sistema de tres fases, también se denomina aguas de lavado. Suele ser de color oscuro con un olor fétido. Suele tener restos de pulpa de la aceituna, sustancias pécticas y mucílagos. El alpechín es considerado un residuo muy contaminante, tiene un DQO entre 40000 y 210000 ppm y DBO entre 10000 y 150000 ppm. Tiene efectos fitotóxicos y un alto poder antibacteriano, lo que impide su rápida degradación microbiana.

2. Orujos (Olive cake), Alperujos (Wet olive cake) y orujillos (Exhausted olive cake): La textura y composición de los orujos suelen ser diferentes dependiendo del sistema de extracción del aceite. En el sistema de dos fases, el orujo posee una textura más pastosa con un mayor porcentaje de humedad al incorporar el alpechín (60%) tomando el nombre de *alperujo* u orujo dos fases. Éstos poseen un alto contenido en azúcares, pectinas y resinas (Alburquerque et al., 2004). En cuanto a su valorización, se conoce la obtención de aceite de orujo empleando procedimientos fisicoquímicos como son el secado y la extracción con n-hexano. El subproducto de este proceso se llama *orujillo* u orujo seco y extractado y se utiliza como biocombustible para la producción de energía y calor. Sin embargo, debido a las altas temperaturas necesarias para proceder a la extracción, los azúcares presentes en el *alperujo* sufren una caramelización, produciendo alteraciones en el aceite extraído teniendo como consecuencia la presencia de sustancias altamente cancerígenas como los benzopirenos. Otros usos que se ha dado al alperujo como subproducto ha sido para la alimentación de rumiantes (Molina Alcaide and Nefzaoui, 1996; Álvarez-Rodríguez; et al., 2009), la obtención de manitol (García-Granados; and Martínez-Nieto, 1993), producción de PHs (biopolímeros de hidroxialcanoatos) y bioplásticos (Cerrone, 2011), producción de pectinas (Cardoso et al., 2003) entre otros. A nivel agrícola, debido a su elevado contenido de materia orgánica, especialmente lignina, hemicelulosa, celulosa, gran contenido en potasio, pH ligeramente ácido y alto contenido de humedad se ha planteado su uso como fertilizante orgánico. Sin embargo, los bajos valores de nitrógeno, fósforo y micronutrientes hace que el *alperujo* sea mezclado con correctores con el fin de considerarlo como enmienda orgánica (Alfano et al., 2008).

2. UTILIZACIÓN DE LOS RESIDUOS ORGÁNICOS COMO ENMIENDAS.

Dentro del proceso de valorización de los residuos orgánicos está su aplicación como enmienda en suelos. Teniendo en cuenta que los niveles de carbono orgánico en suelo (COS) tienen un impacto directo en procesos como la erosión, fertilidad del suelo, el flujo de gases de efecto invernadero y la inmovilización de metales pesados y compuestos tóxicos, es de vital importancia su mantenimiento, ya que precisamente el suelo es el principal reservorio de carbono. La disminución del COS es reconocido por la Unión Europea en la temática de "estrategias para la protección del suelo," como una de las 8 principales amenazas que tiene el suelo. Por lo tanto uno de los objetivos es

mantener y mejorar los niveles de COS (Panagos et al., 2013). En los suelos de clima mediterráneo, la erosión del suelo es uno de los mayores problemas debido a la pérdida de COS y fragmentación del suelo (Figura 5), El uso de enmiendas orgánicas es una de las estrategias más sostenibles para incrementar el carbono orgánico del suelo, especialmente cuando presentan un contenido menor al 2%, como es el caso de los suelos del sureste de España.

Dentro de los beneficios del uso de enmiendas orgánicas está el desarrollo de compuestos humificados como son los ácidos húmicos y fúlvicos. Los ácidos húmicos pueden unirse a los constituyentes minerales del suelo como las arcillas, los óxidos de hierro y aluminio, formando organominerales que harán parte de la estructura del suelo, determinando la capacidad de retención de agua y sorción. A nivel microbiológico, las enmiendas modifican la estructura microbiana del suelo, enriqueciendo grupos dominantes que ayudan a mantener la estructura del suelo y aportando nuevos compuestos orgánicos como fitohormonas que son utilizadas por las plantas (El Azhari et al., 2012). A nivel ambiental, el uso de enmiendas orgánicas ayuda a inmovilizar hidrocarburos aromáticos, metales pesados y por sobre todo compuestos xenobióticos como los plaguicidas (Fernández-Bayo et al., 2009; Moreno et al., 2009).

Teniendo en cuenta que no todos los residuos orgánicos pueden ser utilizados directamente como enmiendas en suelo, ya sea por tener alto nivel de fitotoxicidad, alta carga de metales pesados o patógenos, es necesario pretratar los residuos a través de procesos de estabilización química y biológica. Dentro de las estrategias empleadas está el compostaje, el cual está condicionado al tipo de residuo y los costos económicos durante el proceso, y el vermicompostaje con otras ventajas respecto al compostaje y que es el proceso que se desarrolló durante este trabajo de tesis.

2.1. Definición y requerimientos de los procesos de vermicompostaje.

El vermicompostaje es considerado un proceso biotecnológico cuya finalidad es estabilizar residuos orgánicos mediante la acción de lombrices de tierra con capacidad detritívora; ingiriendo, triturando y digiriendo el residuo orgánico a través de sus enzimas digestivas y mediante la microflora aeróbica y anaeróbica presente en sus intestinos (Edwards and Fletcher, 1988; Aira et al., 2007).

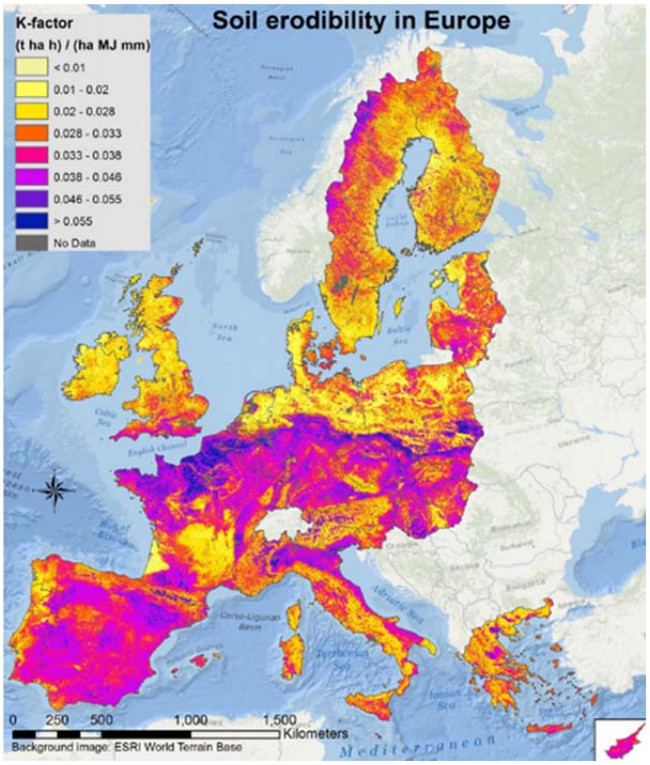


Figura 5. Mapa de erodabilidad del suelo estimado como K-factor en la Unión Europea (Panagos et al., 2014).

Las lombrices utilizadas en vermicompostaje tiene la capacidad de ingerir al día entre el 50% y el 100% de su propio peso, según la especie y tipo de residuo (Aira et al., 2006; Fernández-Gómez et al., 2010) alterando la composición física del residuo al ser fragmentado y química al incrementar la disponibilidad de nutrientes para los microorganismos que facilitan la degradación y estabilización del residuo (Aira and Domínguez, 2009; Domínguez et al., 2010). Como producto final, se obtiene un material altamente humificado (Nogales. et al., 2008; Li et al., 2011; Nogales. et al., 2014) rico en macro y micronutrientes fácilmente asimilables y una microbiota modificada con alta capacidad como biofertilizante (Aira and Domínguez, 2009; Fernández-Gómez et al., 2010). Por otro lado, la biomasa de lombriz puede ser usada como fuente proteica para consumo animal (Nogales. et al., 2008).

Dentro de los requerimientos necesarios para llevar a cabo un proceso de vermicompostaje, se debe tener en cuenta la naturaleza del residuo orgánico. La calidad fisicoquímica del residuo es importante ya que puede afectar el desarrollo natural de la lombriz. Por tal motivo, cuando el residuo orgánico puede llegar a ser letal para la lombriz, este necesita ser corregido ya sea mediante mezcla con otros residuos (Garg et al., 2005; Fernández-Gómez et al., 2010; Yadav and Garg, 2011) precompostaje (Kumar

and Shweta, 2011) o por maceración. No obstante, el residuo para ser vermicompostado deberá comprender las siguientes características:

1. Estructura física. Esta debe permitir el paso del aire y drenaje ya que las lombrices requieren de altas concentraciones de oxígeno (Edwards and Fletcher, 1988).

2. Humedad y temperatura. Debido a que la lombriz realiza el intercambio gaseoso por la epidermis, es necesario que la humedad del residuo esté por encima del 50%. La temperatura óptima para el desarrollo del proceso es 25ºC.

3. pH y Salinidad. Los valores de pH están comprendidos entre 5 y 9 aunque se desarrollan eficientemente en pH cerca a la neutralidad. Ya que las lombrices poseen una baja capacidad de osmorregulación , el contenido de sales del residuo no debe superar los 8 dS cm^{-1} ya que sería letal (Nogales. et al., 2014). Cuando el residuo tiene un alto contenido de sales se recomienda realizar lavados con agua dulce, sin embargo, esto aumentaría los costos del proceso.

4. Contenido carbono/nitrógeno. Se estima que la relación C/N óptima para el desarrollo de las lombrices debe estar entre 20-30. Si el residuo no cumple este requisito, se recomienda mezclar con otro residuo que tenga una relación C/N complementaria (Nogales. et al., 2014).

5. Niveles de amonio y amoníaco. Altos niveles pueden ser tóxicos, por tanto éstos deben estar por debajo de 0.5 mg g^{-1}. Se recomienda saturar el residuo con agua y dejarlo airear durante varios días facilitando la volatilización del contenido de amoníaco (Elvira et al., 1996).

6. Niveles de metales o sustancias tóxicas. Metales pesados, fenoles, residuos de plaguicidas, etc, pueden acumularse en el intestino de la lombriz y ser tóxico no solo afectando el desarrollo de la lombriz, sino también al producto final o si la lombriz va a ser utilizado como pienso (Suthar and Singh, 2009; Singh and Suthar, 2012; Nogales. et al., 2014).

7. Actividad microbiana. Cuando el residuo tiene un alto contenido en nutrientes y microorganismos como es el caso del estiércol de animal, que además presenta una humectación óptima, se puede desarrollar una etapa de pre-compostado cuya mayor característica es el aumento de la temperatura y liberación de sustancias que pueden ser tóxicas a la lombriz (Kumar et al., 2010). En este caso la inoculación de las lombrices debe realizarse después de finalizar dicho proceso.

2.2. Monitorización y control de calidad del proceso de vermicompostaje.

El éxito de los procesos de vermicompost de residuos orgánicos depende del desarrollo eficaz de la lombriz durante su ciclo de vida dentro del residuo. Para tal fin, se debe realizar un seguimiento del proceso (Nogales. et al., 2008; Nogales. et al., 2014). Los parámetros de seguimiento comprenden tanto aquellos involucrados en el desarrollo óptimo de la lombriz, como los físico-químicos y microbiológicos que permiten evaluar la eficacia del proceso:

1. Desarrollo de la lombriz. Durante el proceso se debe evitar que la lombriz alcance elevadas densidades ya que entrarían en competencia por los nutrientes. Por otro lado, un decrecimiento de la población en la fase de aclimatación indica que el residuo no está óptimo para el desarrollo de la lombriz. La pérdida de peso podría indicar que el residuo se encuentra agotado, por tanto se llegaría al final del proceso. Conocer el ciclo biológico de la lombriz también permite conocer el estado del proceso ya que si el residuo permite la viabilidad de la lombriz, éstas alcanzan su madurez sexual que se detecta al encontrarse un mayor porcentaje de individuos clitelados. Cuando el residuo se ha agotado, estas pierden el clitelo.

2. Control físico-químico del residuo orgánico. La monitorización de la temperatura, aireación, pH es fundamental para el desarrollo de la lombriz. Para esto se recomienda llevar a cabo en sitios donde puedan mantenerse estables estas condiciones, además debido a que la lombriz es fotofóbica, se recomienda un sitio donde no esté expuesta a la luz (Edwards and Bohlen, 1995).

3. Monitorización de la actividad enzimática. Se lleva a cabo mediante al análisis de varias enzimas seleccionadas como bioindicadores del proceso de degradación de los residuos (Benitez et al., 2005; Aira et al., 2007; Fernández-Gómez et al., 2010). Dentro de las enzimas más utilizadas para este propósito están: la actividad deshidrogenasa (enzima intracelular de carácter oxidativo bajo condiciones aeróbicas), ureasa y proteasa (cataliza la hidrólisis de enlaces no peptídicos y peptídicos, respectivamente) fosfatasa (mineralización de compuestos organofosforados), β-glucosidasa (hidrólisis de enlaces glucósidos de tipo β) entre otras (Parthasarathi and Ranganathan, 1999; Parthasarathi and Ranganathan, 2000; Benítez et al., 2002; Aira et al., 2007; Shanthi et al., 2010).

4. Seguimiento microbiológico. Como algunos residuos orgánicos suelen contener microrganismos patógenos tanto para plantas como para humanos, el seguimiento de

estas poblaciones es imprescindible. Se sabe por estudios realizados, que el proceso de vermicompostaje reduce estas poblaciones a niveles de bajo riesgo (Monroy et al., 2009; Rodríguez-Canché et al., 2010). Por otro lado, también es importante el seguimiento de las poblaciones microbianas funcionales que son de importancia biológica. Para esto se han utilizado técnicas a nivel molecular basadas en la reacción en cadena de la polimerasa (PCR: *polymerase chain reaction*) de marcadores moleculares como el gen 16S rRNA y el 18S rRNA, las cuales se describirán en detalle en la sección 3.5, y técnicas analíticas, basadas en el análisis de ácidos grasos como FAME (fatty acid methyl ester) y PLFA (Phospholipid fatty acid) mediante cromatografía de gases y espectrometría de masas (Sen et al., 2008; Gómez-Brandón et al., 2011; Fernández-Gómez et al., 2012; Gómez-Brandón et al., 2012).

2.3. Control de calidad del producto vermicompostado.

Según el Real Decreto 506/2013 (BOE num164 del 10 de Julio del 2013) sobre legislación sobre productos fertilizantes, el vermicompost se ubica en el grupo 6 de "Enmiendas Orgánicas" cumpliendo una serie de requisitos para poder ser comercializado. Los microorganismos patógenos y la concentración de metales pesados corresponderían las clases A, B y C y no deberán superar los límites reglamentados con la finalidad de no ser transferidos al suelo y a la cadena trófica (Tabla 1).

3. PLAGUICIDAS.

El concepto de plaga hace relación a cualquier organismo vivo que entra en competición con los humanos o los intereses directos causando daños a cosechas, animales o al propio humano. Por ende, los plaguicidas son sustancias orgánicas o minerales aplicados por humanos para el control del desarrollo de plagas. Según la FAO, se considera plaguicida a cualquier sustancia que previene, destruye o controla cualquier plaga que pueda interferir con la producción, proceso, almacenaje, o transporte de productos de interés comercial. En relación a la protección de cosechas, se llama producto fitosanitario a los compuestos que contengan sustancias activas, protectoras o sinergistas que protejan los productos vegetales (Directiva 91/414/CEE del consejo de la Unión Europea).

Tabla 1. Requerimientos para la utilización de vermicompost para uso agrícola según Real Decreto 506/2013.

Características	**Valores requeridos**		
Propiedades Fisicoquímicas			
Materia orgánica total	≥ 30%		
Humedad	≤ 40%		
Relación C/N	< 20		
N orgánico respecto al N total	Si supera el 1%		
Granulometría	90% de las partículas < 25mm		
Propiedades Microbiológicas			
Escherichia coli	< 1000 número más probable g^{-1} (NPM)		
Salmonela	Ausente en 25 g		
Listeria monocytogenes	Ausente en 1 g		
Enterococaceae	NMP < 10^4 – 10^5 g^{-1}		
Clostridium perfringens	NMP < 10^2 – 10^3 g^{-1}		
Metales pesados	**Clase A**	**Clase B**	**Clase C***
Cadmio	0.7	2	3
Cobre	70	300	400
Níquel	25	90	100
Plomo	45	150	200
Zinc	200	500	1000
Mercurio	0.4	1.5	2.5
Cromo (total)	70	250	300
Cromo (VI)	No detectable	No detectable	No detectable

* Los productos de clase C no pueden aplicarse en dosis superiores a 5 toneladas de materia seca por ha y año.

3.1. Uso de plaguicidas y comercialización

Aunque no existen datos actualizados sobre el mercado y consumo de los plaguicidas, tanto a nivel mundial como Europeo y nacional se ha reportado que el consumo medio en 2005 fue entre 26-29 billones de dólares a nivel mundial, encabezando esta lista Estados Unidos y Canadá con un 29% (Dewar, 2005).

Los herbicidas han sido considerados como el agroquímico con mayor volumen de ventas (45%), seguido por fungicidas e insecticidas (29 y 20%, respectivamente). A nivel Europeo, la European Crop Protection (ECP) ha reportado la venta de 272.568 toneladas de plaguicidas en 2010 para 18 países de la Unión Europea (Tabla 2) (http://www.ecpa.eu/information-page/industry-statistics-eu-15-total), de las cuales

39.043 toneladas fueron vendidas en España, convirtiéndose en el segundo país con mayor consumo de plaguicidas a nivel Europeo, decreciendo un 6% con respecto al año 2005, pero incrementándose un 10% en relación al año 2009.

Cerca del 34% de las ventas de pesticidas en España corresponde a los herbicidas, seguido por los insecticidas y funguicidas con un 31 y 26%, respectivamente (Figura 6).

Tabla 2. Cantidad de plaguicidas utilizados en Europa en 2010

País	Herbicidas (T)	Fungicidas (T)	Insecticidas (T)	Otros (T)	Total (T)
Bélgica	1746	1456	655	614	4471
Dinamarca	2503	738	31	106	3378
Alemania	16675	10431	941	3378	31425
Irlanda	1650	710	19	105	2484
Grecia	1992	2765	1703	732	7192
España	**10060**	**11878**	**14335**	**2770**	**39043**
Francia	22632	29829	1033	8410	61904
Italia	6259	18736	5489	7146	37630
Hungría	4068	2846	443	1334	8691
Holanda	2429	3506	198	3049	9182
Austria	1390	1593	83	217	3283
Polonia	9322	5835	1072	1951	18180
Portugal	5167	11844	2496	1551	21058
Eslovenia	265	780	63	40	1148
Finlandia	1200	310	28	55	1593
Suecia	888	232	17	23	1160
UK	11800	4884	621	2727	20032
Noruega	574	87	5	66	732
Total	100620	108460	29232	34274	272586

T: toneladas, (European Crop Protection)

3.1.1. Consideraciones en el registro de plaguicidas.

La venta y uso de los plaguicidas está sujeto a una autorización oficial que define las condiciones de uso preservando la capacidad de producción de los agrosistemas. Se debe realizar una evaluación por cada ingrediente activo, el cual incluye la caracterización de las propiedades intrínsecas de cada sustancia identificando la peligrosidad para los animales, los humanos y el medio ambiente. La evaluación requiere: **(i)** descripción de las propiedades químicas y físicas del compuesto activo, **(ii)** condiciones de uso y seguridad del formulado, **(iii)** estudio de comportamiento ambiental, **(v)** análisis de toxicidad biológica.

Dentro de la Unión Europea, el registro de plaguicidas está sujeto por el Reglamento (UE) Nº 334/2014 del Parlamento Europeo y Consejo, donde el European Food Safety Authority es el encargado de evaluar los compuestos activos para la Unión Europea. Después de pasar todas las evaluaciones expuestas anteriormente, el compuesto activo pasa a ser autorizado por la EU, luego cada país miembro es libre de decidir si autoriza la comercialización del producto. En España la tramitación de homologación y registro está regido por la Ley 30/1992 modificada luego por la Ley 4/1999 y el Reglamento 334 de la Unión Europea.

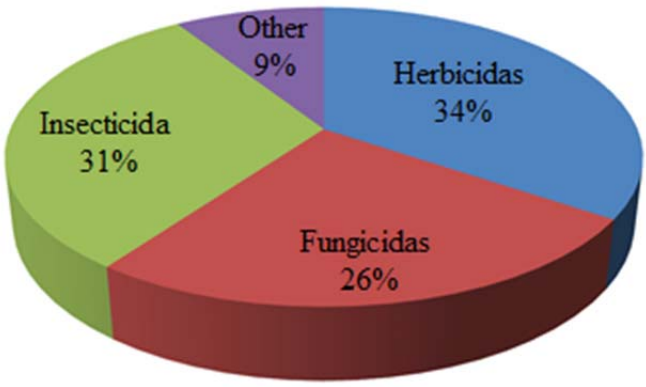


Figura 6. Venta de plaguicidas por tipo de producto en España 2010 (ECP).

3.2. Clasificación y modo de acción de los plaguicidas.

Los plaguicidas pueden ser de origen inorgánico u orgánico. Dentro de los inorgánicos encontramos: el ácido sulfúrico, bórax (Tetrabact de Na), cianamida de

calcio, cianato potásico, cloruro de mercurio, arsenito sódico y cloratos de sodio que actúan como herbicidas. También tenemos polisulfuros compuestos a base de cobre y mercurio que tienen acción como fungicidas y fluoruro de sodio. Otros derivados fluorados actúan como insecticidas. Los pesticidas orgánicos pueden ser derivados de otros organismos ya sean de origen vegetal, animal o microbiano. Como ejemplo está la nicotina que es un alcaloide que se extrae de la planta del tabaco (*Nicotiana tabacum*) o la piretrina extraída de la planta de chrysanthemum (*Chrysanthemum cinerariaefolium*) que funcionan como insecticidas.

En el mundo existen más de 500 formulaciones diferentes que son usadas para el control de plagas (Arias-Estévez et al., 2008) y están clasificadas siguiendo diferentes parámetros: por su modo de acción, según el organismo diana y por su naturaleza química (Tabla 3).

Atendiendo a su composición química y el mecanismo de acción de los herbicidas, se pueden destacar (Labrada et al., 1996):

1. Bipiridilos. Interfiere con el fotosistema 1 (FS1). Desvían el flujo de electrones en el extremo terminal del FS1, son dependientes de la luz para promover el flujo de electrones y del oxígeno para producir peróxido de hidrogeno y el radical libre hidroxilo, que es altamente perjudicial. Estos radicales interactúan con los lípidos de las membranas celulares produciendo una filtración, destruyendo el tejido foliar, provocando necrosis y desecación. Los herbicidas más importantes con este mecanismo de acción son el paraquat y Diquat. Son considerados altamente tóxicos con una dosis letal DL50 en ratas de 120 y 230 mg kg^{-1} de peso corporal. El uso repetido de estos herbicidas en cultivos perennes ha conducido al desarrollo de biotipos tolerantes de 13 especies de malezas (LeBaron, 1991).

2. Triazinas. Son inhibidoras del fotosistema 2, tiene baja volatilidad y fotodescomposición, poseen baja solubilidad en agua y se aplica comúnmente al suelo, donde es absorbido por las raíces. En climas áridos y pH altos, la residualidad de estos herbicidas puede dañar los cultivos subsiguientes, sobre todo en el caso de la aplicación con atrazina y simazina. Si se aplican coadyuvantes, las triazinas pueden aplicarse foliarmente.

Se considera que son herbicidas de amplio espectro de malezas de hojas anchas y gramíneas. Dentro de las triazinas tenemos la atrazina, como el más extensamente usado, con adsorción que va de moderada a alta, según el contenido de materia orgánica

y arcilla. La cianazina que se une con menor fuerza a los coloides del suelo que la atrazina, se aplica en pre y postemergencia en el cultivo del maíz. De todas las triazinas, la cianazina es considerada la más toxica. El metribuzin, posee una alta solubilidad en agua (1200 mg L^{-1}) y al ser relativamente móvil en el suelo puede persistir durante todo el ciclo de los cultivos anuales. La prometrina y propazina, son atrazinas que se fijan fuertemente al suelo, con limitada movilidad. La simazina tiene movilidad limitada y se fija fuertemente a los coloides del suelo por lo que debe ajustarse la dosis en relación al contenido de materia orgánica y arcilla. No se absorbe foliarmente y se aplica en pre y post-emergencia para el control de las malezas en combinación con un herbicida de contacto como el paraquat.

3. Ureas sustituidas y uracilos. También es un inhibidor del fotosistema 2 y guarda muchas características con las triazinas, con la diferencia de tener una menor persistencia en suelos (3 a 6 meses). La adsorción en suelo y la solubilidad está relacionado al número de átomos de cloro. La mayoría no son selectivas a dosis altas, por lo tanto puede usarse para el control de vegetación en general con una persistencia de 2 años. Los uracilos tienden a ser lixiviados fácilmente y son menos selectivos, se usan en cultivos perennes como cítricos. Dentro de este grupo tenemos el **Diuron** que se adsorbe al suelo fuertemente y resiste la lixiviación y se aconseja usarlo en cultivos de raíces profundas. El isoproturon presenta una movilidad limitada. El linuron, se fija fuertemente a la materia orgánica y menos a las arcillas y la dosis se ajusta al contenido de materia orgánica del suelo. El problema más importante encontrado por el uso de estos herbicidas es la contaminación de aguas subterráneas porque se han usado de manera intensiva y repetidamente.

Existen más herbicidas que son inhibidores del fotosistema 2 como los misceláneos de acción foliar, entre estos tenemos la bentazona, bromoxynil y propanil. También existen los inhibidores de la síntesis de clorofila como Aciflourfen, bifenox y oxyfluorfen. Los inhibidores de la síntesis de carotenoides, como Amitrol y Diflufenican. Los inhibidores de la biosíntesis de lípidos, dentro de los cuales tenemos Oximas, esteres de ácidos ariloxi-fenoxialcanoicos y los tiolcarbamatos. También los inhibidores de la división celular como cloroacetamidas, dinitroanilinas y carbamatos.

Tabla 3. Clasificación de los plaguicidas

Por organismo blanco		Por modo de acción		Por estructura química
Tipo	Blanco	Tipo	Acción	
Bactericida	Bacteria	Contacto	Destruye por contacto con la plaga	Orgánica o inorgánica. Mayoría son orgánicos
Fungicidas	Hongos	No-selectivo	Toxico en cosecha y semilla	Comúnmente se usan pesticidas inorgánicos a base a cobre o cal de azufre
Herbicidas	Malas hierbas	Post-emergencia	Efectivo después de cosechar o surgimiento de malezas.	Sulfato de amonio unido a Glifosato. Otros son bipiridilos, triazinas, ureas sustituidas, uracilos, sulfonilureas
Insecticidas	Insectos	Pre-emergencia	Después de la siembra, antes de cosechar o malezas emergentes.	Los insecticidas orgánicos pueden ser naturales (extractos de plantas o bacterias) o sintéticos que son la mayoría y están agrupados según su familia química o su estructura. Entre estos están: Organofosforados, organoclorados, carbamatos, piretroides, nicotínicos.
Acaricidas	Ácaros y garrapatas	Pre-siembra	Efectivo antes de la siembra	
Molusquicidas	Babosas y caracoles	protectores	Efectivo antes que el patógeno infecte la planta	
Nematicida	Nematodos	Selectivo	Toxico solamente para malezas	
Regulador de crecimiento	Procesos de crecimiento de cultivo	Esterilizante del suelo	Toxico para toda la vegetación	
Rodenticida	Roedores	Veneno estomacal	Muere la plaga por ingestión	
Preservador de madera	Organismos que destruyen la madera	Sistémico	Transportado atreves de la cosecha o plaga que luego es absorbido.	

Según el Comité de Acción para la Resistencia a los Insecticidas (IRAC) los insecticidas pueden clasificarse en 28 grupos según su modo de acción, de los cuales nombraremos los primeros 6 por ser los más usados (Sparks and Nauen, 2015):

1. Inhibidores de la acetilcolinesterasa: Afectan al sistema nervioso ya que actúan inhibiendo esta enzima. Dentro de este grupo se encuentran los **carbamatos** que derivan del ácido carbámico, dentro de las materias activas registradas se conoce formetanato, metiocarb, oxamilo y pirimicarb. Los organofosforados derivan del ácido fosfórico y presentan un átomo de fosforo en su estructura en forma de ésteres unido a oxígeno, carbono, azufre y nitrógeno. Dentro de estos se conoce clorpirifos, dimetoato, etoprofós, metil-clorpirifós y metil-pirimifós.

2. Antagonistas del receptor GABA (ácido gamma aminobutírico) en el canal cloro. Estos insecticidas interfieren con los canales de cloruro en la membrana de las células nerviosas, interrumpiendo la transferencia de iones que realiza el neurotransmisor GABA ocasionando hiperactividad neuronal. Dentro de estos encontramos 2 grupos, los ciclodieno organoclorados como clordano, endosulfan y lindano. Los fenilpirazoles como fipronil.

3. Moduladores del canal de sodio. De acción nerviosa, los insecticidas de este grupo interfieren en los canales de sodio, los cuales están involucrados en la propagación de potencialidad de acción a lo largo de las células nerviosas. El efecto producido es una hiperactividad y bloqueo del nervio. Dentro de estos encontramos los piretroides, como piretrinas, acrinatrin, betaciflutrín, ciflutrín entre otros. En otro subgrupo se encuentra el DDT y metoxicloro los cuales fueron prohibidos por la EPA en 1973 por su persistencia y alta toxicidad.

4. Antagonista del receptor nicotínico de la acetilcolina. Los **Neonicotinoides** actúan como antagonista en el receptor nicotínico postsináptico lo que produce una sobreestimulación e hiperactividad. Dentro de estos encontramos acetamiprid, clotianidina, **imidacloprid**, tiacloprid, tiametoxam.

5. Activadores del receptor alostérico nicotínico de la acetilcolina. Mediante un sistema de proteínas se acoplan a los receptores nicotínicos de acetilcolina permitiendo el flujo del ion sodio al interior de la célula post sináptica. Un ejemplo es el spinosan.

6. Activadores del canal de cloro. Tiene acción nerviosa y muscular. Dentro de este encontramos avermectinas y milbemectinas. Éstos estimulan la liberación del neurotransmisor inhibitorio GABA en el axón, potencializando la fijación de éste en los receptores post-sinápticos, inhibiendo la transmisión de los impulsos eléctricos en las uniones neuromusculares, manteniendo activados los canales de cloro por más tiempo provocando la paralización y muerte de los insectos.

3.2.1. Clasificación toxicológica

Teniendo en cuenta que los plaguicidas tienen un efecto letal frente a determinados organismos vivos no diana cuando se aplica la dosis recomendada, es necesario también conocer los efectos adversos que puedan tener en los humanos o demás mamíferos. Por esta razón la Organización Mundial de la Salud (OMS) ha propuesto clasificar los plaguicidas de acuerdo a su toxicidad, composición química y formulación (Tabla 4) a través del conocimiento de la dosis letal media (LD_{50}), que significa la dosis requerida para matar el 50% de la población de prueba, en el caso de la clasificación dada por la OMS, ésta se basa en el peso corporal en ratas.

Tabla 4. Clasificación de los plaguicidas según su toxicidad (OMS)

Clase		LD_{50} en ratas (mg kg^{-1} peso corporal)			
	Categoría	Oral		Dermal	
		Sólido	Líquido	Sólido	Líquido
Ia	Extremadamente peligroso	≤ 5	≤ 20	≤ 10	≤ 40
Ib	Altamente peligroso	> 5	> 20	> 10	> 40
II	Moderadamente peligroso	> 50	> 200	> 100	> 400
III	Ligeramente peligroso	>500	>2000	> 1000	> 4000

3.3. Dinámica de plaguicidas en el suelo.

Debido a la presencia de plaguicidas en aguas subterráneas, cuerpos de aguas (ríos, lagos, mar) así como también en el suelo, es de gran interés estudiar los procesos involucrados en la dinámica de los plaguicidas en el medio ambiente. El

comportamiento de los plaguicidas en el suelo está sujeto a procesos que permiten su retención y persistencia (Pierzynski. et al., 2000). Cuando el plaguicida es aplicado y entra en el suelo, su dinámica dependerá de varios factores como son la volatilización, lixiviación, sorción, escorrentía y degradación, los cuales dependerán también de las propiedades físico-químicas del propio plaguicida (Figura 7).

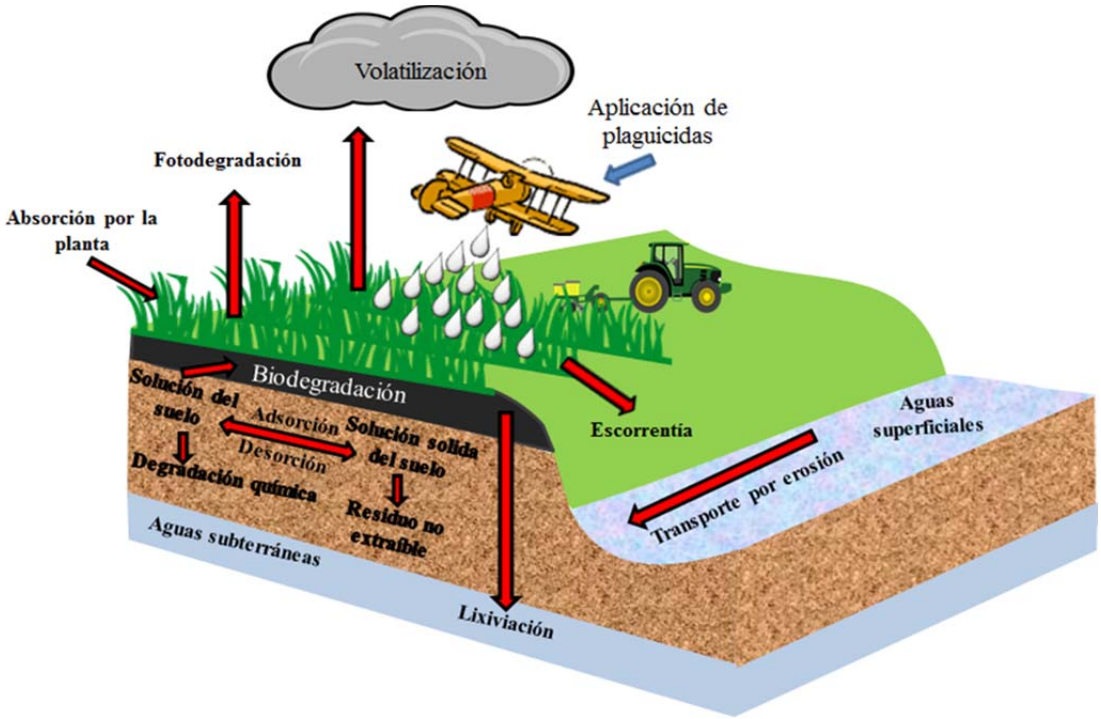


Figura 7. Dinámica de los plaguicidas en el suelo.

3.3.1. Proceso de adsorción y factores involucrados.

Se entiende por adsorción el proceso de adhesión de un soluto en fase líquida a una interfaz sólido-líquido (Calvet, 1989; Weber et al., 1991). Este proceso implica la atracción del compuesto hacia la superficie de la partícula durante un tiempo y depende de la afinidad del compuesto por la superficie, lo que indica que el proceso puede ser reversible. En suelos es difícil experimentalmente establecer diferencias entre la adsorción, absorción (penetración más o menos uniforme en la fase sólida), precipitación (formación de una fase cristalina en la superficie de las partículas del suelo) y los enlaces no reversibles de tipo covalente entre el compuesto y constituyentes del suelo.

Por tal razón el término sorción involucra todos estos procesos señalados (Koskinen and Harper, 1990).

La adsorción de los plaguicidas viene dada por los componentes físicos del suelo, como por ejemplo contenido y naturaleza de la materia orgánica (Wauchope et al., 2002; Gunasekara and Xing, 2003; Ahmad et al., 2006; Fernández-Bayo et al., 2009), el contenido y tipo de arcilla (Dios-Cancela et al., 1990; Murphy et al., 1992; Fogg et al., 2004), los óxidos e hidróxidos de Fe y Al, la asociación órgano-mineral (Szeto and Price, 1991), pH del suelo (Boivin et al., 2005) y la prevalencia de algunos cationes en solución (Murphy et al., 1992). Sin embargo, la fracción adsorbida de un plaguicida puede variar dependiendo de las propiedades físico-químicas del plaguicida, como son la solubilidad, potencial de ionización, longevidad, volatilización, extractabilidad y reactividad (Weber et al., 1991). Cuando un plaguicida es adsorbido se considera que no está disponible para las plantas y microorganismos, no obstante, en algunos casos estos pueden ser descompuestos a otros metabolitos por procesos de transformación química o microbiana (Arbeli and Fuentes, 2007).

La adsorción de plaguicidas por los componentes del suelo está determinada por una serie de interacciones como son las fuerzas de dispersión de London-Van Der Waals, los enlaces hidrógeno, la transferencia de cargas, el intercambio iónico o las interacciones hidrofóbicas (Kah et al., 2007). La metodología para evaluar la capacidad de adsorción del suelo es a través de las cinéticas de adsorción que permiten, a escala de laboratorio, cuantificar la adsorción en función del tiempo requerido para alcanzar el equilibrio de partición del soluto en solución con el adsorbente. Por otro lado, están las isotermas de adsorción, la capacidad adsorbente del suelo frente a diferentes concentraciones del plaguicida a una temperatura constante y considerando el tiempo de contacto sólido-solución donde se alcanza el equilibrio.

3.3.1.1. Isotermas de adsorción.

Los estudios de isotermas de adsorción son llevados a cabo bajo condiciones de presión, temperatura y cantidad de adsorbente constante. Para ello se emplean expresiones matemáticas que relacionan la concentración de plaguicida en las partículas

del suelo en función de la concentración de plaguicida encontrada en la solución del suelo (Ecuación 1).

$$\frac{x}{m} = K_f \times C^{\frac{1}{nf}} \qquad \text{Ec 1}$$

donde *x/m* (µg/g) es la cantidad de compuesto adsorbido, *C* (µg mL^{-1}) es la concentración del plaguicida en solución en situación de equilibrio y *Kf* y 1/*nf* son constantes empíricas de Freundlich, la primera es la capacidad del sorbente para adsorber el plaguicida y la segunda describe la linealidad de la isoterma o afinidad del sorbente por el soluto.

Existen otras aproximaciones para describir la distribución en el suelo, por ejemplo la isoterma tipo Langmuir cuando la isoterma no es lineal y la lineal (Figura 8) las cuales vienen en función del valor de 1/n (Giles et al., 1960).

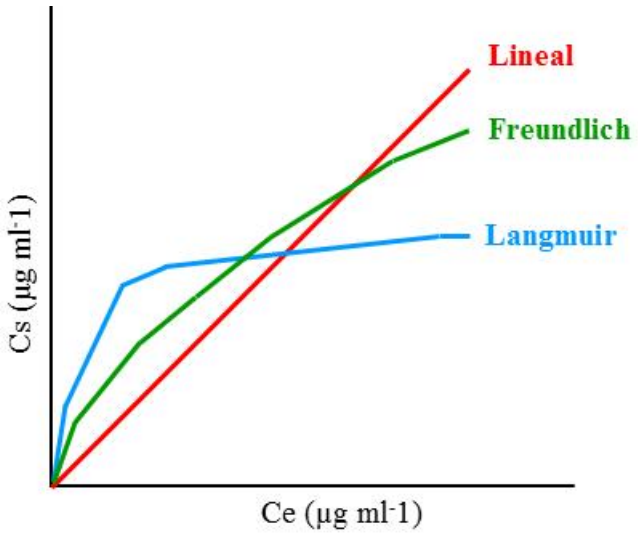


Figura 8. Isotermas de adsorción.

La materia orgánica y el contenido de arcilla del suelo son los factores más relevantes en los procesos de adsorción de plaguicidas en suelos. Numerosos trabajos han descrito una correlación positiva entre el contenido de la materia orgánica y las constantes de partición o distribución (Ahmad et al., 2001; Spark and Swift, 2002; Romero et al., 2006; Fernández-Bayo et al., 2009). Estos valores del coeficiente de distribución (*Kd* o *Kf*) pueden ser normalizados en función del contenido de carbono

orgánico del suelo (*Kco*) (Ecuación 2), reflejando su potencial de adsorción. Sin embargo, cuando el contenido de arcilla es alto, el coeficiente de adsorción puede variar, por tanto debe normalizarse en función del contenido de arcilla del suelo.

$$K_{co} = \frac{K_d}{\%\, CO} \times 100 \qquad \text{Ec. 2}$$

3.3.1.2. Formación de residuos persistentes

Cuando un plaguicida es incorporado al suelo, una fracción de éste es retenido en la fase sólida del suelo de forma irreversible y no puede ser rápidamente desorbido o extraído (Fernández-Bayo et al., 2008). Esta fracción puede ir aumentando con el tiempo dependiendo tanto de las propiedades físico-químicas del plaguicida como del proceso de envejecimiento de estos residuos en el suelo. Debido al poco conocimiento desarrollado en el estudio de estos procesos de envejecimiento o "aging", la persistencia de los residuos de plaguicidas en el medio edáfico se atribuye a la formación de uniones covalentes entre el plaguicida y los componentes del suelo o al secuestro del plaguicida en sus microporos (Gevao et al., 2000; Arias-Estévez et al., 2008). En estos casos, se considera que la manera de poder ser liberados es a través de un cambio de las propiedades físico-químicas del suelo o de la degradación de la materia orgánica, sin embargo estos cambios son muy lentos por lo que la permanencia de estos residuos pueden permanecer durante cientos de años (Gevao et al., 2000; Schnitzler et al., 2007).

3.3.2 Dispersión de los plaguicidas.

La capacidad de dispersión de los plaguicidas es importante ya que de esta manera se asegura el acceso a la plaga a tratar. Sin embargo, el plaguicida puede ser traslocado a otros compartimentos del medio ambiente mediante diferentes procesos:

1. Volatilización. El proceso de volatilización de plaguicidas en suelos está determinado por las condiciones ambientales como son alta humedad y temperatura, velocidad del aire y la textura del suelo cuando el plaguicida no se encuentra fuertemente adsorbido (Taylor and Spencer, 1990; Gish et al., 1995). En cuanto a las

propiedades físico-químicas del plaguicida, es importante conocer la presión de vapor, la cual permite calcular la constante de Henry (H_o) a través de la relación entre la presión de vapor y la solubilidad del plaguicida en agua y el peso molecular del compuesto. Atendiendo a esta propiedad se estima que los plaguicidas con alta presión de vapor (> $1x10^{-3}$Pa) tienen alto potencial para volatilizarse, excepto que también sean muy solubles en agua (Wauchope et al., 1992). Por ejemplo, el lindano, que con una presión de vapor de $1x10^{-5}$ Pa puede perderse en suelos húmedos cerca del 90% de la cantidad aplicada en pocos días (Glotfelty et al., 1984). Aunque existen pocos estudios, se ha descrito también la volatilización de plaguicidas a través de las plantas por procesos de fitovolatilización (Doucette et al., 2004; Jing et al., 2007).

2. Lixiviación y escorrentía. Una mayor fracción de los residuos de plaguicidas está disuelta en la solución del suelo y dependiendo de factores climáticos como la lluvia o la porosidad, textura y estructura del suelo y las características físico-químicas del plaguicida, éste puede transferirse a aguas subterráneas por lixiviación o a aguas superficiales por escorrentía. La presencia de flujos preferenciales que permiten la percolación en el suelo y el movimiento advectivo con los coloides del suelo hacen que los plaguicidas puedan llegar a aguas subterráneas (Sánchez et al., 2003; Arias-Estévez et al., 2008). La Agencia de Protección Ambiental (EPA) de los Estados Unidos, mediante los parámetros físico-químicos de los plaguicidas, determinó unos valores que indican el potencial de los plaguicidas para contaminar aguas subterráneas (Tabla 5).

Tabla 5. Potencial de los plaguicidas para contaminar aguas subterráneas.

Propiedades físico-químicas de los plaguicidas	Valores determinados
Solubilidad en agua	>30 ppm
Constante de Henry	$<10^{-2}$ atm m^{-3} mol^{-1}
Koc	< de 300 a 500
Vida media por hidrólisis	> 25 semanas
Vida media por fotólisis	> 1 semana

Otro indicador del potencial de lixiviación es el Groundwater Ubiquite Score-GUS (Gustafson 1989), que estima de manera empírica la lixiviación atendiendo a parámetros de adsorción y persistencia de los plaguicidas en el suelo (Ecuación 3). Esta ecuación permite clasificar los plaguicidas como lixiviables (GUS>2.8), no lixiviable (GUS<1.8) y de transición (2.8>GUS< 1.8) (Fushiwaki and Urano, 2001).

$$GUS = \log \mathrm{t}\frac{1}{2} * (4 - \log K_{oc}) \qquad \text{Ec. 3}$$

Cuando el suelo llega a su nivel de saturación de agua, ya sea por las lluvias, riego excesivo o escurrimiento, los plaguicidas que son muy solubles y se adsorben con baja afinidad a las partículas del suelo son fácilmente transportados por las aguas de escorrentía a las aguas superficiales. En condiciones agrícolas se ha observado una mayor prevalencia de plaguicidas en aguas superficiales que en aguas subterráneas (Lari et al., 2014).

3. **Absorción por las plantas**. Las plantas de la misma manera que absorben nutrientes de la solución del suelo, también pueden absorber plaguicidas, los cuales se distribuyen a través del xilema a todas las partes de la planta donde pueden ser acumulados o transformados por el metabolismo de la misma planta (Singh and Jain, 2003). Estos procesos están condicionados por la concentración, capacidad de translocación en la planta y biodisponibilidad del plaguicida (de Carvalho et al., 2007).

3.3.3. Degradación de plaguicidas.

El proceso por el cual el plaguicida es transformado a metabolitos se denomina degradación, pero cuando este proceso es llevado a una completa transformación a dióxido de carbono se denomina mineralización. Sin embargo, en algunos casos estos procesos llevan a la formación de metabolitos con mayor toxicidad y persistencia que el compuesto parental en suelos. Por otro lado, una alta tasa de degradación puede perjudicar la eficiencia del control de la plaga a tratar (Arbeli and Fuentes, 2007). La degradación de plaguicidas en suelos está dirigida por procesos abióticos y bióticos, donde la efectividad está asociada a los factores físico-químicos del compuesto y del suelo, como también de la capacidad metabólica de la microbiota del medio edáfico.

3.3.3.1. Degradación abiótica.

Los dos procesos más importantes en la degradación abiótica son la degradación química y la fotodegradación. Estos procesos dependen de las características intrínsecas del compuesto y factores físico-químicos del suelo como son el pH, la humedad, temperatura, contenido de materia orgánica y minerales (Malato et al., 2001; Felsot et al., 2003; Kah et al., 2007). La degradación química de los plaguicidas está sujeta a procesos de hidrólisis, oxidación, hidroxilación, alquilación, isomerización, sustitución, etc (Cathleen, 1992; Salvestrini et al., 2002). Esta degradación es dominante una vez que el plaguicida ha atravesado la zona radicular. La fotodegradación de plaguicidas tiene lugar en los primeros milímetros de suelo y está asociada a la capacidad de absorción de fotones, especialmente los que se encuentran en la longitud de onda del infrarrojo, ultravioleta y luz visible. La mayoría de los plaguicidas en el suelo sufren proceso de degradación por vía indirecta ya que su absorción en esa región del espectro es escasa. Además, la fotodegradación está limitada por el contenido de MO (ácidos húmicos y fúlvicos), la profundidad, textura, temperatura y humedad (Romero et al, 1998; Katagi, 2004). Dentro del tratamiento de residuos de plaguicidas por este proceso, se encuentra la fotocatálisis que es la aceleración de una fotorreacción mediante un catalizador, el fotofenton (Vermilyea and Voelker, 2009), donde la oxidación de hierro ferroso por el peróxido de hidrógeno generan radicales hidroxilo altamente reactivos que aceleran la degradación del contaminante y la fotólisis, que es el la ruptura de la molécula por fotones.

3.3.3.2. Degradación biótica.

Dentro del proceso de degradación biótica encontramos la biodegradación microbiana y la fitodegradación, los cuales son los procesos más dominantes en la dinámica de plaguicidas en el ambiente, especialmente dominante en la zona radicular.

3.3.3.2.1. Degradación microbiana

La degradación microbiana o biodegradación de los plaguicidas, es el principal proceso de disipación de los plaguicidas en suelos. Este proceso es llevado a cabo por reacciones mediadas por enzimas que son producidas por la microbiota del suelo, realizando una conversión a formas inactivas y menos tóxicas (Hussain et al., 2009). Dependiendo de las capacidades bioquímicas, adaptabilidad y resistencia de los microorganismos, en la degradación de plaguicidas pueden darse diferentes vías de degradación por procesos de metabolismo y co-metabolismo. La biodegradación por metabolismo microbiano hace referencia a la completa mineralización ya sea por un solo microorganismo o varias poblaciones microbianas formando un consorcio en la degradación (Pattanasupong et al., 2004; Chirnside et al., 2007). La degradación por co-metabolismo hace relación a la degradación no específica, es decir, los xenobióticos tienen homología con el sustrato original que utilizan las enzimas, sin embargo este tipo de actividad no contribuye al crecimiento del microorganismo degradador (Horvath, 1972; Sylvia, 1998), por el contrario, puede llegar a formar metabolitos que pueden ser más recalcitrantes que el compuesto parental como es el caso del ácido 6-chloronicotinico (Casida, 2011), que es el metabolito producido en la degradación del insecticida imidacloprid, y de la 3,4-dichloroanilina, que es el metabolito más persistente y tóxico producido en la degradación del herbicida diuron (Giacomazzi and Cochet, 2004).

3.3.3.2.2 Fitodegradación

La fitodegradación hace referencia a la modificación que sufre en este caso el plaguicida por efecto del metabolismo propio de la planta. La fitodegradación forma parte del proceso de fitorremediación, el cual involucra también la fitovolatilización y la fitoextracción. El proceso de fitodegradación consiste en un ataque enzimático a los grupos funcionales del plaguicida, seguido de una reacción de transformación donde la enzima adiciona un azúcar al contaminante como glutatión transformándolo en un compuesto menos tóxico y transportable a vacuolas celulares (Hussain et al., 2009). Dentro de las enzimas involucradas tenemos el citocromo P450, la glutation-S-

transferasa, peroxidasas, carboxilesterasas, peroxigenasas (Wolfe and Hoehamer, 2004). El proceso de fitodegradación ha sido estudiado para la descontaminación de aguas por hidrocarburos (Al-Baldawi et al., 2015) y plaguicidas como el DDT (Garrison et al., 2000) y el 2,4-diclorofenol (Laurent et al., 2007).

3.3.3.3. Factores que afectan la degradación de plaguicidas en suelos.

Tanto los procesos de sorción como de degradación de plaguicidas están condicionados a las propiedades físico-químicas del suelo y del plaguicida (Sylvia, 1998). Por tanto podemos agrupar aquellos factores que son propiamente del suelo y los que son intrínsecos de los plaguicidas.

3.3.3.3.1 Propiedades del suelo que afecta la dinámica de los plaguicidas.

Dentro de las propiedades más importantes del suelo que pueden afectar el comportamiento de los plaguicidas en el suelo se encuentran:

1. Textura del suelo. La dinámica de los plaguicidas está afectada por la composición del suelo. Éstos están conformados de acuerdo a la proporción de partículas minerales de diferentes tamaños. Los plaguicidas pueden ser adsorbidos ya sea por adsorción física o partición, o por adsorción química debida al intercambio catiónico o protonación, enlaces hidrógeno y coordinación (complejos metálicos). Los suelos arenosos con un tamaño de partícula entre 2.00-0.05 mm son altamente permeables y presentan escasos coloides, por lo que pueden facilitar el movimiento de los plaguicidas a capas freáticas. Por el contrario, en los suelos arcillosos con un 40% de partículas de arcilla, con un tamaño <0,002 mm, la adsorción de plaguicidas está influenciado por la reactividad del área superficial de los diferentes tipos de arcilla y la presencia de grupos hidroxilo en superficie o cationes de intercambio que permiten establecer diferentes tipos de enlace con los grupos funcionales de los plaguicidas. Cuando la fuerza de enlace en las interláminas de la arcilla es débil, la entrada de moléculas orgánicas es más fácil separando las láminas como ocurre con las montmorillonitas y vermiculitas, pero esto no ocurre con la caolinita cuyo espacio interlaminar es menor (Khan, 1980).

La presencia de cationes como H^+, K^+, Mg^+, Ca^+ y Na^+, los cuales están unidos a los minerales de arcilla por interacciones electrostáticas, pueden pasar a la solución e intercambiarse por otros cationes que se encuentran en dilución. Este proceso de intercambio catiónico es considerado como uno de los mecanismos más importantes a tener en cuenta en la adsorción de plaguicidas (Braschi et al., 2011). Los cationes adsorbidos por la superficie de las arcillas están rodeados de agua de hidratación, la cual es más ácida que la solución del suelo. Este efecto de acidez superficial es un factor importante en los procesos de adsorción de herbicidas que contienen grupos nitrógeno, los cuales se protonan en las superficies ácidas y pueden luego ser adsorbidos por mecanismos de intercambio catiónico.

2. Materia Orgánica. La materia orgánica (MO) está compuesta mayormente por residuos vegetales degradados, biomasa de organismos y material de neogénesis. La descomposición de los residuos vegetales y animales por el ataque enzimático de los microorganismos que habitan en el suelo es lenta. Los productos que se generan en esta etapa de degradación son macromoléculas policondensadas que forman estructuras complejas de las cuales las más conocidas son las sustancias húmicas y fracciones de la materia orgánica disuelta (DOM) (Fernández-Bayo et al., 2008).

La MO ayuda a mantener la estructura del suelo, cubriendo los poros creados por las raíces y la pedofauna del suelo. También afecta el flujo de agua en la porosidad capilar del suelo ya que por la coexistencia de zonas hidrofílicas e hidrofóbicas en la misma estructura de la MO hace que ésta tenga la capacidad de retener la humedad o repeler el agua, decreciendo así el flujo de agua por los poros. Por otro lado, la MO puede formar agregados con especies inorgánicas como óxidos e hidróxidos de Fe y Al dando estabilidad a la estructura del suelo y a su vez puede interactuar con los agroquímicos mediante enlaces de hidrogeno, fuerzas de Van der Waals y uniones hidrofóbicas (Ahmad et al., 2001).

Las sustancias húmicas tiene la capacidad de formar complejos organominerales que condicionan el estado de agregación de las arcillas en el suelo, afectando la persistencia y degradación de los xenobióticos (Calvet, 1989). La materia orgánica disuelta (DOM) es la cantidad de materia orgánica capaz de disolverse en condiciones de campo. Es importante en los procesos biogeoquímicos del carbono, nitrógeno, fósforo y en el transporte de contaminantes (Cox et al., 2000; Solinger et al., 2001; Spark and Swift,

2002). La DOM se genera a partir de los fotosintatos, lixiviación y descomposición de MO por los microorganismos, siendo éstos el mayor agente en la formación de la DOM (Guggenberger et al., 1994; McDowell, 2003).

Algunos estudios reportan la efectividad de la DOM en el aumento de la solubilidad de agroquímicos y está controlado por el tamaño molecular de la DOM y su polaridad (Chiou et al., 1986). Por otro lado, se ha descrito que la naturaleza de la DOM (exógena o endógena) afecta la adsorción y desorción de herbicidas como dimefuron, carbetamida y atrazina (Barriuso et al., 1992). Debido a que los herbicidas dimefuron y atrazina son menos solubles que la carbetamida, estos fueron mayormente adsorbidos después que el suelo fuera pre-tratado con una solución de DOM. Por el contrario, la alta solubilidad de carbetamida hace que decrezca su adsorción en el suelo ya que los compuestos orgánicos de DOM se ubican en los sitios hidrofílicos durante el pretratamiento con DOM. Otros estudios describen la rápida adsorción de diquat y paraquat por DOM y su posterior adsorción en el suelo (Pennington et al., 1991).

En relación al efecto de MO en la biodegradación de plaguicidas, se ha observado que la MO controla también estos procesos ya que suministra los nutrientes que utilizan los microorganismos para crecer (Briceño et al., 2007). La adición de materia orgánica mediante enmiendas de compost y vermicompost se considera como un método de inoculación de microorganismos exógenos y exoenzimas al suelo (Benoit and Barriuso, 1997; El Azhari et al., 2012).

3. pH del suelo. La concentración de iones hidrógeno tiene un efecto complejo sobre los pesticidas. Este puede afectar la estabilidad de los minerales de arcilla, la capacidad de intercambio catiónico y la ruptura química y biológica de los compuestos orgánicos (Werkheiser and Anderson, 1996). Sin embargo, también puede afectar en la solubilidad de la MO haciéndola más hidrofilia y provocando una reducción de la adsorción de compuestos con alta hidrofobicidad como el herbicida diuron (Sheng et al., 2005).

La biodegradación de plaguicidas es un proceso que se ve afectado por el cambio de pH ya que las enzimas involucradas son dependientes de este parámetro (Okeke et al., 2002). Este afecta el estado de ionización de las enzimas, modificando la afinidad de sustrato y afectando la actividad microbiana. Muchos de los grupos de microorganismos están asociados al pH del suelo, por ejemplo los hongos crecen eficientemente a pH bajo, mientras las poblaciones bacterianas son abundantes a pH cerca a la neutralidad

(Smith and Doran, 1996). Por lo tanto, la relación entre estas dos poblaciones las cuales afectan la biodegradación de plaguicidas y la acumulación de metabolitos están reguladas por los cambios de pH.

4. Profundidad del suelo. Se considera que la profundidad del suelo es uno de los factores que regulan la degradación de plaguicidas. La abundancia de las poblaciones microbianas van decreciendo a medida que se profundiza en el suelo, esto es debido a la disminución de la materia orgánica, afectando la mineralización y por ende la degradación de plaguicidas (Bending and Rodriguez-Cruz, 2007).

5. Temperatura y contenido de humedad del suelo. La temperatura y el contenido de agua del suelo están entre los parámetros más importantes en la regulación de la actividad microbiana. El incremento de la temperatura está asociado a la perdida de actividad de algunos plaguicidas y con el incremento de la actividad microbiana, acelerando la degradación de estos compuestos. Se ha observado que la degradación de atrazina es mayor con el incremento de la temperatura y el contenido de humedad. El mismo comportamiento ha sido observado con linuron y metolachlor en suelos franco arcilloso (Walker and Zimdahl, 1981). Sin embargo, con diuron ocurre lo contrario, en algunos suelos donde el incremento de la humedad hace que este herbicida sea arrastrado por lixiviación a aguas superficiales (Sebaı et al., 2010; Broznic and Milin, 2012).

3.3.3.3.2. Propiedades de los plaguicidas que intervienen en su dinámica.

Las propiedades físico-químicas de los plaguicidas son importantes en la dinámica que éstos van a llevar en el medio ambiente. Entre las más importantes encontramos:

1. Volatilización. El grado de volatilización de un plaguicida depende de propiedades físico-químicas como la presión de vapor y la solubilidad como fue explicado en el apartado 3.3.2. La tasa de volatilización está expresada como el tiempo necesario para reducir a la mitad la concentración del plaguicida en agua. Teniendo en cuenta estos valores, se sabe que plaguicidas como Dieldrin tiene una tasa de volatilización media de 327 días, Lindano 115, DDT 45 y disolventes como tolueno y benceno una media de 2.8 horas (Pierzynki et al., 2005). No obstante, hay que considerar que la tasa de

volatilización en el suelo de los plaguicidas es menor, ya que su presión de vapor en el suelo es menor que en agua debido a las interacciones con los componentes del suelo.

2. Solubilidad. Este factor es uno de los más importantes a tener en cuenta cuando va a ser aplicado. La predicción de su comportamiento en agua y su movilidad en suelos depende de otros factores como el pH, la temperatura, la fuerza iónica y además está afectado, como se mencionó en el apartado 3.3.2, por sustancias como la materia orgánica disuelta (DOM). Existen 2 métodos para estimar la solubilidad de una molécula orgánica: **(i)** basado en su estructura química (*Kps*) y **(ii)** en el coeficiente de partición octanol/agua (*Kow*). La solubilidad de los plaguicidas esta correlacionada con la persistencia de éste en suelos. Sin embargo, ésta puede cambiar cuando el plaguicida coexiste con otras sustancias como la DOM (Li et al., 2005).

3. Coeficiente de adsorción de carbono orgánico (*Koc*). Se le conoce como coeficiente de adsorción normalizado con el contenido de carbono orgánico del suelo y mide la tendencia de un compuesto orgánico a ser adsorbido por el suelo, corregido para el contenido de c.o. del suelo. Este valor es específico para cada plaguicida. Un *Koc* elevado (entre 10000 a 100000) indica una adsorción fuerte en la materia orgánica del suelo, por el contrario valores menores a 10 se considera una adsorción débil por tanto el plaguicida puede desplazarse en el medio y llegar a contaminar los recursos hídricos o liberarse a la atmósfera.

4. Coeficiente de partición iónica. Esta propiedad hace referencia a la capacidad de los plaguicidas de comportarse como ácidos y bases débiles en solución. En el primer caso, el compuesto es capaz de aceptar un par electrónico y en el segundo, puede donar un par electrónico. La constante de ionización por tanto hace referencia a un ácido (Ka) o a una base (Kb). Si le sacamos el logaritmo tendremos pKa y pKb, parámetros que definen el pH en el cual la molécula se comporta como especie neutra o ionizada (Ecuación 4),

$$pKa = pH - log\frac{[A^-]}{[AH]} \qquad \text{Ec. 4}$$

donde [A^-] es la forma ionizada y [AH] la no ionizada. Un compuesto básico puede ser considerado 100% asociado o disociado cuando el pH es aproximadamente 2 unidades por debajo o por encima del pKa, respectivamente (Tabla 6).

Tabla 6. Consecuencias ambientales relacionadas a las propiedades ácidas o básicas de los plaguicidas (Hornsby et al., 1996).

Valores de pKa o pKb	Especies dominantes del plaguicida dentro de un rango de pH normal del suelo (5-8)	Consecuencias ambientales
$pK_a < 3$	X^- (Anión)	Altamente móvil en suelos, a menos que forme un complejo químico. Menos móvil en condiciones muy ácidas, muy soluble y no volátil.
$pK_a > 10$	XH (neutro)	Comportamiento no iónico, excepto en condiciones extremadamente alcalinas. Menos soluble y movible que el anión. Posiblemente volátil.
pK_a 3-10	Ratio X^-/XH	Si el pH es cerca al pK_a, la movilidad, solubilidad y volatilidad será sensible al cambio de pH.
$pK_a < 4$	XH^+ o X^+ (catión)	Muy movible (adsorbido en la superficie de las arcillas), muy soluble. Al ser altamente adsorbido su vida media es larga y escasa la actividad biológica. No-volátil.
pK_b 4-11	$(XH)^+/X$ $X^+/X(OH)$	Si el pH está cerca al pK_b, la solubilidad, volatilidad y movilidad serán sensibles al pH. La adsorción extrema provoca una acidez superficial del suelo.
$pK_b > 11$	X o X(OH) (neutro)	Se comporta como material no iónico excepto en condiciones extremadamente acida. Es más móvil y menos soluble que el catión. Posible volatilización.

3.4. Técnicas analíticas para determinación de plaguicidas en suelo.

Existe una diversidad de técnicas analíticas que permiten monitorizar el comportamiento de los plaguicidas en diferentes ambientes. Entre las más ampliamente usadas en química analítica están las técnicas cromatográficas como cromatografía de gases (GC) y cromatografía líquida de alta eficiencia (HPLC).

Estas técnicas analíticas son las más utilizadas para el análisis de plaguicidas. La utilización de una u otra técnica depende de varios factores como son las propias del compuesto, la selectividad, sensibilidad, disponibilidad y costes. Debido a que GC y HPLC fueron las técnicas empleadas durante este trabajo de tesis, vamos a describir brevemente cada una de ellas**:**

1. Cromatografía de Gases (GC): Es una técnica muy sensible que ha permitido separar una gran cantidad de plaguicidas gracias al desarrollo de detectores muy sensibles y selectivos como el de captura de electrones (ECD), el nitrógeno-fósforo (NDP) y el de espectrometría de masas (MS). Esta técnica permite la determinación de compuestos estables térmicamente pero que pueden ser volátiles o moderadamente volátiles (van der Hoff and van Zoonen, 1999). Aunque los dos primeros detectores son ampliamente usados, el detector MS que utiliza como técnica de ionización el impacto de electrones, ha permitido identificar varios compuestos de plaguicidas (Lehotay, 2000; Castillo et al., 2011; He et al., 2015; Nan et al., 2015) o la elucidación estructural empleando espectrómetros de masas en tándem, donde la detección del compuesto es comparado con los espectros de estándares.

2. Cromatografía líquida de alta eficacia (HPLC): Debido a que algunos plaguicidas son de elevado peso molecular, baja volatilidad, alta polaridad e inestabilidad térmica, el uso de HPLC ha permitido analizar dichos compuestos. Los detectores más comunes son: ultravioleta (UV) con diodos integrados (UV-DAD (UV), fluorescencia (FD) y MS. Esta técnica permite acoplar una gran variedad de columnas para la separación de diferentes compuestos y además es posible realizar gradientes de elución permitiendo separar varios compuestos a la vez (Gámiz-Gracia et al., 2005).

La eficiencia de la detección de un compuesto por cromatografía depende mucho del éxito en la extracción que se realiza. Dentro de las más habituales están:

a) **Extracción Líquido-Líquido (LLE)** que se basa en la separación de los componentes de la muestra en dos disolventes inmiscibles, generalmente una fase acuosa y una orgánica (diclorometano, hexano, etc.).
b) **Extracción Sólido-Líquido (SLE)** donde los plaguicidas en muestras sólidas son separados al entrar en contacto con uno o varios disolventes. Para forzar la eficacia de extracción, la muestra con el extractante se somete a procesos de agitación, calentamiento, ultrasonido, etc, con el fin de lograr extraer la mayor cantidad de soluto de la muestra.
c) **Extracción en Fase Sólida (SPE)** se basa en la extracción y pre-concentración del analito de una fase líquida o gaseosa al pasar a una fase estacionaria sólida seguido de una elución del compuesto en un disolvente orgánico apropiado (Wells, 2003). Estas fases estacionarias están compuestas por partículas empaquetadas en el interior de pequeñas columnas, discos o cartuchos cerrados compuestas principalmente de silicato de magnesio (Florisil), carbón activado, alúmina o polímeros orgánicos.
d) **Microextracción en fase sólida (SPME)**, permite realizar un pretratamiento de la muestra, también puede ser utilizado como técnica de muestreo ya que permite la extracción y preconcentración de la muestra en un solo paso (Wells, 2003; Kudlejova et al., 2012; Ouyang, 2012). La extracción es realizada por una fibra de sílice recubierta de un adsorbente compuesto por diferentes polímeros. Esta fibra va incorporada en la aguja de una jeringa que se acopla al sistema inyector del equipo (Figura 9). Por tanto la extracción se lleva a cabo sumergiendo la fibra directamente inmersa en la muestra o manteniéndola en el espacio en cabeza que está en equilibrio con la muestra. Después se realiza la desorción que puede ser térmica para su uso en GC o mediante disolventes orgánicos para su uso en HPLC (Gaurav et al., 2009).

Figura 9. Detalle de la microfibra para la determinación de compuestos mediante microextración en fase sólida (SPME).

3.5. Herramientas moleculares para evaluar la degradación de plaguicidas.

Debido a que los microorganismos poseen una amplia batería enzimática con capacidad de mineralizar una gran variedad de compuestos xenobióticos, el uso de técnicas moleculares ha permitido caracterizar los genes y enzimas que median el clivaje de grupos funcionales específicos de los plaguicidas para su detoxificación (Karpouzas and Singh, 2006). Estas enzimas son generalmente hidrolasas, dehalogenasas, ureasas, deaminasas, dioxigenasas y reductasas (Tabla 7).

Los genes que codifican estas enzimas en algunos estudios han sido empleadas como marcadores moleculares para evaluar de forma indirecta la biodegradación de plaguicidas (El Azhari et al., 2012). Por otro lado, debido al amplio progreso en el campo de la ecología microbiana, muchas técnicas moleculares se han desarrollado con la finalidad de describir y caracterizar la diversidad funcional y filogenética de los microorganismos en diferentes ambientes, bien de manera parcial o total (Figura 10).

3.5.1. Técnicas basadas en una aproximación parcial de la comunidad microbiana.

Estas técnicas se basan en el análisis de fragmentos de genes microbianos que están presentes en todos los organismos. Estos métodos incluyen el método de amplificación basado en la reacción en cadena de la polimerasa (PCR). Mediante esta técnica, se amplifica por PCR fragmentos de genes conservados en la comunidad microbiana como el 16S rRNA en procariotas como las bacterias, y el 18S rRNA en eucariotas como los hongos.

Estos genes son ampliamente utilizados en ecología microbiana ya que son ubicuos, están estructuralmente y funcionalmente conservados con regiones variables y otras altamente conservadas (Hugenholtz, 2002; Fernández-Gómez et al., 2012; Castillo et al., 2013).

Tabla 7. Genes y enzimas microbianos involucrados en la degradación de plaguicidas Tabla extraída de Hussain et al. (2009).

Plaguicida	Género	Gen	Enzimas
Insecticida			
Organoclorinados			
Hexaclorociclohexano	*Mirobacterium, phingomonas Sphingobium*	*lin, lin*A, *lin*B, *lin*C, *lin*D, *lin*E, *lin*X	Dehalogenase, Dehidrogenasa, Dehidroclorinasa, Hidrolasa.
Imidacloprid	*Bradyrhizobium*	*cch1, cch2*	Hidrolasa
Organofosforados			
Organofosforo	*Achromobacter, Clavibacter Delftia, Escherichia Flavobacterium, Pseudomonas*	*mpd, opd, phn*E, *glp*T, *pde*A	Hidrolasa
Fenitrothion	*Burkholderia*	*mpd,* pNF1, d pNF2	Hidrolasa
Metilparation	*Plesiomonas*	*mpd*	Hidrolasa
Carbamato	*Arthrobacter, Methylobacteria Pseudomonas, Methylomonas*	*pcd*	Hidrolasa
Carbaril	*Arthrobacter*	pRC1, pRC2, pRC3	Hidrolasa
Carbofuran	*Achromobacter*	*mcd*	Hidrolasa
Thiacarbamato	*Rhodococcus*	*thc*F, *thc*G	Haloperoxidasas Hidrolasas
Herbicidas			
Atrazina/ Simazina	*Alcaligenes, Agrobacterium Aminobacter,Arthrobacter Burkholderia, Variovorax Pseudomonas, Ralstonia Rhizobium, Sphingomonas Stenotrophomonas*	*Atz* ABCDEF, *trz*ND, *psb*A1, *trz*A, *smz*A	Hidrolasa, ureasa, citocromo P-450
2,4-diclorofenoxiacético	*Brevundimonas, Stenotrophomonas Ochrobacterium, Pseudomonas,*	*tfd*A, *tfd*B, *tfd*C, ORF1, ORF6	Dioxigenasa, isomerasa, reductasa
Diuron	*Arthrobacter*	*puh*A	Hidrolasa

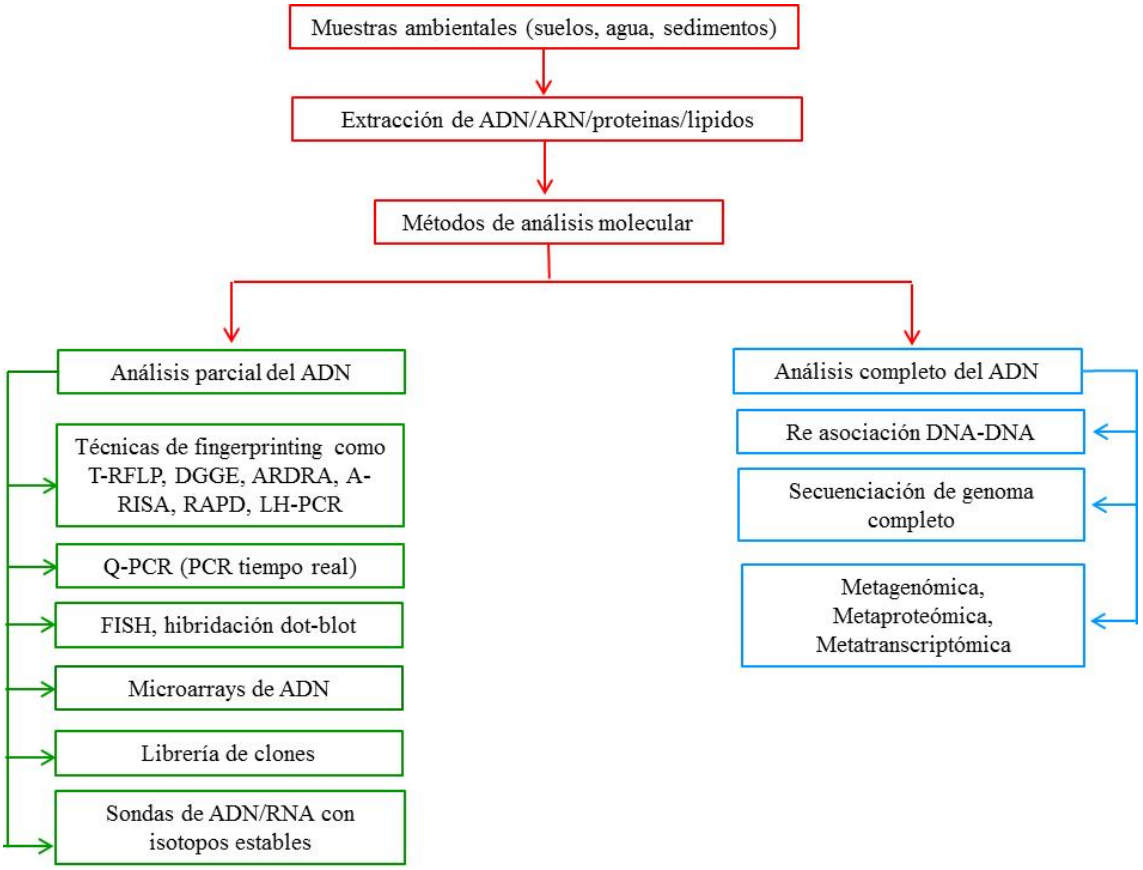


Figura 10. Técnicas moleculares utilizadas en el análisis de AND/RNA.

Los productos de PCR de muestras ambientales pueden ser analizados por diferentes técnicas:

1. Técnicas genéticas de fingerprinting. Estas técnicas permiten obtener un perfil genético basado en el análisis directo de los productos de PCR del ADN ambiental (Muyzer, 1999). Entre éstas cabe mencionar:

1.a Electroforesis en gel con gradiente desnaturalizante (DGGE) o térmico (TGGE). En el caso de DGGE (Denaturing Gradient Gel Electroghoresis) los productos de PCR son obtenidos de marcadores moleculares como los genes 16S o 18S rRNA. Éstos son electroforados sobre un gel de poliacrilamida con un gradiente compuesto por urea y formamida que permite la desnaturalización de los fragmentos de ADN (Muyzer et al., 1993). En el segundo caso, el TGGE (Temperature Gradient Gel Electrophoresis), la separación de los fragmentos de ADN es a través de un gradiente de temperatura. La variación de guanina-citosina en las secuencias de los diferentes fragmentos de PCR

determinara el comportamiento de fusión y por ende la posición de los diferentes amplicones en el gel. Para llevar a cabo todo este proceso, previamente al cebador forward de PCR se le añade un anclaje de GC en la región 5'. Esto permite la separación de las dos hebras, quedando ancladas por la cola de GC que permanece sin desnaturalizar. Luego de este proceso las bandas pueden ser cortadas del gel, reamplificadas, secuenciadas o colocadas en una membrana de nylon para ser hibridadas con una sonda específica para cada grupo taxonómico. Sin embargo, estas 2 técnicas pueden tener algunas desventajas como son: (i) los pequeños fragmentos que son menores a 500 pares de bases (pb), limitando la información de la secuencia. (ii) los diferentes fragmentos de ADN pueden llegar a tener puntos de fusión similar parando la migración en el mismo sitio del gel, (iii) heterogeneidad de secuencias entre múltiples operones de rRNA en un mismo microorganismo, lo que provoca múltiples bandas en el gel, sobrestimando el análisis de diversidad. Por lo tanto, las técnicas de DGGE/TGGE podrían ser relativamente adecuadas solamente en el análisis de cambios estructurales de la comunidad bacteriana en el medio ambiente.

1.b Polimorfismo de Conformación de Cadena Única (SSCP). El método de fingerprinting SSCP (Single-Strand Conformation Polymorphism) se basa en la desnaturalización de los productos de PCR seguido de una digestión con exonucleasas dejando libre una sola cadena de los fragmentos de ADN con tamaños entre 150 y 400 pb, los cuales son separados sobre un gel de poliacrilamida no desnaturalizante por la forma de plegamiento que es dependiente de la secuencia de nucleótidos, donde un solo par de bases puede cambiar el plegamiento y diferenciación de otros fragmentos con igual tamaño. Esta técnica suele tener algunos inconvenientes como es la alta tasa de reanillamiento de las bandas de ADN después de una desnaturalización inicial durante la electroforesis, esto se puede evitar usando un primer fosforilado durante la PCR y la exonucleasa lambda que digiere la hebra fosforilada.

1.c Polimorfismo de longitud de fragmentos de restricción terminal (T-RFLP). Esta técnica se caracteriza por el marcaje fluorescente de uno de los primers en la región 5'. Los productos de PCR son digeridos por enzimas de restricción y los fragmentos son purificados y separados en un secuenciador automatizado de ADN, donde los fragmentos marcados serán detectados dando un patrón de picos separados. Esta técnica es un avance en la caracterización de las comunidades microbianas donde el tamaño del

fragmento, altura del pico y número de picos son usados para calcular índices ecológicos como son la abundancia y riqueza de las especies microbianas (Thies, 2007). Cada fragmento es considerado una única unidad taxonómica operacional (Operational Taxonomic Unit, OTU), lo cual permite en algunos casos conocer la secuencia de los fragmentos permitiendo identificar los microorganismos presentes mediante la comparación con librería de clones.

1.d Análisis del Espacio Intergénico Ribosomal Automatizado (ARISA). Una de las técnicas más sensibles en estudios de estructura microbiana es ARISA (Automated Ribosomal Intergenic Spacer Analysis) que hace relación a la amplificación por PCR de la región que involucra el espacio intergénico entre las subunidades ribosomales 16S y 23S. El espacio Intergénico ribosomal (ISR) es una región bastante heterogénea en la secuencia de nucleótidos y en la longitud de éste. Esta técnica permite generar perfiles de los grupos bacterianos más dominantes en la muestra ambiental, donde cada banda corresponde a un organismo dentro de la comunidad microbiana. El método automatizado incluye el marcaje del primer forward con un fluoroforo y los fragmentos son detectados por un detector laser que detecta diferentes tamaños de fragmentos, siempre y cuando estén marcados. Esta técnica ha sido ampliamente utilizada en estudios de ecología microbiana (Ranjard et al., 2001) y en estudios de agroecología (Petric et al., 2011; Niepceron et al., 2014).

1.e Microarrays de ADN. Esta técnica se basa en la hibridación de los productos de PCR con sondas moleculares marcadas con un fluoróforo que están unidas a una matriz. Al hibridar la sonda con el producto de PCR marcado, es excitado y capturado por un escáner laser unido a un microscopio confocal. La intensidad de la señal es proporcional a la abundancia del organismo diana.

Esta técnica ha permitido desarrollar una gran cantidad de sondas asociadas al gen ribosomal 16S rRNA de grupos taxa de bacteria y archea. Este microarray es conocido como PhyloChip y ha sido utilizado para evaluar comunidades bacterianas en muestras ambientales (DeSantis et al., 2007; Rastogi et al., 2010). Otros chips han sido desarrollados como el compochip utilizado para el estudio de poblaciones microbianas en compost y vermicompost (Fernández-Gómez et al., 2011).

2. PCR cuantitativa. Es la técnica más utilizada para cuantificar la abundancia y expresión de genes funcionales y de taxonomía como el 16S rRNA y el 18sRNA para

bacterias y hongos (Chemidlin Prévost-Bouré et al., 2011; Philippot et al., 2011). Esta técnica utiliza colorantes fluorescentes como SYBR Green o sondas marcadas como TaqMan que se intercalan en la cadena de ADN de los fragmentos de PCR que al ser excitados, la señal de los amplicones es medida durante cada ciclo de PCR mediante un programa que registra la concentración durante la fase exponencial de la amplificación. Esta técnica puede ser acoplada a la reacción de transcripción reversa para evaluar la expresión directa de genes mediante el ARN que es transcrito a ADN y posteriormente cuantificado. Esta técnica ha permitido cuantificar la expresión de genes involucrados en los ciclos biogeoquímicos del Nitrógeno (López-Gutiérrez et al., 2004; Philippot et al., 2013) y como marcador molecular en degradación de xenobióticos.

3. **Hibridación fluorescente In Situ (FISH).** Esta técnica molecular permite localizar un determinado fragmento de ADN. Se puede aplicar sobre extensiones celulares, cortes de tejido, muestras ambientales previamente tratadas. Las sondas son pequeñas secuencias de cadena sencilla de ADN, complementarias a la secuencia de interés (Amann et al., 1995). Las sondas para 16S rRNA suelen tener una longitud entre 18-30 nucleótidos, conteniendo un colorante fluorescente en la posición 5' y se pueden observar por microscopia de epifluorescencia. La intensidad de la señal está relacionada al contenido de rRNA y a la tasa de crecimiento de los microorganismos.

3.5.2 Técnicas basada en el análisis completo de la comunidad microbiana.

Dentro de las técnicas más conocidas esta la cinética de hibridación DNA-DNA, que esencialmente consiste en la desnaturalización del ADN separando ambas hebras para luego incubarlo en condiciones de reasociación o hibridación. La tasa de reasociación del ADN vendría a estar correlacionado con la diversidad genómica presente en la muestra. Otra técnica es el fraccionamiento del contenido de Guanina más Citosina (G+C). Ya que los grupos taxonómicos microbianos difieren en el contenido de G+C, el ADN total puede ser separado mediante un fraccionamiento total de la comunidad por gradiente de centrifugación basado en el contenido de G+C (Nüsslein and Tiedje, 1999), luego la banda generada puede ser extraída y tratada para hacer otra separación por DGGE u otra técnica de fingerprinting (Bulgarelli et al., 2012).

Aunque estas técnicas no conllevan realizar un trabajo muy laborioso, no son muy utilizadas ya que recientemente se han desarrollado nuevas técnicas de secuenciación masiva que permiten obtener una información más amplia y precisa de la diversidad microbiana o de genes implicados en procesos metabólicos.

4. DIURON

4.1. Uso y modo de acción de Diuron.

Es un herbicida sistémico que pertenece al grupo de las fenilureas (Figura 11). Se considera un herbicida no-selectivo que es fácilmente absorbido por las raíces de las plantas y transportado por el xilema al resto de la planta mediante el sistema de transpiración. Al ser sistémico, inhibe la reacción Hill en la fotosíntesis por disrupción de la transferencia de electrones en el fotosistema II, limitando la fijación de CO_2 y la producción de ATP, importantes en el metabolismo de las plantas (Allen et al., 1983).

El Diuron es utilizado para el control de malas hierbas en pre-emergencia y pos-emergencia. Se ha utilizado en viñedos, cultivos de espárragos, árboles frutales, olivos, caña de azúcar, algodón, y varios cereales en dosis de 1.8 kg ha^{-1} $año^{-1}$. También ha sido utilizado como alguicida y en áreas urbanas cerca de líneas de trenes y zonas no cultivables en concentraciones de 38 kg ha^{-1} $año^{-1}$. (Giacomazzi and Cochet, 2004; Tomlin, 2011).

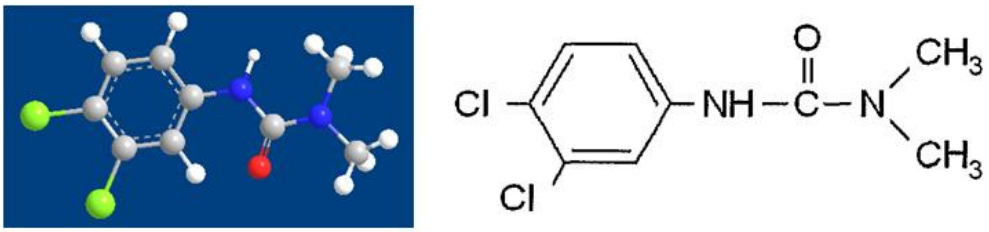


Figura 11. Molécula de Diuron

4.2. Propiedades fisicoquímicas de diuron

El diuron es una fenilurea que tiene 2 cloros en el anillo fenilo y esta característica lo diferencia de otros herbicidas de este grupo como el isoproturon, monuron, etc.

(Sørensen et al., 2003). El Diuron es un compuesto sólido a temperatura ambiente, no posee olor y es de color blanco cristalino. Puede usarse emulsificado. Debido a la baja presión y constante de Henry, se considera que tiene baja volatilidad y es relativamente soluble en agua (Tabla 8). El Diuron tiene estabilidad hidrolítica a pH neutro pero es inestable cuando el pH del medio es ácido o básico.

Tabla 8. Propiedades físico-químicas de Diuron (Tomlin, 2011).

Propiedades físico-químicas	Medida
Peso molecular	233.10 g mol^{-1}
Solubilidad en agua	36.4 ppm (25ºC)
Presión de Vapor	6.90 $x10^{-8}$ mm Hg (25ºC)
Vida media	1490 días (pH 5)
	1240-1330 días (pH 7)
	2020 días (pH 9)
Fotólisis en agua (vida media)	43.1-2180 días (pH 7 y 25ºC)
Degradación aeróbica en suelos	372 días
Degradación anaeróbica en suelos	995 días
Fotólisis en suelo (Vida media)	173 días
Disipación en campo (vida media)	99.9-134 días
Constante de Henry	5.10 x 10^{-10} atm m^3 mol^{-1} (25ºC)
Coeficiente Octanol/agua (Kow)	648-747
Coeficiente adsorción en suelos (Koc)	418-560

4.3. Ecotoxicología de Diuron

La aplicación intensiva de diuron y otros plaguicidas fenilureas está correlacionada con el incremento de residuos de diuron en aguas subterráneas o superficiales, probablemente por la moderada solubilidad que tiene en agua. Se ha detectado en ríos, lagos, manglares (Green and Young, 2006; Lapworth and Gooddy, 2006; Abbot and Marohasy, 2011) incrementando la preocupación sobre los efectos que pueda tener sobre

el ecosistema y la toxicidad que pueda tener frente a organismos vivos. Por ejemplo, se ha detectado la presencia de 3,4-dicloroanilina (3,4-DCA) que es considerado más tóxico que diuron debido a que es más apolar y presenta una alta adsorción (Claver et al., 2006; Fernández-Bayo et al., 2008; Fernandez-Bayo et al., 2015) afectando no solamente a la flora y fauna donde se encuentra, siendo a su vez altamente tóxico en humanos (Giacomazzi and Cochet, 2004; Lopez-Doval et al., 2010). Como consecuencia, la EPA ha clasificado al Diuron toxicológicamente como clase III, tóxica y ligeramente peligrosa, de la misma manera la unión europea incluyó a diuron en el anexo I de la Directiva del Consejo 91/414/EEC después de la entrada en vigor de la Regulación (EC) No 396/2005, donde involucra la toxicidad de los metabolitos y principalmente 3,4-DCA definiendo un límite de cuantificación en aguas, grasas y en materias primas de 0.01mg kg^{-1} (European Food Safety Authority (EFSA), 2011).

4.4. Dinámica de diuron en suelos.

Después de ser aplicado, el diuron puede sufrir varios procesos que dependen de las condiciones físico-químicas del suelo (Gooddy et al., 2002). Se ha encontrado en aguas superficiales y subterráneas (Claver et al., 2006). Diuron es considerado de persistencia moderada (Tabla 8) sin embargo, dependiendo de las condiciones físico-químicas del suelo, se ha observado que el coeficiente de partición en suelos de diuron es fuertemente dependiente del contenido y composición del carbono orgánico (Ahangar et al., 2008; Fernández-Bayo et al., 2008; Fernández-Bayo et al., 2009).

Aunque Diuron tiene una moderada persistencia, se ha observado que la adición de algunas enmiendas orgánicas promueve la lixiviación al formarse un complejo móvil entre el diuron y la materia orgánica disuelta (Thevenot et al., 2009).

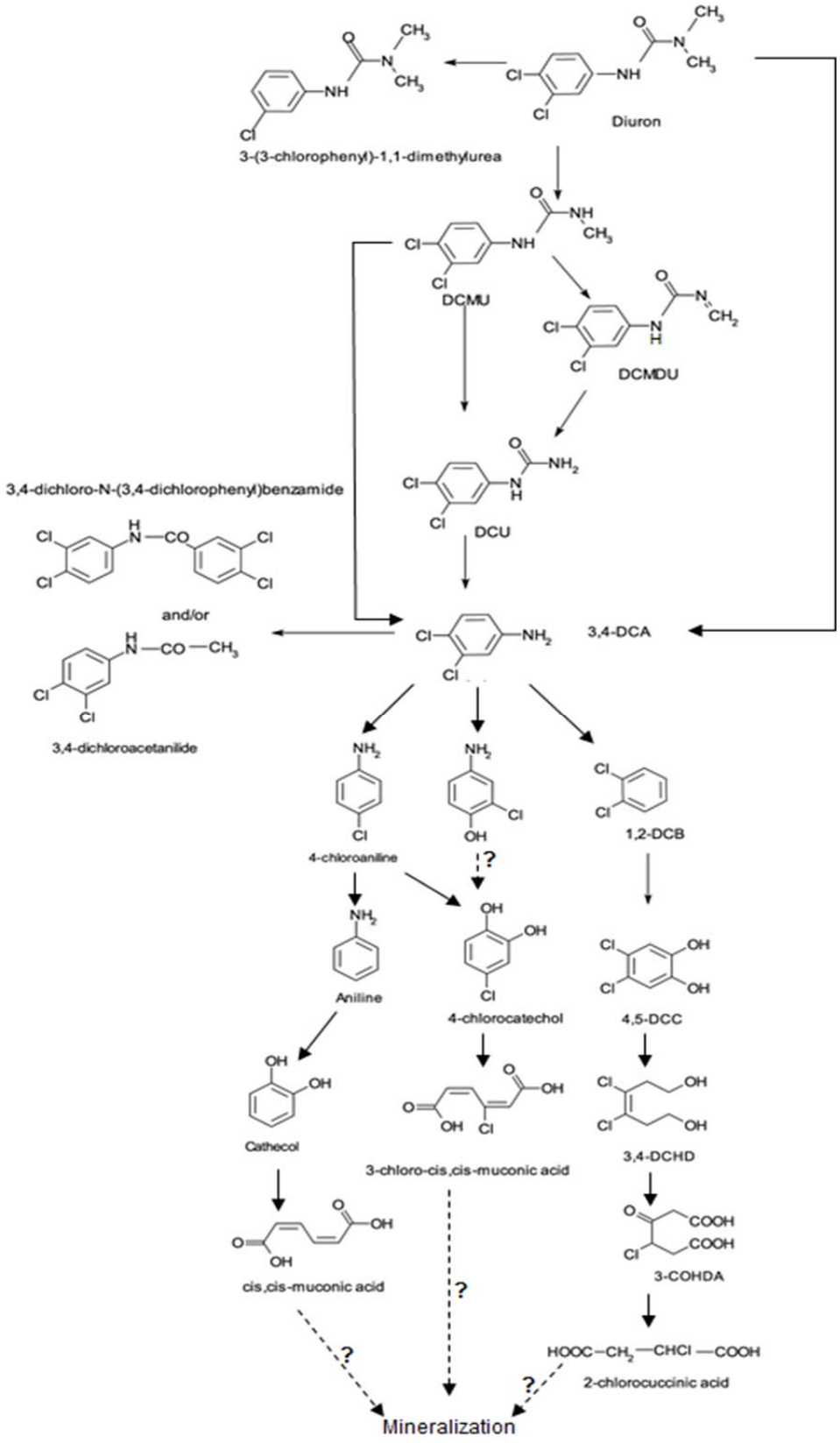


Figura 12. Vía metabólica propuesta para diuron

4.5. Degradación de Diuron

Diuron es estable a la degradación química cuando se encuentra con valores de pH entre 4-10. Por tal razón, la degradación abiótica es baja en suelos agrícolas. Aun así, se ha observado la transformación de diuron por procesos de fotólisis (Giacomazzi and Cochet, 2004; Katsumata et al., 2009) y de oxidación (Acero et al., 2007). Sin embargo, la mayor degradación es biológica. Se ha descrito que algunas plantas como trigo y rábano pueden adsorber diuron y linuron formando un conjugado de azúcares para luego hacer una N-demetilación y N-demetoxilación (Pascal-Lorber et al., 2010). Pero son los microorganismos los encargados de realizar la mayor degradación (El Sebai et al., 2010; Pesce et al., 2010), donde los principales metabolitos están citados en la ruta metabólica propuesta por Giacomazzi and Cochet (2004) y Hussain et al. (2015) (Figura 12).

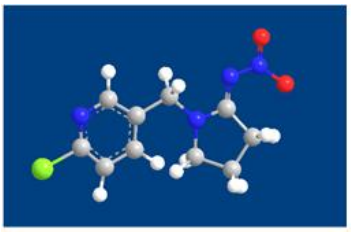
5. IMIDACLOPRID

5.1. Uso y modo de acción de Imidacloprid

El Imidacloprid es un insecticida sistémico perteneciente al grupo químico de los cloronicotinilo (nitroguanidina cloronicotinilo) (Figura 13). Sus propiedades físico-químicas, le permiten ser traslocado por la planta a la superficie de las hojas. Posteriormente éste entra a la plaga por ingestión o contacto directo afectando el sistema nervioso del insecto al unirse a los receptores post-sinápticos nicotínicos de acetilcolina, provocando parálisis y muerte del insecto.

El uso de imidacloprid es amplio, desde el control de insectos chupadores, termitas, insectos del suelo y masticadores, es efectivo en todas las fases larvarias de los insectos diana. Imidacloprid es aplicado a plantas o directamente en semillas (El-Hamady et al., 2008). Puede ser aplicado por aspersión de forma foliar o por inyección en árboles, también en forma granular en suelos. Imidacloprid desde su salida en 1991 ha sido utilizado ampliamente en más de 120 países de los 140 donde se ha registrado, con ventas anuales de 1091 millones de dólares en 2009 (Scholer and Krischik, 2014). En Estados Unidos, de 178 millones de hectáreas cultivadas, en 58 millones de ellas se ha

aplicado 907158 kg de imidacloprid con otros insecticidas neonicotinilos (Jeschke et al., 2011).



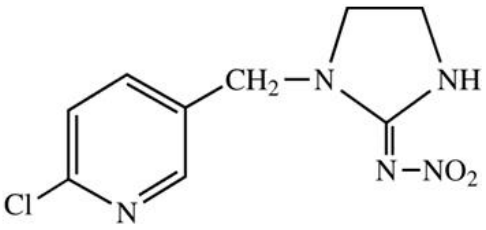


Figura 13. Molécula de Imidacloprid

5.2. Propiedades fisicoquímicas de Imidacloprid

El Imidacloprid está sintetizado como un cristal incoloro e inodoro. La baja presión de vapor y constante de Henry indican que tiene baja volatilidad en agua (Tabla 9). Imidacloprid al tener un bajo coeficiente de adsorción (Koc) y ser altamente soluble en agua, sugiere que es potencialmente lixiviado a aguas subterráneas.

Tabla 9. Propiedades físico-químicas de Imidacloprid

Propiedades físico-químicas	Medida
Peso molecular	255.7 g mol^{-1}
Solubilidad en agua	514 mg L^{-1} (20ºC a pH7)
Presión de Vapor	1.00 x10^{-7} mm Hg (20ºC)
Hidrólisis (vida media)	>30 días (25ºC a pH 5)
Fotólisis en agua (vida media)	<1 hora (pH 7 y 24ºC)
Anaeróbica (vida media)	27.1días
Aeróbica (vida media)	997 días
Fotólisis en suelo (Vida media)	38 días
Disipación en campo (vida media)	26.5-229 días
Constante de Henry	6.5 x 10^{-11} atm m^3 mol^{-1} (20ºC)
Coeficiente Octanol/agua (Kow)	3.7
Coeficiente adsorción en suelos (Koc)	132-310

5.3. Ecotoxicología de Imidacloprid

La Comisión Europea restringió el uso de imidacloprid a partir del 2013 (Regulación EU 485/2013), sin embargo, esta será revisada dentro de 2 años. Su uso es prohibido en cultivos como cereales donde las abejas puedan verse afectadas (Scholer and Krischik, 2014). Sólo se permite su uso bajo invernadero y en condiciones controladas.

Principalmente la toxicidad de imidacloprid es por ingesta, más que por vía cutánea. La DL50 en ratas es de 450 mg kg^{-1} y se ha encontrado que puede afectar a las lombrices (Fernández-Gómez, et al., 2011 y Dittbrenner et al., 2011). También se ha observado que algunos de los metabolitos de degradación pueden llegar a ser más tóxicos que el compuesto parental. Por ejemplo, el metabolito ácido 6-cloronicotinico (6-CNA) es más tóxico debido a su alta solubilidad en agua (2 g L^{-1}) lo que conlleva a una mayor lixiviación (Malev et al., 2012).

5.4. Dinámica de Imidaclorpid en suelos

La adsorción de imidacloprid depende de las propiedades del suelo. Estudios a escala de laboratorio han determinado que la adsorción de imidacloprid incrementa cuando la concentración del insecticida decrece (Cox et al., 1998a). Por otro lado, el contenido de materia orgánica puede incrementar la adsorción (Cox et al., 1997; Cox et al., 1998a; Cox et al., 1998b). Sin embargo, en otros estudios, esta relación no se ha encontrado (Flores-Cespedes et al., 2002), lo que indica que la adsorción depende de la composición y estructura de la materia orgánica.

En aguas superficiales, la degradación de imidacloprid está asociada a procesos de fotólisis. En aguas subterráneas, son puntuales los estudios que han descrito la lixiviación de imidacloprid (Krohn and Hellpointner, 2002). Según la Agencia de Protección Ambiental (USA) en 2003 se detectó imidacloprid en aguas subterráneas en New YorK en concentraciones de 6,69 ppb, sin embargo, es considerado de bajo riesgo ambiental y alimentario. Imidacloprid se degrada más rápidamente en suelos agrícolas con cubierta que en suelos desnudos, con una vida media $t_{1/2}$ de 48 a 190 días, respectivamente (Scholz and Spiteller, 1992).

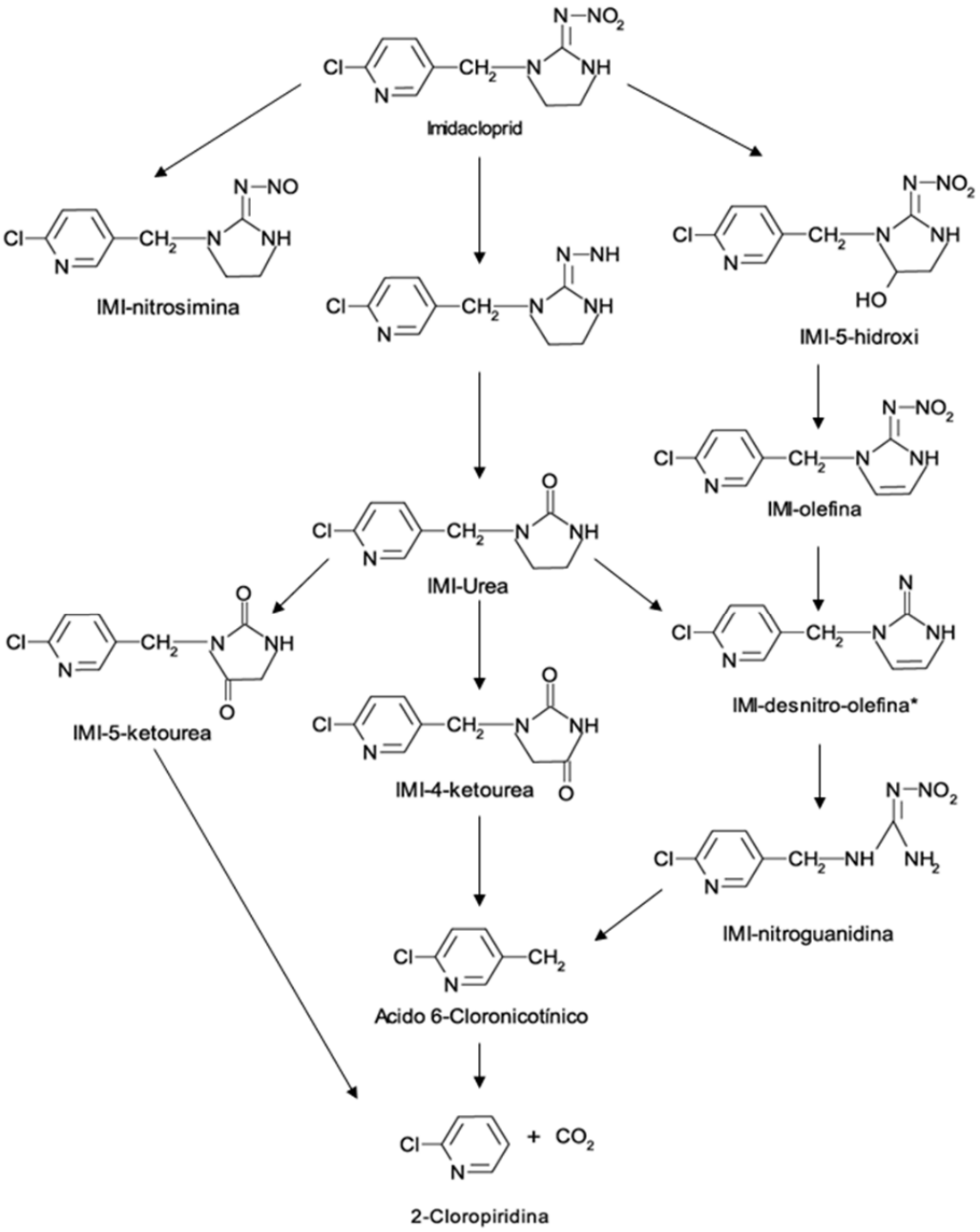


Figura 14. Vía metabólica de imidacloprid (Krohn and Hellpointner, 2002).

Suele degradarse rápidamente en suelos por fotólisis ($t_{1/2}$ 39 días) y suele incrementarse la persistencia en suelos a medida que se incrementa el pH del suelo (Sarkar et al., 1999).

5.5. Degradación de Imidacloprid

La biodegradación, después de la fotólisis, es el proceso más dominante en la degradación de imidacloprid. Se conoce, por estudios de laboratorio, la capacidad de degradación de imidacloprid de microorganismos como *Leifsonia* sp, *Pseudomonas* sp. 1G y *Stenitrophomonas maltophilia* CGMCC1.1788, transformando este insecticida a metabolitos secundarios como 6-CNA y metabolitos de urea (Figura 14) (Dai et al., 2006; Anhalt et al., 2007; Pandey et al., 2009; Phugare et al., 2013).

OBJETO DEL TRABAJO

La contaminación de las aguas subterráneas por residuos de plaguicidas se consideró, durante muchos años, como improbable debido a que el perfil del suelo actúa como un filtro de purificación (Aharonson et al., 1987). Además, se estimó que la contaminación de las aguas superficiales por estos residuos de plaguicidas era transitoria debido a su baja solubilidad en agua como es el caso del DDT. Sin embargo, en los años 90, se detectaron tanto en aguas superficiales como subterráneas algunos compuestos herbicidas, los cuales eran más solubles en agua que los organoclorados y más ampliamente utilizados en la agricultura. Es entonces cuando se desarrollaron políticas para reducir la contaminación del suelo y se establecieron límites de máxima concentración admisible (MCA) para las aguas potables (Directivas 2000/60/EC, 2006/118/EC) Así como nuevas directivas relativas a las normas de calidad ambiental en el ámbito de la política de las aguas (2008/105/CE). Dado que algunos de estos plaguicidas son necesarios en la agricultura actual, es necesario desarrollar tecnologías de bajo coste que permitan reducir los efectos adversos que conlleva el uso de estos compuestos en el medio y en la salud humana.

En trabajos anteriores realizados en el proyecto nacional REN2003-04693, se iniciaron estudios encaminados a valorizar residuos de la agroindustria del aceite y del vino, muy abundantes en la agricultura de clima mediterráneo, para obtener vermicomposts como enmiendas orgánicas sostenibles que permitan aumentar el contenido en materia orgánica de suelos agrícolas de zonas del sureste de Europa, caracterizados por su bajo contenido en carbono orgánico (Zdruli et al., 1999) y controlar la disipación de residuos de plaguicidas. Como resultados más relevantes, se obtuvo que los vermicomposts tienen una gran capacidad para retener los plaguicidas (Romero et al., 2006); y como enmienda orgánica en el suelo, el vermicompost permite reducir la disponibilidad de los residuos de plaguicidas (Fernández-Bayo et al., 2007, 2008), la cual se ve afectada por el tiempo de envejecimiento de los vermicomposts en el suelo tras su aplicación y por el tipo de vermicompost aplicado (Fernández-Bayo et al., 2009).

En relación a lo anteriormente expuesto, el objetivo general del presente trabajo de Tesis Doctoral fue profundizar en el conocimiento de los efectos que promueve la aplicación de vermicomposts oleícolas y vitivinícolas en suelos con bajo contenido en carbono orgánico, como tecnología sostenible medioambiental de bajo coste, que

permita retener y favorecer la degradación de los plaguicidas en el medio edáfico, contribuyendo de esta forma a reducir su impacto en el suelo minimizando la transferencia de los plaguicidas o de sus metabolitos a los recursos hídricos y promoviendo así el uso de estas enmiendas como alternativa frente a la mitigación de plaguicidas en el medio ambiente.

En este estudio, a diferencia de los resultados obtenidos en el proyecto REN2003-04693, se planteó realizar ensayos de adsorción y degradación con un suelo que había sido sometido a una doble enmienda orgánica que conlleva un tiempo de estabilización antes de aplicar el plaguicida, como ocurre en la práctica agrícola, como también evaluar el efecto de la enmienda en un suelo que fue previamente contaminado con plaguicidas.

Para profundizar en el conocimiento adquirido anteriormente, en este estudio de Tesis Doctoral se propuso investigar aspectos físico-químicos, bioquímicos y especialmente microbiológicos empleando herramientas moleculares, de forma conjunta tanto en los procesos de vermicompostaje, como en estudios que se iniciaron para conocer el potencial de biodegradación de microorganismos aislados de estos vermicomposts y para el uso de estas enmiendas orgánicas, evaluando su efecto en la disponibilidad, retención y degradación de los plaguicidas Diuron e Imidacloprid en suelo. Los objetivos específicos propuestos en esta Tesis Doctoral fueron:

- **Objetivo 1.** Obtener vermicomposts de los residuos agroindustriales generados en la producción del aceite de oliva y del cultivo de la vid (*alperujo* y sarmientos), prestando especial atención a la dinámica de la población microbiana presente durante el desarrollo de este proceso.

- **Objetivo 2.** Conocer la capacidad que la microbiota autóctona de estos vermicomposts, en su etapa de preacondicionamiento, tiene en la degradación de residuos de plaguicidas difícilmente biodegradables.

- **Objetivo** 3. Evaluar la eficacia de los vermicomposts de residuos agroindustriales bajo diferentes condiciones agronómicas para mitigar el impacto del herbicida Diuron en el suelo y su repercusión en la dinámica de este agroquímico, analizando

paralelamente la presencia de marcadores moleculares implicados en la degradación de este herbicida.

- **Objetivo 4.** Estudiar el efecto de estos vermicomposts en la distribución de Imidacloprid en las diferentes fracciones del suelo, evaluando a su vez la dinámica de este insecticida bajo diferentes condiciones agronómicas, haciendo énfasis en su disipación y su relación con la función biológica del suelo.

Un resumen gráfico de los objetivos que fueron perseguidos en los diferentes capítulos que conforman el apartado de resultados de la Tesis Doctoral se expone en la figura 1.

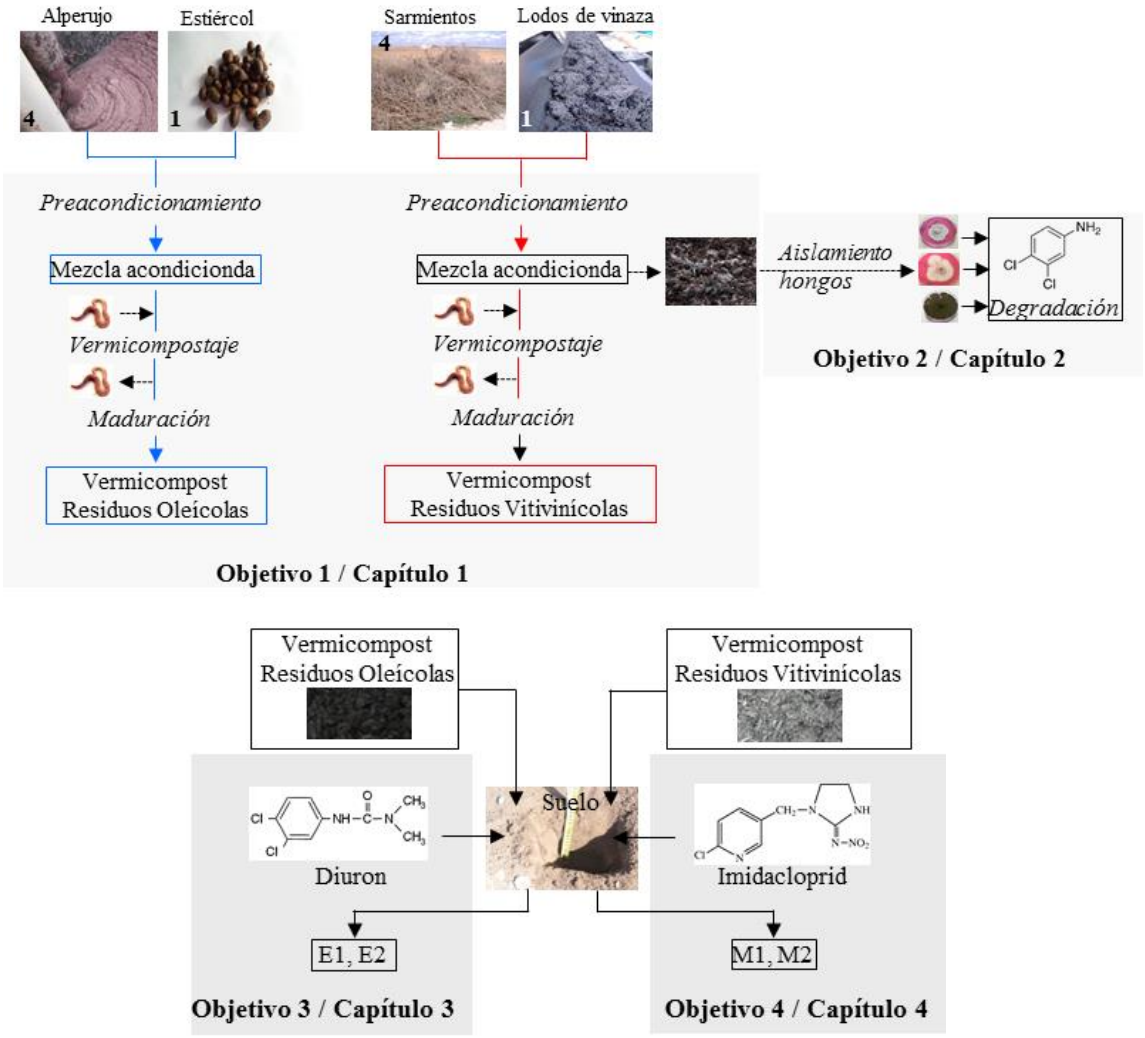


Figura 1. Objetivos y capítulos del apartado de resultados que conforman la memoria de esta Tesis Doctoral.

MATERIALES

1. **Residuos orgánicos utilizados**
2. **Lombrices epigeas utilizadas en los procesos de vermicompostaje**
3. **Preacondicionamiento de residuos organicos y procesos de vermicompostaje desarrollados**
4. **Plaguicidas y metabolitos ensayados**
5. **Suelo**

Para la realización de los estudios científicos que constituyen la Tesis Doctoral, que se recogen en los capítulos 1 al 4 del apartado de Resultados de la presente memoria de Tesis Doctoral, se han utilizado diferentes residuos orgánicos agroindustriales con el fin de obtener diferentes tipos de vermicomposts. Los plaguicidas y productos de degradación analizados en los estudios de degradación, así como el suelo seleccionado para los estudios de disipación de los plaguicidas empleados (Diuron e Imidacloprid) se encuentran descritos a continuación.

1. RESIDUOS ORGANICOS UTILIZADOS

- **Alperujo/alperujo or wet olive cake:** Suministrado por la Empresa olecícola ROMEROLIVA, Deifontes, Granada. El alperujo (Figura 1a) fue utilizado en el proceso de vermicompostaje de residuos oleícolas desarrollado en el Capítulo 1.
- **Estiércol de cabra /goat manure:** Suministrado por el grupo de Investigación de Pequeños Rumiantes (IFNA, EEZ-CSIC). El estiércol de cabra (Figura 1b) fue utilizado en el proceso de vermicompostaje de residuos oleícolas desarrollado en el Capítulo 1.

Figura 1. Residuos orgánicos utilizados. a) alperujo, b) estiércol de cabra, c) sarmientos, d) lodos de vinaza

- **Sarmientos/Vine shoots**: Procedentes de la poda de cepas de viñas de la zona de Valdepeñas (Ciudad Real). Los sarmientos (Figura 1c), después de su astillado en piezas de 2 a 5 cm fueron utilizado en el proceso de vermicompostaje de residuos vitivinícolas desarrollado en el Capítulo 1.
- **Lodo de Vinazas/Biosolid vinasse:** procedentes de la depuración de las vinazas generadas durante la destilación de alcohol a partir de vino, orujos frescos y lías y suministrado por la Estación Depuradora de Vinazas, Tomelloso, Ciudad Real. El lodo de vinaza (Figura 1d) fue utilizado en el proceso de vermicompostaje de residuos vitivinícolas desarrollado en el Capítulo 1.

2. LOMBRICES EPIGEAS UTILIZADAS EN LOS PROCESOS DE VERMICOMPOSTAJE

Las lombrices utilizadas en los procesos de vermicompostaje pertenecen a la especie *Eisenia fetida* (Savigny, 1826) (Figura 2) de la familia Lumbricidae, orden de los Haplotáxidos y subclase de los oligoquetos. Las lombrices fueron suministradas por la Empresa Humus-Fertil (La Roda, Albacete, España)

Figura 2. Lombrices de la especie *Eisenia fetida* sobre (a) mezcla de *alperujo* y estiércol de cabra y (b) mezcla de sarmientos y lodos de vinaza

3. PREACONDICIONAMIENTO DE RESIDUOS ORGANICOS Y PROCESOS DE VERMICOMPOSTAJE DESARROLLADOS

- **Vermicompost de residuos oleícolas/ Vermicompost from olive wastes:** Vermicompost obtenido a partir de una mezcla de *alperujo* y estiércol caprino en proporción 4:1 (ps:ps) mediante un proceso de vermicompostaje que se llevó a cabo en una litera de 2 m^2 de superficie construida de madera, la cual se mantuvo inclinada (5%) para facilitar el drenaje. La litera se rellenó con la mezcla de *alperujo* y estiércol (Figura 3a), que inicialmente fue preacondicionada durante un periodo de 15 días. Posteriormente, en el lateral de la litera se colocó un cordón de amortiguación consistente en vermicompost de estiércol, lugar en el cual se procedió a la inoculación de las lombrices (*E. fetida*). Durante el periodo de vermicompostaje la humedad se mantuvo entre 70-80% con riegos periódicos. Transcurrido ese periodo, las lombrices fueron separadas manualmente, y el vermicompost obtenido fue madurado durante 2 meses.

Figura 3. Literas utilizadas para los procesos de vermicompostaje de residuos agroindustriales (Capitulo 1). a) mezcla de alperujo con estiércol de cabra b) mezcla de sarmientos con lodos de vinazas,

- **Vermicompost de residuos vitivinícolas / Vermicompost from winery wastes:** Vermicompost obtenido a partir de una mezcla de sarmientos con lodos de vinazas en proporción 4:1 (ps:ps) que fue vermicompostada en literas de madera de 2 m^2, la cual se mantuvo inclinada (5%) para facilitar el drenaje. La litera se rellenó con la mezcla de

sarmientos y lodos de vinazas (Figura 3b) que inicialmente fue preacondiconada durante un periodo de 15 días. Durante este periodo, la temperatura de la mezcla aumentó durante los primeros 5 días hasta 50°C y posteriormente, durante 10 días, disminuyó a condiciones mesófilas (25°C). A los 15 días, la mezcla se encontraba completamente cubierta de micelios fúngicos (Figura 4), recogiéndose cuatro muestras representativas de este sustrato orgánico, que después de su mezcla y homogeneización, constituyo el material utilizado para el aislamiento de hongos cuyo estudio se expone en el Capítulo 2. Posteriormente la mezcla de residuos fue vermicompostada y madurada con la misma metodología que la desarrollada en el vermicompostaje de los residuos oleícolas.

Figura 4. Detalle de la proliferación de hongos en la mezcla de sarmientos con lodos de vinaza

En los capítulos 3 y 4 se utilizaron vermicomposts de residuos oleícolas (vermicompost from olive wastes, O) y vermicomposts de residuos vitivinícolas (vermicompost from winery wastes, W).

4. PLAGUICIDAS Y METABOLITOS ENSAYADOS

Los principios activos de los plaguicidas y metabolitos utilizados en los estudios recogidos en los capítulos 2, 3 y 4, fueron provistos por Dr. Ehrenstorfer (Augsburg,

Germany), ACROS Organics (Geel, Belgium) y por Fluka (Steinheim, Germany) y se exponen, respectivamente, en las tablas 1 2 y 3.

Tabla 1. Estructura química de la 3,4-dichloraniline (DCA) y de los productos de degradación analizados en el estudio del capítulo 2.

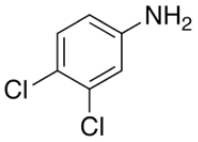
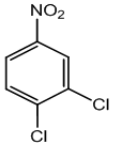
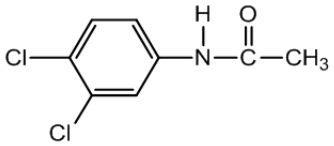
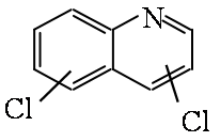
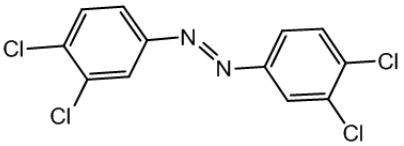
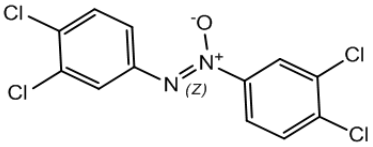
Producto	Acronimo	Molecula
3,4-dicloroanilina ***3,4-dichloroaniline***	**DCA**	
3,4-dicloronitrobenceno ***3,4-dichloronitrobenzene***	**DCNB**	
3,4-dicloroacetanilido ***3,4-dichloroacetanilide***	**DCAN**	
4,7-dicloroquinolina ***4,7-dichloroquinoline***	**DCQ**	
3,3´4,4´-tetracloroazobenceno ***3,3'4,4'tetrachloroazobenzene***	**TCAZB**	
3,3´,4,4´-tetracloroazoxybenceno ***3,3',4,4'-tetrachloroazoxybenzene***	**TCAXB**	

Tabla 2. Estructura química del Diuron y de sus metabolitos de degradación utilizados en el estudio del capítulo 3

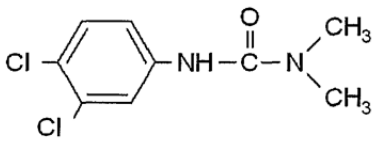
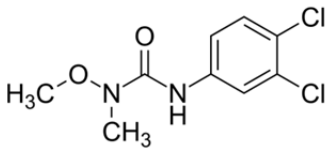
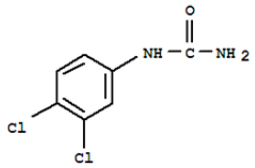
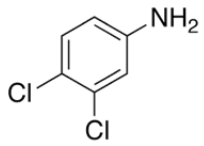
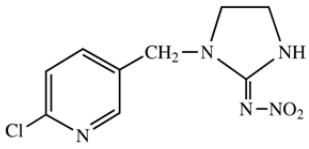
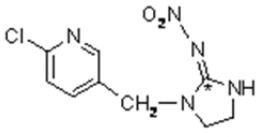
Producto	Acronimo	Molecula
Diuron ***Diuron***	**D**	
3-(3,4-diclorofenil)-1-metilurea ***3-(3,4-dichlorophenyl)-1-methylurea***	**DPMU**	
3,4-diclorofenilurea ***3,4-dichlorophenylurea***	**DPU**	
3,4-dicloroanilina ***3,4-dichloroaniline***	**DCA**	

Tabla 3. Estructura química del Imidacloprid utilizado en el estudio del capítulo 4

Producto	Acronimo	Molecula
Imidacloprid ***Imidacloprid***	**IMI**	
Imidazolidina -^{14}C-Imidacloprid, con 3.7 MBq actividad ***Imidazolidine ring-^{14}C-Imidacloprid, with 3.7 MBq activity***	**14CIMI**	

5. SUELO/ SOIL (S)

Para los estudios de biodisponibilidad de los plaguicidas en el suelo de los capítulos 3 y 4, se utilizó la capa arable de un suelo calcáreo clasificado como Cambisol calcárico (Figura 4), localizado en las cercanías de Deifontes (Granada), en el Cortijo la Parra. El análisis químico y textural de la capa arable del suelo calcáreo utilizado en el experimento se expone en la tabla 4. La descripción del perfil del suelo se expone a continuación:

Figura 4. Suelo (Cambisol calcárico) utilizado en los capítulos 3 y 4.

Coordenadas: 37º 22´19.41´´N, 3º 36´5.54´´O.
Altitud: 855 m
Posición fisiográfica: Pendiente convexa
Topografía del terreno circundante: ondulado
Pendiente: llano o casi llano, <2%
Uso del suelo: Cultivo de cereal
Material original: conglomerado calizo
Drenaje: Clase 2, perfectamente drenado.
Condiciones de humedad: seco
Pedregrosidad: Muy poca pedregosidad, Clase 0
Afloramientos rocosas: Ninguna, clase 0
Erosión: ligera

Descripción de los horizontes:

Horizonte Ap (0-14 cm) "capa arable"

Color 7.5YR 5/4 en húmedo, en seco 10YR 7/3. Textura franco-limosa. Estructura granular media bien desarrollada. Adherente, plástico, friable y ligeramente duro. Sin cútanes. Poros frecuentes, finos y medianos. Fragmentos rocosos moderados, gravas. Calcáreo. Raíces relativamente abundantes, medianas y finas.

Horizonte Bw (13-40 cm)

Color 7.5YR 5/4 en húmedo, en seco 10YR 7/4 Textura franca. Estructura granular media con tendencia a masivo. Adherente, plástico, friable y ligeramente duro. Sin cútanes. Poros pocos y finos. Fragmentos rocosos moderados, gravas. Calcáreo. Raíces comunes y finas.

Horizonte BC (28-5 cm)

Color 5YR 5/6 en húmedo, en seco 7.5YR 8/2. Textura franca. Estructura masiva. Ligeramente adherente, plástico, friable y duro. Fuertemente cementado. No hay poros. No hay fragmentos rocosos. Fuertemente calcáreo. Raíces muy pocas y muy finas.

Horizonte Ckm (>50cm)

Conglomerado calcáreo

Tabla 4. Análisis químico y textural de la capa arable del suelo básico.

Arena	Limo	Arcilla	pH	CE	Carbonatos
(g kg^{-1})	(g kg^{-1})	(g kg^{-1})		(dS m^{-1})	(g kg^{-1})
111	489	400	8.3	0.3	430
CIC	COT	NKT	C/N	33kPa	1500kPa
$cmol^{+}$ kg^{-1}	(g kg^{-1})	(g kg^{-1})		(%)	(%)
15.5	20.7	2.7	8.3	33.2	17.9

RESULTADOS

1. **Dinámica de las comunidades microbianas relacionadas con parametros bioquímicos durante el vermicompostaje y maduración de residuos lignocelulosicos**

2. **Degradación de 3,4 dicloroanilina por hongos aislados durante el pretratamiento de residuos vitivinícolas por vermicompostaje**

3. **Evaluación multidisciplinar de la reducción de los plaguicidas en suelo enmendados con vermicompost de residuos agroindustriales**

4. **Efecto de los vermicomposts sobre el comportamiento de Imidacloprid en suelo y riesgos asociados en la funcionalidad del suelo**

CAPÍTULO 1

Dinámica de las comunidades microbianas relacionadas con parametros bioquímicos durante el vermicompostaje y maduración de residuos lignocelulosicos

Dynamics of microbial communities related to biochemical parameters during vermicomposting and maturation of agroindustrial lignocellulose wastes.

El capitulo 1 ha sido publicado en la revista Bioresouce Technology

Castillo-Diaz, J.M., Romero, E., Nogales, R. 2013. Dynamics of microbial communities related to biochemical parameters during vermicomposting and maturation of agroindustrial lignocellulose wastes. Bioresource Technology, 46, 345-353. DOI: 10.1016/j.biortech.2013.07.093

Resumen

Existe una escasa información sobre los cambios de la abundancia de taxones microbianos que tienen lugar durante el proceso de vermicompostaje. El estudio tuvo como objetivo evaluar, mediante PCR cuantitativa y análisis de DGGE, los cambios de la estructura microbiana, de la bacterias y hongos y de la abundancia relativa de cuatro clases bacterianas durante un proceso de vermicompostaje y posterior maduración, utilizando, independientemente, dos residuos orgánicos lignocelulósicos, alperujo (O) y sarmientos (W). El análisis de la correlación multivariante entre la estructura y la abundancia microbiana, la biomasa de lombrices y las actividades enzimáticas dejó patente interacciones similares y divergentes en ambos procesos. Aunque el desarrollo de *Eisenia fetida* fué diferente, se encontraron correlaciones significativas con la actividad β-glucosidasa y con la estructura de bacterias y hongos. Durante el período de vermicompostaje de O y de W, se observó una disminución del número de bacterias (94% y 77%), hongos (93% y 94%), y Gammaproteobacterias (56% y 71%) y un aumento de Betaproteobacterias y Actinobacterias (62 % a 79%). Las Alphaproteobacterias únicamente aumentaron en alperujo (26%). Los vermicomposts maduros obtenidos tuvieron propiedades microbiológicas y bioquímicas similares, pese a que se utilizaron diferentes residuos lignocelulósicos

Palabras clave: Biomasa de *E. fetida*; actividades enzimática; Q-PCR; DGGE; test de Mantel.

Abstract

Scarce information is available on the changes in abundance of microbial taxa during vermicomposting. Quantitative PCR and DGGE analysis were used to monitor variations in the microbial structure, relative abundance of four bacterial classes and fungi over the vermicomposting and maturation period of wet olive cake (O) and vine shoots (W). Multivariate correlation analysis between microbial structure and abundance, earthworm biomass and enzyme activities revealed similar and divergent interactions in both processes. Although E. fetida development was different, significant correlations were found with β-glucosidase activity and with bacterial and fungal structure. In the vermicomposting period of O and W, a decline was found in bacteria (94% and 77%), fungi (93% and 94%), and Gammaproteobacteria (56% and 71%) but an increase in Betaproteobacteria and Actinobacteria (62% to 79%). Alphaproteobacteria increased only in O (26%). Despite the different initial lignocellulose wastes, the mature vermicomposts were similar in microbial and biochemical properties.

Keywords: E. fetida biomass; enzyme activities; Q-PCR; DGGE; Mantel test.

1. INTRODUCTION

Vermicomposting is an eco-biotechnological mesophilic process that transforms complex organic wastes into a stabilized humus-like product, consists of two different periods related to the earthworm activity: (i) a vermicomposting period when earthworms process the waste for growth, modifying its physical and chemical properties as well as its microbial composition; and afterwards (ii) a maturation period, following the removal of the earthworms, when microorganisms take over decomposition of the waste processed up to then by the earthworms. Also, organic wastes with a high C:N ratio require a preprocessing or preconditioning period (before vermicomposting) to make them acceptable to earthworms. Such preliminary treatments can involve mixing with other organic wastes with low C:N ratio and/or predecomposition with microorganisms naturally present in the wastes or inoculated (Ndegwa and Thompson, 2000 and Kumar and Shweta, 2011).

Lignocellulosic wastes are composed of complex heteropolymers that confer recalcitrant characteristics, becoming a challenge for microorganisms due to the inhibition of cellulosic enzymes (DeAngelis et al., 2011). Composting and vermicomposting are two of the best-known processes for the biological degradation and stabilization of these wastes. Most studies concerning these processes have focused on changes of physical-chemical properties and biochemical (enzymatic) parameters. These parameters reflect the earthworm and microbial activity. Hydrolytic enzymes such as dehydrogenase, β-glucosidase, urease, and phosphatase, involved in the C, N, and P cycle, as well as phenol oxidases involved in lignin degradation, have been studied elsewhere but their relationship with different microbial taxa over the vermicomposting process has not been extensively studied (Sen and Chandra, 2009).

Limited information is available on changes in abundance and structure of microbial taxa and the dynamics of each process. Thermophilic composting and mesophilic vermicomposting can determine the microbial communities and thus the decomposition of the organic matter (Lazcano et al., 2008). In regard to lignin decomposition, Proteobacteria and Actinobacteria are two major taxa involved in this process, where Alpha- and Gammaproteobacteria classes are two of the most important degraders (DeAngelis et al., 2011). Bacteria from the class Actinobacteria are fundamental in

lignin and polyphenol degradation (Kirby, 2005) as well as in the production of antibiotics and enzymes such as chitinases, which are able to degrade the fungal-cell membranes (Jayasinghe and Parkinson, 2009). Recently, molecular techniques such as denatured gel gradient electrophoresis (DGGE) and single-strand conformational polymorphism (SSCP), based on the PCR-amplification of small ribosomal subunit, have been used to analyse of microbial community structure during vermicomposting (Sen and Chandra, 2009). The analysis of phospholipid fatty acid (PLFA) has revealed that earthworms have a diverse pool of digestive enzymes that can digest the microbiota, reducing the microbial populations (Gómez-Brandón et al., 2012). Real time quantitative PCR (Q-PCR) has recently become a valuable molecular tool for quantifying indigenous organisms in environmental samples directly from environmental DNA extracts. This method is powerful, accurate, and culture independent (Prevost-Boure et al., 2011).

The use of agroindustrial wastes to produce organic fertilizers is growing as a profitable and sustainable solution to the high prices of fertilizers, which also cause soil organic-matter loss. Some of the most abundant agroindustrial wastes in the world come from the olive-oil and wine production. Spain has more than 25% of the world's olive growing surface area, with an average production of 0.7-1 x 10^6 t $year^{-1}$ of olive oil, generating 3-4 x 10^6 t $year^{-1}$ of wet olive cake or *alperujo*. In addition, Spain has a 14% of the total world surface area of vineyards, being the world's third wine producer (34 – 37x 10^6 HL), resulting also in abundant waste of grapevine shoots (3 x 10^6 t $year^{-1}$). Both wastes contain recalcitrant lignocellulose materials with high C:N ratios that need to be mixed with other organic wastes with low C:N ratios and preprocessed in order to optimise the biological transformation and to produce an adequate organic fertilizer.

Therefore, the present study firstly evaluates the changes in earthworm biomass, chemical properties, and biochemical functions (dehydrogenase, β-glucosidase, acid phosphatase, urease, and ortho-diphenol oxidase). Secondly the changes in the bacterial and fungal structure are assessed during vermicomposting and after a maturation period. In addition, the total abundance of bacteria and fungi as well as microbial taxa (Alpha-, Beta- and Gammaproteobacteria and Actinobacteria) are quantified, using taxa-specific real-time PCR assay. The final aim is to elucidate the interactions among those parameters in relation to the microbial dynamics during the biotransformation of those complex organic wastes.

2. MATERIALS AND METHODS

2.1. Organic-waste collection and earthworms

Vine shoots collected from a vineyard had 4% moisture, 490 g kg^{-1} organic carbon (o.c.) and 6 g kg^{-1} total Kjeldahl nitrogen (TKN). This waste was air-dried and chipped into pieces of 2 to 5 cm. Biosolid vinasse (85% moisture, 440 g kg^{-1} o.c. and 75 g kg^{-1} TKN) was collected from a vinasse wastewater-treatment plant (Tomelloso, Spain). The wet olive cake from a commercial olive-oil manufacturer (ROMEROLIVA, S.L. Deifontes, Spain), contained 65% moisture, 540 g kg^{-1} o.c. and 10 g kg^{-1} TKN. Goat manure had 71% moisture, 394 g kg^{-1} o.c. and 22 g kg^{-1} TKN. Earthworms (*Eisenia fetida*) from Humus-Fertil (La Roda, Spain) were used.

2.2. Experimental setup

The vermicomposting process was conducted at a pilot scale using wooden boxes (1.7m x 1.4m x 0.25 m) sloped 5% for drainage under semi-controlled field conditions, as reported previously (Melgar et al., 2009). The winery substrate (W) consisted of 46 kg of vine shoots mixed at 4:1 (dw:dw) with biosolid vinasse, while the olive-mill substrate (O) was composed of 49 kg of the wet olive cake mixed with goat manure at 4:1 ratio (dw:dw). Two wooden boxes were used for each substrate, which underwent an initial pre-processing time of two weeks (initial periods, I). Subsequently, two narrow rows of vermicomposted manure were placed on both sides of each mixture to serve as an initial habitat for the earthworms and also as a source of microbial inoculum. An earthworm biomass equivalent to 3% of the dry organic material was inoculated.

During the vermicomposting period, no new substrate was added and the moisture content was kept at 70-80% by irrigation. From each box, four samples were collected by using a sampler of 20 x 20 cm and 25 cm deep. The sampling timetable was: at 1, 3, 5, 7, 11, and 15 weeks to follow the earthworm biomass; when the vermicomposting period was finished (FV =15 wks in the W substrate and 18 wks in the O substrate) the earthworms were removed by hand, and the vermicomposted compounds were matured

for 2 months without water addition. Finally, these samples were stored in plastic bags at 4ºC for enzyme-activity analysis or were frozen in liquid nitrogen at -80ºC until DNA extraction. Representative samples were also collected at the initial time (I), and after the maturation period (M) they were air-dried and finely ground to determine the changes in the chemical properties of these substrates.

2.3 Chemical and enzyme analysis

The pH and electrical conductivity (EC), total organic carbon (TOC), total Kjeldhal nitrogen (TKN), total extractable carbon (TEC), carbon contents in humic and fulvic acids (HAC, FAC) and water-soluble carbon (WSC) were determined according to established methods (Fernández-Gomez et al., 2011). Hemicellulose, cellulose, lignin, and total phenolic compounds were determined according to validated methods described by Romero et al. (2006). The activities of certain enzymes (dehydrogenase, β-glucosidase, acid phosphatase, and urease) were analysed in triplicate using 0.2 g of organic samples, as described elsewhere (Fernández-Gómez et al., 2011). Orthodiphenol oxidase (o-DPO) activity was determined using catechol as the substrate (Perucci et al., 2000).

2.4. Genomic DNA extraction

Total DNA was extracted from 250 mg of each of three representative organic samples collected following the ISO-11063 procedure described by Petric et al. (2011). For purification, aliquots (100 μL) of crude DNA extracts were loaded onto PVPP (polyvinyl polypyrrolidone) minicolumns (BIORAD) and centrifuged for 4 min at 1000 g. This step was repeated to ensure the complete removal of PCR inhibitors when necessary. The quality of the DNA extracts was checked by electrophoresis on 1% agarose gel and quantified at 260 nm using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA), and later were stored at -20ºC until used.

2.5. PCR-DGGE analysis of bacterial and fungal communities

Table 1. Primers and thermal cycling conditions used for PCR-DGGE and quantification by real-time Q-PCR of the different classes.

Primers	Sequences (*5′-3′*)	Thermal conditions
Total bacteria 341F* 518R	 CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	95ºC, 15min, one cycle. 95ºC for 15s, 50ºC for 30s, 70ºC for 30s,72ºC for 60s, 35 cycles.
Total fungi FR1* FF390	 AXCCATTCAATCGGTAXT CGATAACGAACGAGACCT	95ºC, 15min, one cycle. 95ºC for 15s, 57ºC for 30s, 72ºC for 30s,75ºC for 30s, 35 cycles.
Alphaproteobacteria E338F Alfa- 685R	 ACTCCTACGGGAGGCAG CAG TCTACGRATTTCACCYCTAC	95ºC, 15min, one cycle. 95ºC for 15s, 60ºC for 30s, 72ºC for 30s,75ºC for 30s, 35 cycles.
Betaproteobacteria E338F Beta-680R	 ACTCCTACGGGAGGCAG CAG TCACTGCTACACGYG	95ºC, 15min, one cycle. 95ºC for 15s, 55ºC for 30s, 72ºC for 30s,75ºC for 30s, 35 cycles.
Gammaproteobacteria Gamma 395f Gamma 871r	 CMATGCCGCGTGTGTGAA ACTCCCCAGGCGGTCDACTTA	95ºC, 15min, one cycle. 95ºC for 15s, 56ºC for 30s, 72ºC for 30s,75ºC for 30s, 35 cycles.
Actinobacteria Actino 235f E518r	 CMATGCCGCGTGTGTGAA ACTCCCCAGGCGGTCDACTTA	95ºC, 15min, one cycle. 95ºC for 15s, 60ºC for 30s, 72ºC for 30s,75ºC for 30s, 35 cycles.

*Primers with a GC clamp at the 5’ end for DGGE.

The 16S rRNA and 18S rRNA genes were amplified from 25 ng of DNA in a final volume of 25 µL using 0.5µM of each primer (341F-GC/518R and FR1GC/FF390) for bacteria and fungi, respectively (Table 1), 2.5 mM MgCl2, 0.5 mM dNTPs, 1µL of T4 gen 32 (Qbiogene, France) and 1 U of Taq DNA polymerase (Eurotaq). Denaturing gradient gel electrophoresis (DGGE) was conducted by loading 200 ng of PCR products onto 9% and 8% (w/v) polyacrylamide containing 45-70% and 30-60% of denaturant gradients for bacteria and fungi, respectively [100% denaturant contained 7M urea and

40% (vol/vol) formamide]. The banding patterns of DGGE gels were analysed using the GelCompar II software (Applied Maths, Ghent, Belgium). The band profiles were converted into a binary matrix representing the occurrence of DGGE bands (band presence/absence).

2.6. Real-Time PCR Quantification (Q-PCR) of bacteria and fungi

The total abundance of bacteria and fungi as well as Alpha-, Beta-, Gammaproteobacteria, and Actinobacteria classes in the organic samples were quantified using the taxon-specific 16S rRNA or 18S rRNA, respectively. For this purpose, a set of primers were selected (Table 1). Standard curves were drawn for plasmids containing cloned the16S rRNA gene from *Sinorhizobium meliloti*, *Burkholderia* sp., *Pseudomonas putida* KT2440, and *Arthrobacter* sp, which represent these bacterial taxa, as well as the 18S rRNA from a pure culture of *Fusarium oxysporum* (EEZ-CSIC strain collection). The bacterial and fungal Q-PCR conditions were according to Philippot et al. (2011) and Prevost-Boure et al. (2011), respectively, with some modifications (Table 1). The PCR reactions were conducted in an iCycler MyiQ™ Systems (Bio-rad, USA) using SybrGreen® as the detection system. The reaction was performed in a final volume of 15 µl containing 7.5 µl of iQ™ SYBR® Green Supermix, 0.5 µM of each primer, 0.5 µl of T4 gen 32 (Qbiogene, France) and 2 ng of template DNA. The function that described the relationship between Ct (threshold cycle) and the number of sequences of each bacterial and fungal taxon, ranged between -3.40 to -3.27 with a PCR efficiency of 95.6% to 99.9%, respectively. Controls without templates gave null or negligible values.

2.7. Statistical analysis

Earthworm biomass, biochemical and microbiological data during the vermicomposting process were analysed by repeated-measures analysis of variance (RM-ANOVA). Violation of the sphericity assumption (Maulchy's test) was amended by application of the Huynh-Feldt correction to the significance level. The Least Significant Difference test (LSD) was used to compare the different means in each treatment (W and

O) and each period. Physico-chemical properties were analysed using Student's t-test between the final vermicompost (FV) and mature vermicompost (M) periods. All these analyses were made using the SPSS version 15.0 (IBM, Chicago, IL, USA). Bacterial and fungal-DGGE profiles were analysed by principal components analysis (PCA) on the covariance matrix that was performed on a data matrix (sampling time of the vermicomposting periods as rows and presence/absence bands as columns) created byusing the GelCompar II software. This ordination method provided a factorial map of the genetic structure of the microbial community based on similarities. The PCAs (Canoco, Windows 4.5) and a multivariate analysis of Variances (MANOVA) were employed to test significant differences among the PC scores closely associated. MANOVA is a sensitive analysis for the inequality of variances represented by Box's test of equality of covariance matrices. In cases where more than two sampling times were compared, Tukey's *post hoc* test was employed. The correlation between microbiological and biochemical variables as well as *E. fetida* biomass during the vermicomposting period was assessed using Mantel's multivariate test and corroborated using Pearson's correlation (PCORD 5). With Mantel's test, distance matrices for DGGE profiles of 16S rRNA and 18S rRNA were determined using a Jaccard distance transformation, and for the rest of variables, the Euclidian distance were inferred.

3. RESULTS AND DISCUSSION

3.1. Evolution of total earthworm biomass during the vermicomposting period

The colonization of earthworms in the mixture of vine shoot and biosolid vinasse (W) was very fast (Figure 1). Three weeks after the inoculation, the earthworm biomass reached a maximum value of 23±4 g kg^{-1} substrate. Thereafter, the worm biomass declined significantly until the week 7 as available nutrients in the organic mixture became depleted, remaining constant until the end of the vermicomposting period. By contrast, the mixture of wet olive cake and goat manure (O) remained uncolonized until the 3rd week, when low earthworm biomass was recorded (0.96±0.1 g kg^{-1} substrate). Afterwards, earthworm biomass increased significantly, reaching the maximum (21±3 g kg^{-1} substrate) at week 15 of vermicomposting.

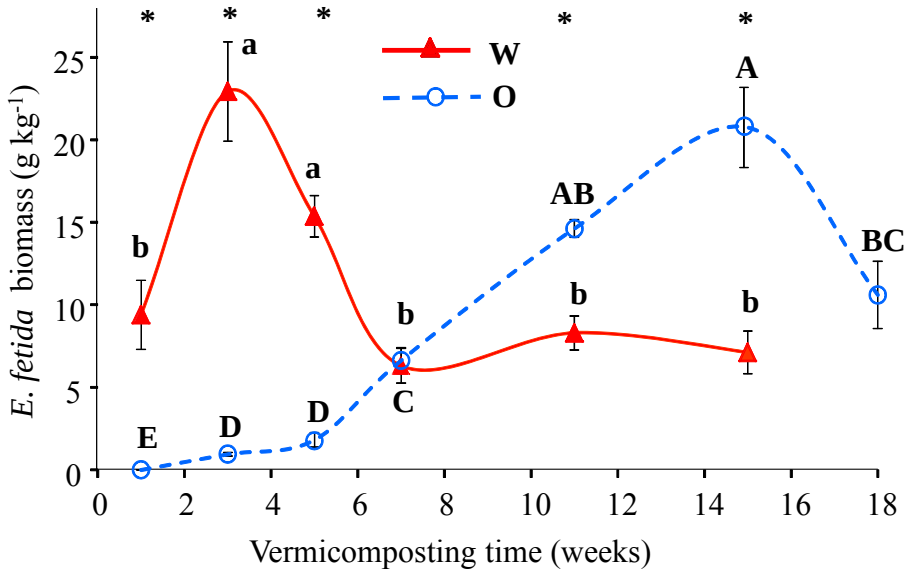


Figure 1. Earthworm biomass during the vermicomposting period. Vertical bars represent the standard deviation. Same letter (lowercase W or Capital O) are not significantly different ($p < 0.05$). The asterisk (*) denotes a significant difference between substrates at a given time.

From 15 to 18 weeks, earthworm biomass diminished significantly as a consequence of depletion of the nutrient content in this feed substrate. At week 15 in W and 18 in O, no clitellated earthworms were found, implying that more substrate would be necessary in order to sustain earthworm growth and reproduction. In previous vermicomposting processes using wet olive cake, alone or mixed with other organic wastes, but inoculated with a higher earthworm density, the substrate was also colonized in a slow manner, and increments in earthworm biomass were found after at least 2 months post-inoculation (Melgar et al., 2009). The high levels of fatty and oily compounds in the wet olive cake may initially favour a microaerophilic environment in the O substrate, and therefore the earthworms are deprived of oxygen and/or exposed to transitory toxic substances produced under those conditions. The fast increase of earthworm biomass in the W may have been due to the addition of small vine-shoot fragments (< 5 cm) that promoted the rapid colonization of filamentous fungi on the wood substrate, creating a favourable environment for bacterial growth during the preprocessing period (Figure 1). Wal et al. (2007) describe the size as a factor affecting the rate of initial wood decomposition, as small fragments have a relatively large surface area for interaction with the surrounding

microbiota. Given that untreated wood wastes are unsuitable for the earthworm development (Kumar and Shweta, 2011), the rapid growth of these microorganisms must have helped to reduce the predecomposition time in W substrate (McMahon et al., 2008). In fact, the wide set of enzymes capable of decomposing recalcitrant lignocellulose wastes and the networks of hyphae of most fungal species may allow the translocation of nutrients from N-rich to N-poor substrates (Chigineva et al., 2011), stimulating microbial activity and promoting earthworm colonization, since fungal mycelium constitute a nutritional source for these Oligochaeta.

3.2. Chemical changes after the vermicomposting-maturation period

The chemical properties of the initial substrates (I) were significantly altered after the vermicomposting process and the maturation (M) period (Table 2). The pH values changed slightly, significantly rising only in the olive-oil-waste mixture (O). The electrical conductivity, 3-fold higher in O than in the winery mixture (W), decreased significantly, whereas in the W substrate the opposite occurred. In either case, total organic carbon (TOC), water-soluble carbon (WSC), cellulose, hemicellulose, lignin, and polyphenols significantly decreased. The reductions in the WSC, lignin, cellulose, and hemicellulose content in W were greater (64%, 33%, 73%, and 45%, respectively) than in O (41%, 20%, 65%, and 39%, respectively). However, the reduction in TOC content recorded in the olive substrate (O) was greater (45%) than in W (30%), due to the presence in O of specific organic compounds, such as carbohydrates or fats (7-19%) (Alburquerque et al., 2009), which were degraded during the vermicomposting and maturation processes. The total extractable carbon (TEC), as a measurement of the total carbon in the humic substances, was similar in the initial substrate and the mature vermicomposts. Nevertheless, the humic acid fraction (HAC) was significantly higher in the vermicomposted and matured product (M). The total N contents in the mature vermicomposts were higher than initial substrate, although significant differences were detected only in O. The significantly lower C/N values and the higher HAC:FAC ratio and percentage of humification (HR), in both mature vermicomposts (M) with respect to the initial values, indicate a high degree of stability and an extended synthesis of organic compounds resistant to microbial degradation (i.e. humification). Comparatively, the

humification process was more widespread in the olive substrate (O), presumably related to its greater mineralization (% of TOC lost).

Table 2. Physico-chemical analysis of initial substrates (I) and mature vermicomposts (M) of vine shoots mixed with biosolid vinasse (W) and wet olive cake mixed with goat manure (O).

	W		O	
	I	MV	I	MV
pH	7.8±0.2	7.6±0.1	7.3 ± 0.1	7.8 ± 0.0 *
EC (dS m^{-1})	1.4±0.0	4.4±0.2*	4.4 ± 0.2 *	3.2 ± 0.2
TOC (g kg^{-1})	454±1*	317±6	496±2 *	274±1
WSC (g kg^{-1})	15.6±2.4 *	5.6±0.2	13.1±0.4 *	7.6 ± 0.0
Cellulose (g kg^{-1})	248±1*	66±1	193±1*	68±1**
Hemicell. (g kg^{-1})	186±1*	102±2	161±1*	115±1*
Lignin (g kg^{-1})	240±2*	160±1	189±1*	151±1.0
Polyphenols (g kg^{-1})	3.5±0.1*	1.4±0.0	4.8±0.6*	1.9±0.1
Total N (g kg^{-1})	26±0.8	28±0.5	17±0.6	19±0.3*
C/N	17±0.5*	11±0.0	30±1*	14±0.2
TEC (g kg^{-1})	55±1	55±3	142±4	151±1
HAC (g kg^{-1})	16±2	30±0.3*	87±2	105±0.7*
HR %	12±0.2	17±1*	29±1	55±1
HAC/FAC	0.43±0.11	1.2±0.2*	1.6±0.0	2.2±0.0*

EC: Electrical conductivity, TOC: Total organic carbon, WSC: water-soluble carbon, TEC: Total extractable carbon, HAC: Humic acid carbon, FAC: Fulvic acid carbon, HR: Humification ratio=100×TEC/TOC. The asterisk (*) denotes, in each substrate, significant differences ($p < 0.05$, paired-sample t-test) between the initial substrate and mature vermicompost for each parameter

3.3. Enzyme activities during the vermicomposting and maturation time.

In W, Dehydrogenase (Dhase) activities related to the metabolic state of microbial population showed significant differences (F=60.3 p<0.001). Values were highest during the first three weeks and afterwards fell significantly to remain almost constant until the end of the vermicomposting period (FV). However, the Dhase activity remained constant the first 5 weeks in O, and then intensified until FV (Fig 2a). In both process, the Dhase activity in the maturation time (M) was significantly lower than in FV. The o-DPO enzyme, which is related to the capacity of the microorganisms to break down recalcitrant organic compounds, followed a similar pattern in both substrates, increasing during the first three weeks when the amount of these compounds was larger, then declining, and finally increasing significantly in O until FV (figure 2b). In M, the activity diminished significantly for W and in O showed similar activity to the 1th week (F=26.3 p=0.14). In both organic substrates, the o-DPO activity decreased significantly in the maturation period. In the W, the highest values of β-glucosidase and urease activities were recorded in the first week of the vermicomposting period, diminishing until the 5th wk and then remaining constant. By contrast, β-glucosidase activity intensified significantly during the vermicomposting period in the O, while urease activity reached the highest values after 3 weeks, and then declined later until FV. In general, these both enzymes significantly declined after the maturation period (figure 2c, d), except to β-glucosidase, where FV and M no showed differences in O (p=0.54). In relation to the acid phosphatase activity, vermicomposting and maturation time did not significantly differ over the time course (F=2.55 p=0.12) in W. However, O substrate registered higher acid phosphatase activities at FV and M times with respect to the first five weeks. In general, all these enzymes were significantly higher at the first week in the W than in O mixtures. This reveals that the W contained greater amounts of easily available organic compounds than did O. Thus, the higher activity of β- glucosidase can be explained by the higher hemicellulose and cellulose content in W (Table 1), in which hydrolysis produced cellobiose, this enzyme being responsible for converting cellobiose into glucose. In addition, the higher content in organic N and the addition of a substrate with an easily metabolizable organic-N, via vinasse biosolids, as has been reported in

other biosolids (Parmar et al., 2001), would induce the urea production, thereby leading to the notable urease activity recorded at the beginning of the vermicomposting process.

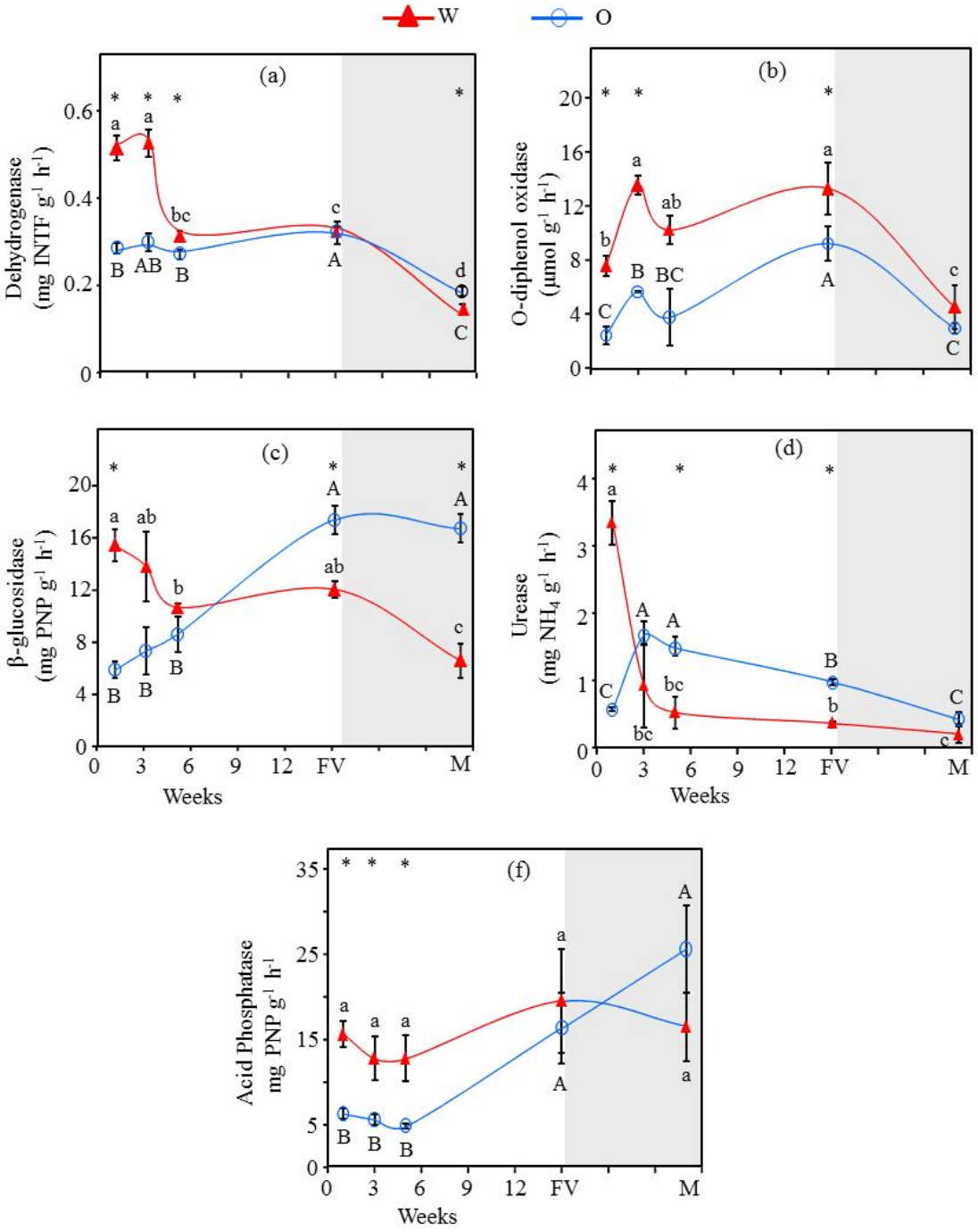


Figure. 2. Enzyme activities during the vermicomposting period (blank) and after maturation period (grey). Error bars represent the standard deviation. Data with the same lowercase (W substrate) or capital letter (O substrate) are not significantly different ($p < 0.05$). The asterisk (*) denotes a significant difference between substrates at a given time.

Consequently, a high ammonium content accumulated in this substrate (4 mg g^{-1} determined in the blank samples from the urease-activity measurements), which would strongly inhibit the urease activity (Castaldi et al., 2008), and hence a pronounced decrease occurred after one week. Likewise, it is well known that biosolids contain large amounts of organic phosphate compounds which induce acid-phosphatase enzyme synthesis. Due to high amounts of easily available organic compounds in W substrate, a greater dehydrogenase activity was recorded with respect to the O substrate.

Despite these clear differences, in both vermicomposting processes, although the evolution of *E. fetida* biomass was also different (Fig. 1), Mantel's test revealed a significant correlation between the worm biomass and the β-glucosidase and o-DPO activities (Tables 3). This implies that both enzyme activities were directly related to *E. fetida* activity and depended on its population. The enzyme β-glucosidase can be strongly activated in the earthworm gut, as suggested above, resulting in greater cellulolytic activity in castings than in undigested material (Parthasarathi and Ranganathan, 2000). Therefore, this activity abates when the worm biomass decreases and less casting would be released into the organic substrate. Similar results were described in the vermicomposting of other wastes such as pig slurry (Aira et al., 2007) and tomato-fruit wastes (Fernández-Gómez et al., 2010). In addition, β-glucosidase was correlated with Dhase activity, since the vermicast released has a larger surface area, higher humidity, and greater nutrient concentrations, resulting in more vigorous microbial activity. Therefore, the enzyme β-glucosidase is a good biomarker to monitor the time course of an earthworm population in the vermicomposting processes using lignocellulose wastes as feed substrates. Finally, the enzyme o-DPO was related to the relative abundance of the Actinobacteria class and to worm biomass (Table 3). Thus, both would be expected to contribute to the degradation and/or stabilization of the phenolic compounds (Table 2). It has been reported that *E. fetida* has the capability to bioaccumulate high amounts of harmful chemicals (including phenol) in their tissues and either biodegraded or biotransformed by o-DPO enzymes, which are involved in the formation of melanin, a key compound in cellular pathogen defence (Procházková et al., 2006). On the other hand, the intimate mixing during passage through the earthworm gut encourages the stabilization of phenolic compounds by the stimulation of the microbial activity in castings (Sabrina et al., 2012).

Table 3. Mantel's test of dissimilarity matrices obtained from multiple comparisons of worm biomass, enzyme activities, abundance, and structure of bacteria and fungi and relative abundance of microbial taxas during the vermicomposting period.

O / W	Worm Biomass	Enzymes					Abundance		Relative abundance				Structure	
		Dhase	o-DPO	Gluco	Urease	Phosp	Bacteria	Fungi	Alpha	Beta	Gamma	Actino	Bacteria	Fungi
Worm bio.		0.34*	0.60**	0.90**	ns	0.92**	-0.43*	ns	ns	0.72*	ns	0.70**	0.61**	0.64**
Enzyme														
Dhase	ns		ns	0.32*	ns	0.30*	ns	ns	ns	ns	ns	ns	ns	ns
o-DPO	0.33*	ns		0.68*	ns	0.63*	-0.43**	-0.41*	ns	0.58**	ns	0.57**	0.57**	0.62**
Gluco	0.33*	0.54**	ns		0.02	0.87**	-0.52**	-0.40*	ns	0.80**	ns	0.80**	0.64**	0.68**
Urease	ns	ns	-0.47**	ns		ns	0.48*	ns	ns	ns	ns	ns	0.36**	0.53*
Phosp	ns	ns	ns	ns	ns		0.48*	ns	ns	0.70**	ns	0.66**	0.57**	0.56**
Abundance														
Bacteria	-0.44*	0.25*	-0.53**	0.35*	0.55**	ns		0.57**	ns	-0.53**	ns	-0.50**	0.40**	0.40**
Fungi	ns	0.69**	ns	ns	0.22*	ns	0.25*		0.34*	-0.40*	0.34*	-0.43**	0.27*	0.35*
R.Abundance														
Alpha	ns	0.40*	ns	ns	0.62*	ns	0.42*	0.51**		0.35	Ns	0.32*	ns	ns
Beta	ns	ns	ns	ns	-0.51**	ns	ns	ns	ns		Ns	0.69**	0.55**	0.58**
Gamma	ns	ns	ns	ns	0.60**	ns	0.57**	ns	0.60**	0.31		-0.32*	0.28*	0.33*
Actino	ns	ns	0.47**	ns	-0.46**	ns	-0.42**	ns	ns	0.68**	0.26**		0.71**	0.66**
Structure														
Bacteria	0.34*	0.56**	ns	0.37*	0.38**	ns	0.35*	n.s	0.39**	0.53**	0.30*	0.44**		0.92**
Fungi	0.68**	0.31**	0.50**	0.35**	0.71**	ns	0.66**	n.s	0.34*	0.32*	0.41**	0.54**	0.77**	

*significant correlation at 0.05 level; **significant correlation at 0.01; ns: no significant

The maturation period in vermicomposting processes is a final period that needs no treatment, which involves only microorganisms, and is related to the stabilization and humification of the organic matter contained in the substrates. However, the biochemical and microbiological changes occurring at this stage are still not well known. In W and O mixtures after maturation (M), significant decreases in all the enzymes activity were found with respect to the final time of the vermicomposting period (FV), except for acid phosphatase (Figure 2). These reductions indicate depressed microbial activity due partially to the scarce available C and N sources after the vermicomposting period (FV) as well as to the partial drying over this phase (from 76% at FV to 30% after maturation). It is well known that drying depresses microbial activity, particularly the metabolic activity of bacteria, also inducing the inactivation of extracellular hydrolytic enzymes (Dick, 1994). The higher β-glucosidase, urease, and phosphatase activities in O with respect to W in the mature vermicomposts (M) suggest larger fractions of extracellular enzymes-humic acid bonds and more remains of inducers to these enzymes (Burns, 1982). This may be supported by the higher humic acid contents and humification ratio measured in the O substrate at this phase (Table 2).

3.4. Abundance and structure of bacterial and fungal communities during the vermicomposting and maturation period

In the W substrate, the total bacterial abundance was the highest in the first week ($46.1 \times 10^9 \pm 7.3 \times 10^9$ copies g^{-1}), decreasing significantly from 1 to 3 weeks (Fig. 3a). In the O substrate the total bacterial abundance did not show significant differences (F=2.55 p=0.12), however in the first week the abundance was significantly lower ($5.6\ 10^9 \pm 1.6 \times 10^9$ copies g^{-1}) than in W. Nevertheless, a similar value (2.5×10^9 -1.3×10^9 copies g^{-1}) was reached at the end of the vermicomposting period (FV). Total abundance of fungi, followed a similar pattern to that of total bacteria (Fig. 3b). In fact, a significant correlation (Table 3) was found between the abundance of bacteria and fungi during the vermicomposting period, values being higher in O (0.58, $p<0.01$) than in W (0.25, $p<0.05$).

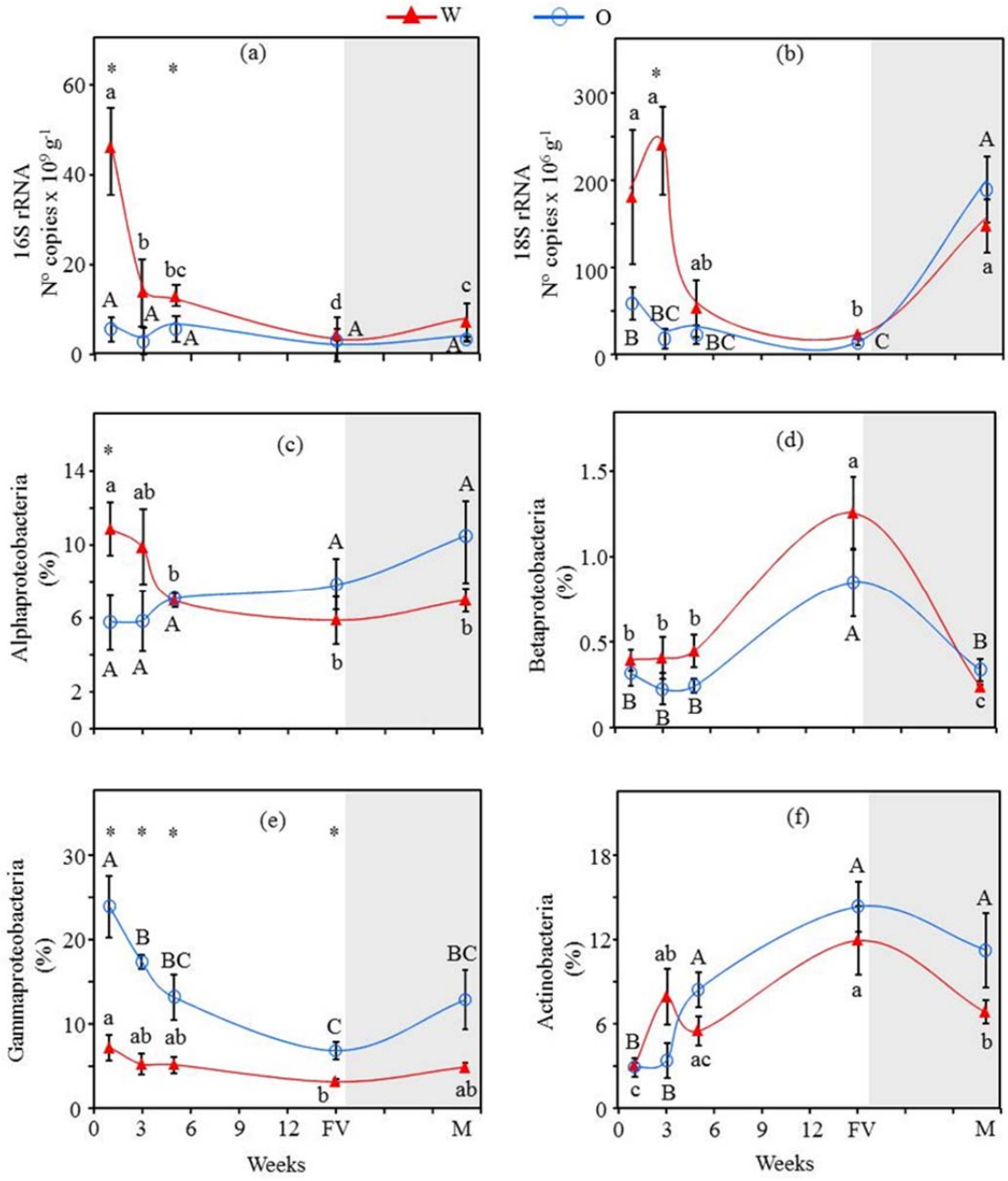


Figure 3. Total abundance of bacteria and fungi, relative abundance of microbial taxa during the vermicomposting period (blank) and after maturation period (grey). Error bars represent the standard deviation. Data with the same lowercase (W substrate) or capital letter (O substrate) are not significantly different ($p < 0.05$). The asterisk (*) denotes a significant difference between substrates at a given time.

The bacteria: fungi abundance ratio tended to decrease during the vermicomposting period in the W substrate (255 in the 1st week, 204 at FV) but increased significantly in the O substrate (95 in the 1st week, 365 at FV). This was due to a similar reduction of bacteria and fungi with respect to the initial population in W (95% and 93%, respectively),while in the O substrate showed a more severe decline in fungal abundance with respect to bacterial abundance (94% and 77% respectively). Bacteria and fungi are considered important dietary sources for earthworms, and therefore, the digestion of the organic wastes by these Oligochaetes would have a negative effect on total bacteria and fungi abundance (Table 3). However, Sen et al. (2008), using the real-time PCR method, observed that total bacterial abundance remained constant during the vermicomposting of industrial sugar wastes, varying within the range of 1.7×10^8 - 6.8×10^8 16S rRNA copies g^{-1}. By contrast, Pramanik and Chung (2011) found increases in the total number of bacteria and fungi during the vermicomposting process. These contrasting results may be related to the carbon available. Tiunov and Scheu, (2004) have reported a strongly decrease in microbial biomass under carbon-limited conditions, where earthworms and microorganisms compete for easily available carbon. On the other hand, the addition of labile carbon increases N conversion into microbial biomass, becoming a source of N available to earthworms.

The fungi abundance in both process increased significantly throughout the maturation period (Figure 3a,b). These pronounced surge in fungi at this stage would confirm that these microorganisms have the ability to grow on recalcitrant compounds after the vermicomposting period under a low-moisture regimen (<30-40%).

The discrete DGGE band patterns found during the vermicomposting period were used as genetic fingerprints of bacterial and fungal community structure. The complex banding pattern with 35 and 25 bands per line in the two vermicomposting processes, respectively. Mostly, the bands were similar between treatment replicates, indicating good reproducibility of extraction, PCR amplification, and electrophoretic separation. (Fig. 4a-d). The principal component analysis (PCA) of the DGGE fingerprinters of bacteria in W substrate (Fig. 4a) revealed that the first principal component (PC1), with 30% of the variance, distinguished the bacterial structure at the early times of vermicomposting (1st and 3rd weeks) from that of the final vermicomposting time (FV).

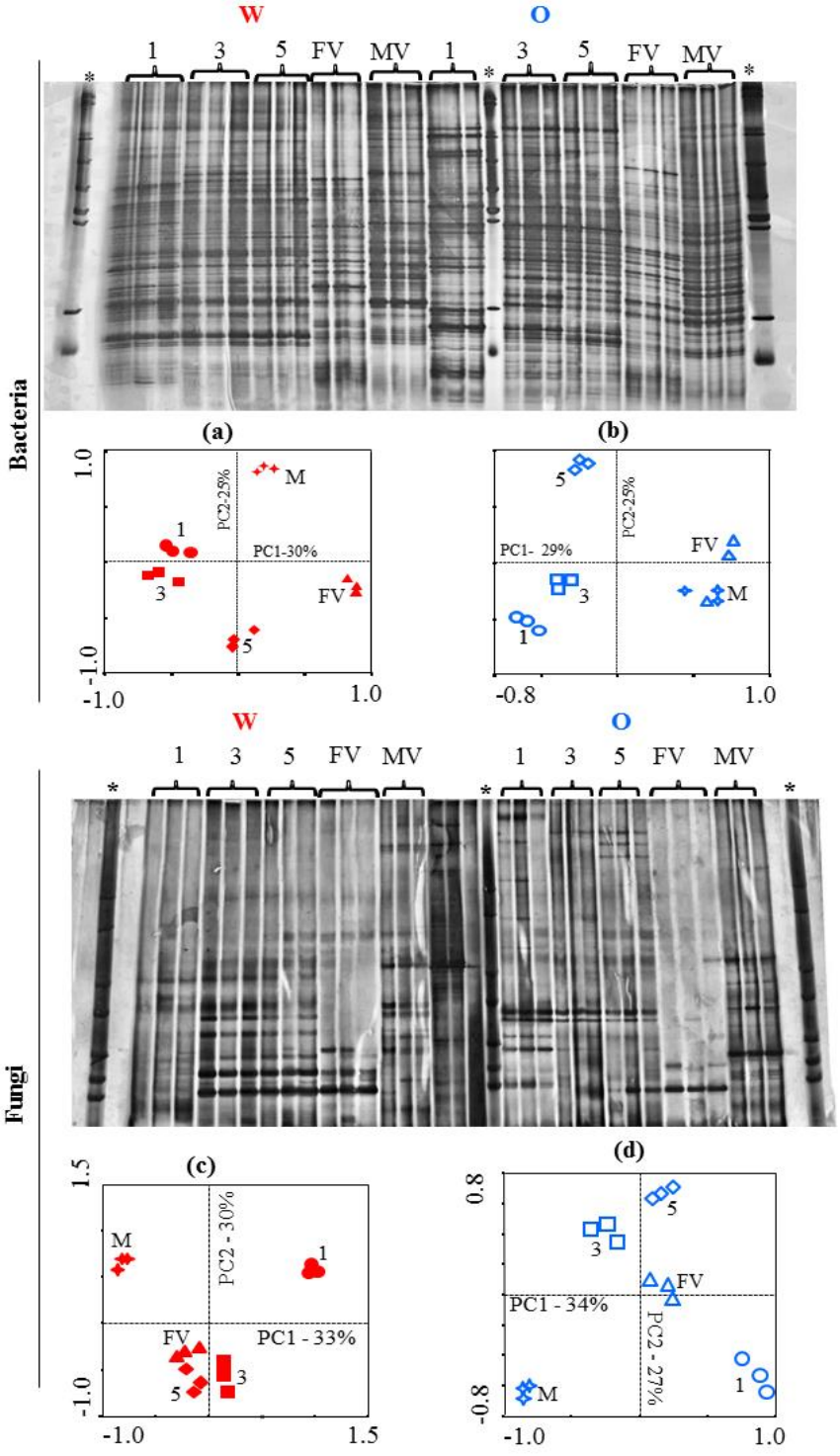


Figure 4. Grey-scale image of DGGE gels and principal-component analysis of bacterial (a and b) and fungi (c and d) in W and O substrates during the vermicomposting period (1,3, and 5 weeks and FV) after maturation (M). The asterisk (*) represents the ladder line.

The second component (PC2) explained the 25% of the variance, discriminating thebacterial structure at the 5th week from the mature vermicompost (M). In the O substrate (Fig. 4b), the PCA of the bacterial structure revealed that 29% of the variance was explained by PC1 but with short spatial distances between the 1st, 3rd, and 5th weeks and between the FV and M. Multivariate analyses of variance (MANOVA) in these PC scores showed that the bacterial structure at the 1st and 3rd weeks significantly differed ($p<0.01$) according to Tukey's test. PC2 explained 25% of the variability, discriminating the bacteria structure at the 5th week from that of the other times. The fungal-structure analysis by PCA in the W substrate showed that PC1 explained 33% of variance data (Fig. 4c), discerning clearly between the 1st week and M. In addition, the MANOVA analysis revealed a significant spatial separation ($p<0.001$) between the fungal structure in the 3rd week and those corresponding to the 5th and FV. These fungal structures were also differentiated from the 1st week and M in the PC2 with 30% variance. The fungal structure analysis in the O substrate with variance data in PC1 and PC2 of 34 and 27%, respectively, was similar to that described in the W substrate (Fig. 4d). The bacterial and fungal structure in both substrates was closely correlated with the time course of worm biomass over the vermicomposting period (Table 3). This supports the idea that the digestion of these organic wastes by *E. fetida* significantly modified the microbial structure, by discriminating feed and/or by stimulating or depressing specific microorganism taxa. Likewise, the bacterial and fungal structures were correlated with each other and with their respective abundances (Table 3). In addition, Mantel's test highlights the correlations between bacterial and fungal structure with β-glucosidase and urease activities in both substrates. This indicates that the structure was related to labile C and N sources.

3.5. Relative abundance of microbial taxa communities during the vermicomposting and maturation period

In both substrates, the abundance of Alpha-, Beta-, and Gammaproteobacteria as well as Actinobacteria classes (Fig. 3) were correlated with bacterial structure (Table 3), indicating that these play a significant role in bacterial diversity changes occurring during the vermicomposting.

At the beginning of the vermicomposting period, the Alpha- and Gammaproteobacteria classes (Fig. 3c and e) were dominant, respectively, in winery wastes (10.9±0.8%) and olive-oil wastes (23.9±0.8%), diminishing significantly ($p<0.001$) with vermicomposting time. In the W substrate, the relative abundance of the two bacterial groups were correlated with each other ($r = 0.60$, $p<0.01$) and with the total bacterial abundance ($r = 0.42$, $p<0.05$ Alphaproteobacteria; $r = 0.57$, $p<0.01$ Gammaproteobacteria), but in the O substrates this did not occur. Alpha- and Gammaproteobacteria taxa are related to the degradation of lignin (DeAngelis et al., 2011) and both with the fungi would be responsible for the decline in these organic winery wastes during the early stages of the vermicomposting. This is supported by the correlation of the urease activity with the abundance of both bacterial groups in this substrate ($r=0.62$, $p<0.05$ Alphaproteobacteria; $r = 0.60$, $p<0.01$ Gammaproteobacteria).

However, this did not occur in the O substrate, where the abundance of the Gammaproteobacteria class was considerable. This class is also an abundant group in microcosms treated with oil products (Viggor et al., 2013), due to the ability the some bacteria to produce biosurfactants and to survive in this substrate type. Betaproteobacteria and Actinobacteria classes, which initially were less abundant, proliferated significantly with vermicomposting time, correlating with each other in both substrates ($r=0.68$, $p<0.01$ in W, $r=0.69$, $p<0.01$ in O). At the end of the vermicomposting (FV), the Actinobacteria became predominant, their relative abundance reaching 11.9% in W and 14.3% in O, exceeding even the sum of the rest of the classes determined. Actinobacteria class have enzyme activity against a wide variety of substrates, as reflected by the significant correlations with o-DPO activity ($r=0.47$, $p<0.01$ in W; $r=0.57$, $p<0.01$ in O). This enzyme has been reported in Actinobacteria such as *Nocardia* sp. and several species of *Streptomyces* (Kirby, 2005).

Some genera of the Actinobacteria class are abundant in the *E. fetida* gut, and it is known that some produce chitinase enzymes to degrade chitin, which is a component of the cell wall of fungi (Yasir et al., 2009). This may explain the negative correlation between the abundance of fungi and the Actinobacteria class in the olive substrate (O) ($r=-0.43$, $p<0.01$). By contrast, in the winery substrate (W), this negative correlation was not found, due presumably to the higher abundance of fungi after the preconditioning period, as mentioned above. Nevertheless, in the W and O vermicomposting, the relative

abundance of Actinobacteria class was negatively correlated with the total bacterial abundance (r=-0.42, p<0.01 in W; -0.50, p<0.01in O) and particularly with the relative abundance of Gammaproteobacteria (-0.26, p<0.01 in W; -0.32, p<0.05 in O). These results must be related with the colonization ability of this taxa in the worm gut and castings (Jayasinghe and Parkinson, 2009) and by its antagonistic activity against other bacterial taxa. It is well known than many genera of Actinobacteria produce antibiotics, which depress or eliminate other bacteria and fungi, including pathogenic bacteria such as *Salmonella* sp., *Vibrio cholerae*, *Pseudomona* sp., and *Escherichia coli*, which belong to the Gammaproteobacteria class (Monroy et al., 2009). The absence of *Salmonella* and *E. coli* determined in the O product, as a consequence of the addition of manure (< 1000 NMP g^{-1} product; Real Decreto 824/2005, Orden PRE/630/2011) leads to the hypothesis that the abundance of Actinobacteria class in the O vermicompost product (Fig. 3f) contributed to the reduction of human pathogens. Thus, the presence of a large abundance of Actinobacteria community when a potential toxic substrate (manure, sludges, etc.) is submitted to vermicomposting would ensure the quality and safety of the product. At the end of maturation period, the relative abundance of Betaproteobacteria and Actinobacteria classes suffered a reduction, whereas Gammaproteobacteria had a significant increment (Figure 3d,e,f).This bacterial taxa must be one of the most resistant bacteria under this maturation condition. On the other hand, the reduction of relative abundance of Actinobacteria class can minimize their antagonistic action against fungi and other bacteria (Jayasinghe and Parkinson, 2009).

4. CONCLUSIONS

Novel information is presented concerning the relationships between microbial communities, enzyme activities and worm biomass in vermicomposting and maturation periods of dissimilar lignocellulosic wastes. In the vermicomposting period of both substrates, the total bacterial abundance diminished but Actinobacteria class proliferated, this being the main microbial taxa at the end of this period. In addition, the bacterial community-structure changes were correlated to biomass worm and β- glucosidase activity. The maturation period resulted in a subsidence of microbialactivity while the

total abundance augmented. Actinobacteria constitute good indicators to ensure the safety of the vermicomposts, although further studies are needed in this sense.

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CAPÍTULO 2

Degradación de 3,4 dicloroanilina por hongos aislados durante el pretratamiento de residuos vitivinícolas por vermicompostaje.

Degradation of 3,4 dichloroaniline by fungal strains isolated from the preprocessing of winery wastes for vermicomposting.

El capitulo 2 ha sido publicado en la revista Journal of Hazardous Materials

Castillo-Diaz, J.M., Nogales, R., Romero, E. 2014. Biodegradation of 3,4 dichloroaniline by fungal isolated from the preconditioning phase of winery wastes subjected to vermicomposting. Journal of Hazardous Materials, 267, 119-127. DOI: /10.1016/j.jhazmat.2013.12.052

Resumen

El contaminante 3,4-dicloroanilina (DCA) se encuentra muy extendido en el medio ambiente debido a su amplio uso en la fabricación de productos químicos y su utilización en los distintos sectores. El estudio tuvo como objetivo investigar la capacidad que tienen diferentes hongos que crecen durante el periodo de preacondicionamiento de un proceso de vermicompostaje de residuos vitivinícolas para degradar DCA. Se aislaron tres hongos filamentosos (F1, F2 y F3), identificados como como *Fusarium sp* (F1 y F2) y *Aspergillus niger* (F3). En medios de cultivo con cada uno de los hongos o en consorcio (Fmix) con DCA como fuente de nitrógeno fueron analizaron mediante microextracción en fase sólida y cromatografía de gases-espectrometría de masas (SPME-GC/MS). La velocidad de degradación de DCA fue más rápida en el consorcio de hongos (Fmix) con un DT50 de 0.85 día^{-1}. El metabolismo del DCA fue diferente para *Fusarium* sp. y *A. niger*. Se identificaron cinco metabolitos como resultado de reacciones de oxidación, co-desnitrificación, N-acetilación y polimeriazación. Los principales metabolitos fueron 3,4 dicloroacetanilida y dichloroquinolinas. Los azo-metabolitos tetracloroazobenceno y tetracloroazoxibenceno y el 3,4-dicloronitrobenceno se encontraron en cantidades menores, pero parecían ser los más persistentes en los cultivos de *Fusarium* (vidas medias que van desde 8,3 a 30,9 días). El estudio puso de manifiesto el potencial metabólico de los microorganismos aislados durante el período preacondicionamiento del proceso de vermicompostaje y su posible aplicación en estrategias de biorremediación in situ.

Palabras claves: 3,4-Dichloroanilina, vías de biodegradación, hongos, vermicompostaje de residuos vitivinicolas, SPME-GC/MS.

Abstract

A hazardous contaminant, 3,4-dichloroaniline (DCA) is widespread in the environment due to its extensive use in the manufacture of chemicals and its application in different sectors. The ability of fungi grow on in winery wastes in the preconditioning period of vermicomposting to degrade DCA was investigated. Three filamentous fungi (F1, F2, and F3) were isolated and one identified as *Aspergillus niger* and two as *Fusarium* sp. strains. The culture media with the fungus alone or in consortium (Fmix) with DCA as the nitrogen source were analyzed by solid-phase microextraction and gas chromatography–mass spectrometry (SPME-GC/MS). The fastest degradation rate was measured in Fmix with a DT50 of 0.85 day^{-1}. *Fusarium* sp. and *A. niger* differed in the metabolism of DCA. Five metabolites were identified as a result of oxidation, co-denitrification, N-acetylation, and polymerization reactions. The major metabolites were 3,4-dichloroacetanilide and dichloroquinolines. The azo-metabolites tetrachloroazobenzene and tetra-cloroazoxybenzene and 3,4-dichloronitrobenzene were found in minor amounts but appeared to be the most persistent in the *Fusarium* cultures (half-lives ranging from 8.3 to 30.9 days). This study highlights the metabolic potential of microorganisms in the preconditioning period of the vermicomposting process and its possible application for in situ bioremediation strategies.

Keywords: 3,4-Dichloroaniline, biodegradation pathway, fungi, winery wastes-vermicompost, SPME-GC/MS.

1. INTRODUCTION

The compound 3,4-dichloroaniline (DCA) is used in the synthesis of a wide range of substances. DCA residues from the chemical industries are released via wastewater into the hydrosphere and are again incorporated into soil or water compartments when pesticides such as vinclozolin or phenylureas are biodegraded by microorganisms [1-3]. This compound remains highly persistent in soils and waters [4-6], affecting the soil microbial population [7] and aquatic species [8]. Tetrachloroazobenzene (TCAZB) and tetrachloroazoxybenzene (TCAXB) are also formed as unwanted by-products from compounds containing the DCA moiety [3]. Martins et al. [9] have reported that aromatic amines (AA) have genotoxic and cytotoxic potential towards most living organisms. Therefore, exposure to this toxic contaminant has raised increasing concern over its fate and persistence in the environment.

Fungi, in contrast to bacteria, are capable of degrading a wide range of organic pollutants such as pesticide residues even at high concentrations because they have a complex enzymatic system which is usually induced by nutrient depletion, not by a particular pollutant. Basidiomycetes, white rot fungi (*Chrysosporium lignorum*, *Trametes versicolor*, *Phanerochaete chrysosporium*) have physiological capacity to degrade lignin and many xenobiotic compounds with a variety of structures due to its ligninolytic system [10]. However, the effectiveness of these fungi in xenobiotic degradation under environmental conditions is questionable, because the pH and C:N ratio are not always optimal for growth [11]. To overcome these disadvantages, the isolation of imperfecti fungi of lignocellulose wastes may be an excellent alternative. Imperfecti fungi have ability to grow and occupy high volumes of wood and tolerate water stress [10]. Moreover, their lignin-degradation systems are not very substrates specific and therefore can oxidize a great variety of compounds, including environmental pollutants such as dye or anthracene [12, 13]. Imperfecti fungi play an important role in the pre-composting period from vermicomposting of lignocellulose organic substrates, contributing to the optimal conditions for earthworm growth [14, 15]. However, no information is available on the biocatalyst potential of these imperfecti fungi during the first step of vermicomposting despite that they may offer an efficient alternative for bioremediation techniques meant to degrade and minimize the

environmental impact of DCA, providing a low-cost environmentally friendly technology

The objectives of this study were: (a) to isolate fungi that often grow in winery wastes submitted to preprocessing before vermicomposting, selecting and identifying those able to survive in DCA media; (b) to evaluate their growth capacity, tolerance, and biodegradation potential for DCA, determining the presence of metabolites of this compound; and (c) to elucidate the mechanisms responsible for DCA degradation.

2. MATERIAL AND METHODS

2.1. Chemicals

The chemical compound 3,4-dichloroaniline (DCA, 99.5% purity) was supplied by Chem Service. The metabolite standards, 3,4-dichloronitrobenzene (DCNB, 95% purity) was from ACROS Organics (Geel, Belgium), 3,4-dichloroacetanilide (DCAN) from MP Biomedicals, LLC (Illkirch, France), 4,7-dichloroquinoline (DCQ, 99% purity) was from Sigma-Aldrich, 3,3´4,4´-tetrachloroazobenzene (TCAZB, 98% purity) and 3,3´,4,4´-tetrachloroazoxybenzene (TCAXB, 99% purity) were supplied by Dr. Ehrenstorfer GmBH (Ausburg, Germany). 2,4,5-trichloroaniline (99.0 % purity) from Fluka (Steinheim, Germany) was used as internal standard. HPLC-grade acetonitrile from Scharlau Chemie, S.A (Barcelona, Spain) was used. Water was purified using the Milli-Q water-purification system (Millipore, MA, US).

2.2 Fungal Isolation and culture media

One gram of a representative organic sample from the pre-processing period of the vermicomposting of vine-shoot (Appendix A) was inoculated into 9 ml of peptone water and homogenized for 1 min by vortexing. Serial dilutions were made (10^{-1}, 10^{-3}, and 10^{-5}). Aliquots of 1 ml from each dilution were inoculated on potato dextrose agar (PDA) medium supplemented with streptomycin (30 mg l^{-1}) and incubated at 28°C for 4 days. Colonies with the presence of mycelia were isolated from PDA medium. For degradation studies, a minimal salts medium (MM) was used, composed of (g l^{-1})

glucose 5.0, NH_4SO_4 7.5, K_2HPO_4 4.8, and $MgSO_4 \cdot 7H_2O$ 0.2, and supplemented with streptomycin (30 mg l^{-1}) and trace elements (mg l^{-1}): $MnSO_4$ 20, $FeSO_4 \cdot 7H_2O$ 1, $CuSO_4 \cdot 7H_2O$ 0.7, and NaCl 0.7. All media were adjusted to pH 7.0 and sterilized by autoclaving at 121°C for 20 min.

2.3 Tolerance of the isolated fungi to DCA

For an assessment of the ability of the strains to grow on MM with DCA, the isolated fungi were inoculated into 50 ml of MM and incubated at 28°C at 110 rpm for 4 days. For the elimination of any trace of C and N, cultures were centrifuged at 4500 rpm for 15 min and washed twice with phosphate buffer at pH 6.5 (KH_2PO_4 6.8 g l^{-1} + K_2HPO_4 8.8 g l^{-1}). Subsequently, 0.5 ml of the suspension was inoculated into solid MM (agar 15 g l^{-1}) that contains DCA at 20 mg l^{-1} as a carbon source (MMCS). DCA was also included in the solid MM as nitrogen source using in this case glucose (5 g l^{-1}) as a carbon source (MMNS). These were maintained at 28ºC under darkness for 5 days. The strains that grew were selected for degradation studies.

2.4 Identification of fungal strains

Chromosomal DNA was extracted from 20 mg of mycelium from each fungus according to Sambrook and Russell [16] with some modifications as an increment of 100 µl in SDS (20%) and 30 µl in proteinase K (20 mg ml^{-1}). Due to PCR inhibition factors, DNA was diluted to 10 ng μl^{-1}. The small subunit (SSU) 18S ribosomal RNA gene was amplified by PCR using 5 ng of DNA as template, 10 µM concentration of each primer un-SSU-0817F (TTAGCATGGAATAATRRAATAGGA) and nu-SSU- 1536R (ATTGGCAATGCYCTATCCCCA) described by Borneman and Hartin [17] and 1 U of Taq DNA polymerase (BioCat GmbH, Germany) in a 25 µl final reaction volume. Amplifications were performed in a Multigene gradient cycler (Labnet International, USA) under the following conditions: 5 min at 95ºC, 30 cycles of 45 sec at 95ºC, 1 min at 58ºC and 1 min at 72ºC, plus an additional 7 min cycle at 72ºC. The amplicon size was 762 bp. The PCR-amplified fragments were cloned in pGEMT-Easy vector (Promega) and sequenced using ABI PRISM 3130x l Genetic Analyzer. Sequences were

compared with those available on the database using the BLAST program at the National Center for Biotechnology Information (NCBI). Molecular sequence data were analysed using MEGA 5.05 package [18]. Those sequences were aligned and phylogenetically analysed with the 18S SSU ribosomal DNA sequences from other DCA-degrading fungi using the MUSCLE program (multiple sequence comparison by log-expectation) and choosing Neighbour Joining as clustering method to produce the distance matrices. The phylogenetic trees were plotted using Likelihood as the statistical method. The alignment was bootstrapped using 100 replications. The model of nucleotide substitution was selected [19]. The consensus tree was constructed following the distance tree.

2.5. Pre-inoculums preparation and degradation experiments

Three fungi (F1, F2, and F3) selected by their tolerance to DCA were inoculated in Erlenmeyer flasks of 500 ml with 100 ml of MM for 4 days to grow abundant mycelia. Fungal biomass was washed twice with phosphate buffer as was described above.

Fungal biomass of F1, F2 and F3 (0.30 g l^{-1} dry weight) individually and three fungi mixture (Fmix; 0.1 g l^{-1} d.w. each) were inoculated in Erlenmeyer flasks of 250 ml containing 50 ml of the MMNS and supplemented with 15 μg ml^{-1} of DCA, respectively. Controls (F1C, F2C, F3C, and FmixC) without DCA and heat-killed inoculum (121ºC by 15 min) were set up to control the abiotic degradation and sorption effect. The treatments were incubated in triplicate at 28ºC on a rotary shaker at 130 rpm in darkness. The fungal growth, pH, and concentrations of DCA were analysed at 0, 3, 5, 7, 15, 29, and 42 days.

2.5.1 Evaluation of growth of fungal strains on DCA

For the determination of the growth of these fungal strains on DCA, the fungal culture from each flask was harvested by using a pre-weighted filter paper (Whatman GP filters) and dried for 24 h at 105ºC. Fungal growth was quantified as gram of dried mycelium per litre of culture medium. Different parameters were measured in the exponential phase [20]: biomass productivity (BP), specific growth rate (μ), biomass

duplication time. All culture media were buffered at pH 7.0, this being similar to the pH measured in the winery wastes in the pre-processing period of the vermicomposting process.

2.6. Chemical analysis.

Liquid media contained in the flasks at different times were centrifuged (8000 rpm) and supernatants were filtered through 0.22 μm PTFE syringe filter (Fisher scientific) to be analysed by solid-phase micro-extraction coupled to a gas chromatograph with a mass-spectrometry detector (SPME-GC/MS), followed the methodology of Risticevic et al. [21]. Two fibres, 85 μm polyacrylate (PA)-coated fibre and a 100μm polydimethylsiloxane (PDMS)-coated fibre from Supelco (Bellefonte PA, USA) were employed for DCA and metabolite analysis (Figs. A.1 and A.2). The fibres were conditioned at 300ºC for 1 h (PA) or 250ºC for 0.5h (PDMS) prior to use. The fibres were immersed directly into 9 ml of each sample for 10 min (PA) or 1 h (PDMS), immediately drawn back into the needle and transferred without delay (less than 5 s) into the injection port of the GC. The incubation and extraction temperature was 35ºC. The speed of the magnetic stirring was 250 rpm. A desorption time of 7 min at 280ºC (PA) or 10 min at 250ºC (PDMS) was used. Blanks were periodically desorbed. The 2,4,5 trichloroaniline at 30 μg l^{-1} was used as an internal standard. Splitless injection was employed. The analyses were performed on a Varian Model 450GC coupled to a 240 MS detector.) Two columns (Varian FactorFourTM 5ms) of different sizes (30m x 0.25mm x 0.25μm film and 20m x 0.15mm x 0.15μm film) from Agilent (Waldbronn, Germany) were used for the metabolite analysis with the PA and PDMS fibres, respectively. Helium was the carrier gas. The GC oven program for the PA fibre was: initial temperature 50ºC held for 7 min, ramped at 20ºC min^{-1} to 250ºC and held for 2 min, for a total run time of 19 min. For the PDMS fibre the oven program was: 50°C for 10 min, ramped at 20°C min^{-1} to 250°C, ramped at 30°C min^{-1} to 300°C and held for 2 min, for a total run time of 23 min. The interface was kept at 280ºC, the ion trap at 240ºC. The mass spectra were determined at electron energy of 70 eV. The analyses were operated in full scan/SIM mode method. The parent compound and metabolites were quantified by using calibration curves ranging from 5 to 100 μg l^{-1} from the

corresponding analytical standards (R2 > 0.994). The most probable metabolite structures were based on their retention times, fragment ions of the standard (Table A.1) and by using NIST library spectra included in the MS Workstation software 6.9.1.

2.7 Kinetic Models

The experimental data of DCA degradation were fitted to two kinetics models [22]: the single first-order model (SFO) and the first-order multi-compartment model (FOMC). The SFO model assumes that the number of pesticide molecules is small relative to the number of degrader microorganisms and their enzymes, or water molecules in the case of hydrolysis. The first-order multi-compartment (FOMC) corresponds to bi-phasic parameters; a quick initial decrease in pesticide concentrations is often followed by a slower decline. DCA and metabolite persistence were calculated by using the DT50 and DT90 values. The chi-squared test (*X2*) was used to evaluate the goodness of fit for each model and the quality of the measured data. A significance level of α=0.05 was used to validate the models. The model with the smallest $\chi 2$ values described the experimental data best. The statistical t-test was used to check the value found for the degradation rate constant (k).

It is important to point out that when metabolites were determined in quantities of less than 10% of the parent compounds, i.e. < 1.5 μg ml^{-1}, they were considered minor metabolites. Furthermore, the model fit gives more weight to parent compound and major metabolites; hence those minor metabolites cannot be optimised together within the model. In these cases, only the experimental declination curve was considered for modelling.

2.8 Statistical analysis

Statistical differences among the biomass productivity for each treatment were performed using Kruskal Wallis non-parametric test. A *post hoc* pairwise comparison was performed by using Tukey's multiple-comparison test at a risk level of α = 0.05. Analysis of variance (ANOVA) was used to determine significant differences in the

specific growth rate (μ) and duplication time (Td), where Tukey's parametric test was used to compare each treatment.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of DCA degrading fungi

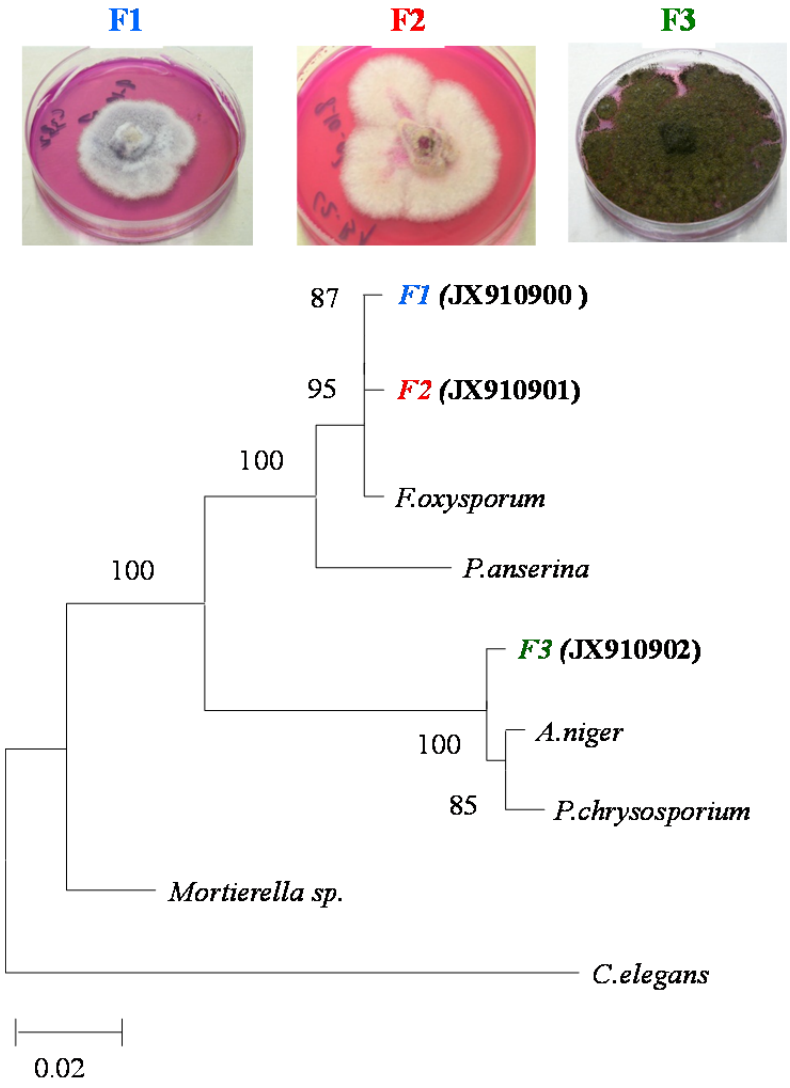

Figure 1. Morphological characteristics of the fungal F1, F2 and F3 isolated at the preconditioning period from the winery waste vermicomposting and phylogenetic tree. The branch support was estimated using 100 bootstrap replicates.

Three fungi (F1, F2, and F3) were selected for their growth on DCA as a nitrogen source. After 7 days from inoculation, F1 and F2 strains showed cottony mycelia white or grey on top and pale pink underneath whereas colonies from F3 had black mycelia (Figure 1). These fungi are part of the aerobic microbial population capable of growth in competition with other microorganisms inhabiting the winery wastes. Under the assayed experimental conditions, the liquid media from F1 and F2 proved blue in colour but the F3 media did not. However, the three fungal strains used glucose as carbon and energy source because the 3,4-DCA was not an easily available carbon source. Emtiazi et al. [23] reported that *Fusarium* sp. isolated from activated sludge was able to utilize aniline as the sole nitrogen, carbon, and energy source with the production of acetanilide and catechol but its chloroaniline derivatives were utilized as a nitrogen source only in the presence of glucose.

The sequences of the small-subunit (SSU) 18Sr ribosomal RNA gene of these fungi were compared with those available in the GENEBANK database by using the Blast program (Figure 1). The sequences of F1 and F2 with 764 bp were 99% identical to that of *Fusarium oxysporum* and were deposited in NCBI database (F1 and F2 Accession Number JX910900 and JX910901, respectively). The sequence from F3 with 767 bp was 99% identical to that of *Aspergillus niger* (Accession No. JX910902). The phylogenetic analysis of these sequences by distance methods showed that F1 and F2 were closely related to *F. oxysporum*, which is also a DCA-degrading fungus [24]. The branches of these strains were related with the *Podospora anserina* strain (Figure 1). On the other hand, F3 was closely associated with *A. niger* which in turn had a short branch distance from *P. chrysosporium*. The relationship among branches of these fungi and the capacity of growth in the presence of DCA indicated that these have a common ancestor, a contention supported by high bootstrap values (100).

3.2 Growth of fungal strains on DCA

In all treatments, there was a lag phase of 3 or 5 days before the exponential phase, which may be associated with adaptation to the culture medium (Figure 2). A significant decrease in mycelia on the 3rd day was measured for F3. The exponential fungal-growth phase reached its maximum value on the 7th day of incubation for F1 and for all the

controls without DCA (F1C, F2C, F3C, and FmixC) while it was reached a week later (15 d) for F2, F3, and Fmix. An increment was detected after 42 days in the Fmix culture, probably due to the slower F3 growth.

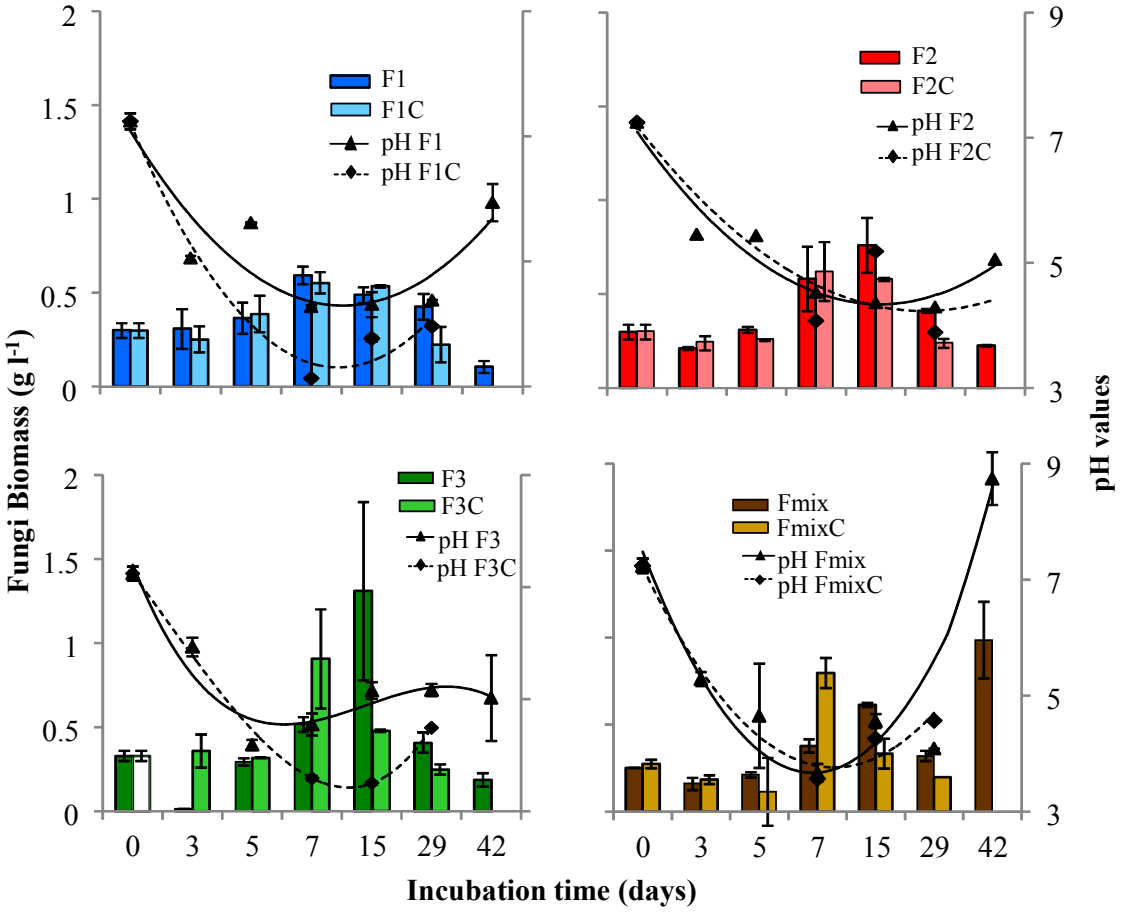

Figure 2. Growth and pH values of the isolated and the consortia fungal incubated in culture media with (F1, F2, F3 and Fmix) and without (F1C, F2C, F3C and FmixC) 3,4-dichloroaniline.

Significant differences in biomass production (Table 1) were found with the Kruskal- Wallis statistical test ($p < 0.05$). Pairwise comparisons between treatments by Tukey's test showed that BP from F1 and F1C was different from F2, F3, F3C, and FmixC.

The specific growth rate (μ) values revealed that when DCA was added to the media, fungal growth decreased significantly only for F3 and Fmix cultures, these

having the longest duplication times. These results suggest that DCA significantly affects fungal growth, especially for F3 and Fmix.

A decrease in pH values during the exponential growth stage gave values between 3.0 and 4.0 (Figure 2). This may be due to the release of organic acids as the result of the glycolysis during fungal growth. In fact, *A. niger* is used in industry to produce citric acid. Except for F3, after the exponential phase the pH rose, reaching the highest value in Fmix culture.

Table 1. Biomass productivity (BP) and kinetic parameters (μ and Td) for the fungal growth with (F1, F2, F3 and the mixture consortia Fmix) and without (F1C, F2C, F3C and FmixC) DCA.

Treatment	BP ($g\ l^{-1}d^{-1}$)	Mean Rank	F1	F1C	μ $days^{-1}$	Td days
F1	0.02 ± 0.01	4.3			0.17±0.11 c,d	3.12±0.64 b,c
F1C	0.01 ± 0.02	4.0	0.30		0.19±0.04 c,d	3.79±0.77 b,c
F2	0.05 ± 0.01	18.3	14.00*	14.33*	0.42±0.24 b,c	2.38±1.92 b,c
F2C	0.03 ± 0.01	9.7	5.33	5.67	0.44±0.13 b,c	1.68± 0.50 b,c
F3	0.07 ± 0.05	18.0	13.67*	14.00*	0.10±0.05 d	8.28±4.26 a
F3C	0.04 ± 0.02	15.7	11.33*	11.67*	0.50±0.17 b	1.48±0.49 c
Fmix	0.03 ± 0.00	12.0	7.67	8.00	0.13±0.11 d	6.13±2.33 a,b
FmixC	0.05 ± 0.01	18.0	13.67*	14.00*	0.96±0.06 a	0.73±0.08 c

* significant differences in BP from Kruskal-Wallis statistic analyse lower case letters are significant differences from Tukey test.

3.3 Biodegradation of DCA and analysis of metabolites

Despite the lag phase detected in fungal growth (Figure 2), the biodegradation of DCA occurred mainly in the first 7-15 days of incubation, either separately or in the

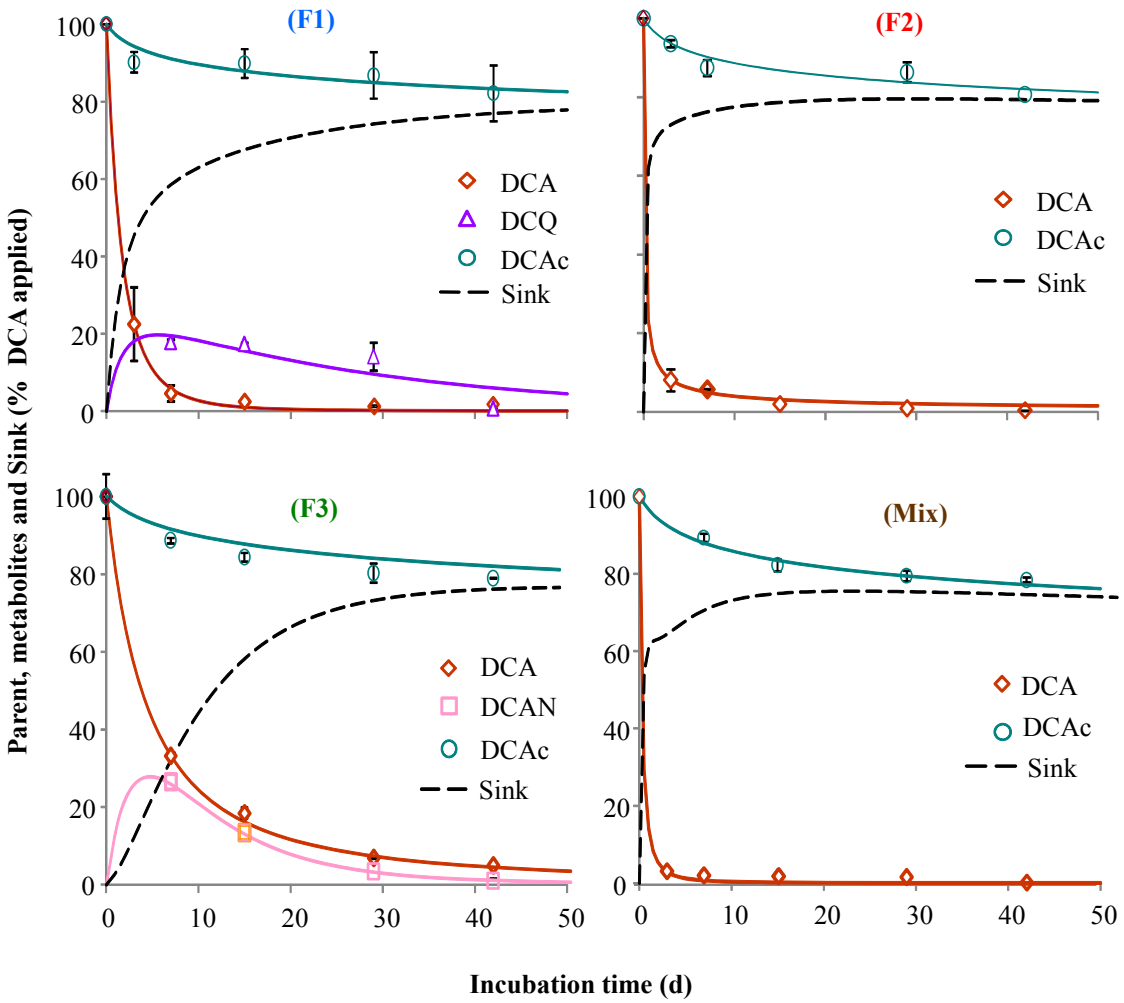

Figure 3. Biodegradation of DCA and of the metabolites DCQs and DCAN by *F. oxysporum* strains (F1 and F2), *A. niger* (F3) and the fungal consortia (Fmix). Controls of heat-killed mycelium were made for each treatment. Error bar are standard deviations in the triplicate samples.

consortium (Figure 3). The amount of DCA degraded after 7 days was higher than 90%, except for F3, with 70% degradation, However, the mass balance (quantification of DCA adsorption, metabolites, and remaining DCA concentration) indicated a progressive increment of unidentified degradation products known as Sink (Figures 3 and 4) . This rapid degradation of DCA by *Fusarium* species, and to a lesser extent by *A. Niger*, must be related to different strategies that *F. oxysporum* has for survival under limited conditions such as denitrification, heterolactic acid fermentation or ammonia

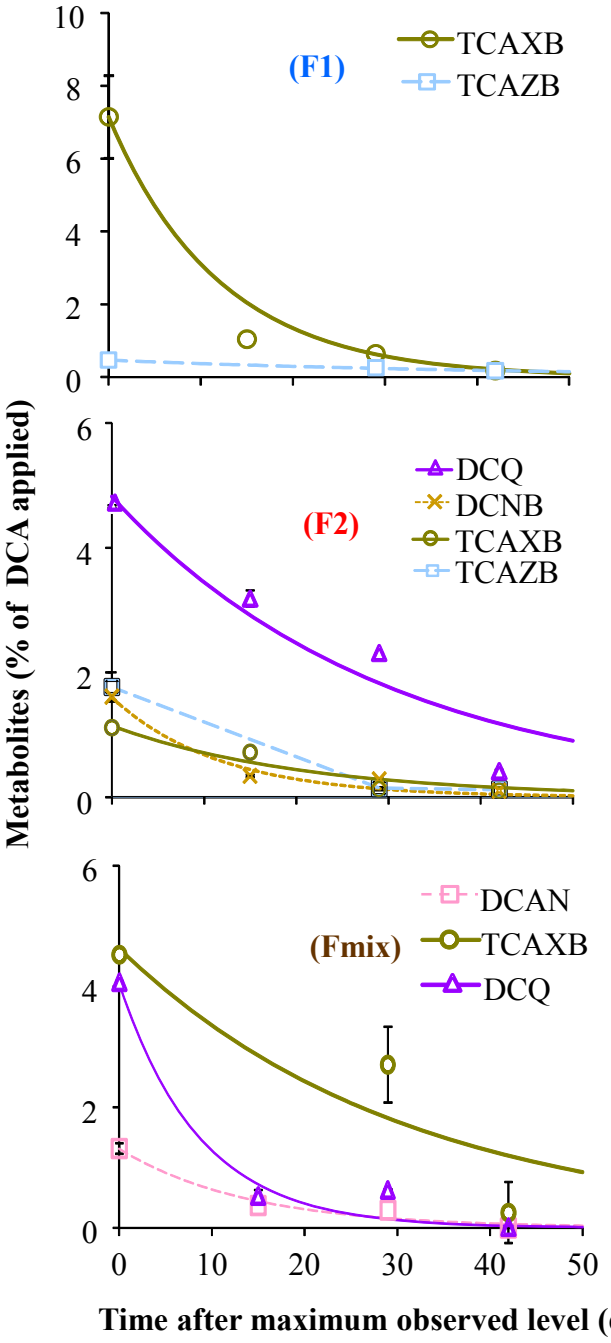

Figure 4. Decline of the metabolites DCAN, DCNB, DCAXB, DCAZB and DCQs from the maximum observed during the biodegradation of DCA by the fungi strains. Error bar are standard deviations in the triplicate samples.

fermentation [25], implying a high metabolic potential against a variety of xenobiotics [12, 26]. Despite the larger amount of DCA remaining in F3, the biomass productivity in this medium was higher than in the media inoculated with F1 and F2 (Table 1). This

may be due either to the transformation of DCA into less toxic compounds by F3 or else to its higher tolerance to DCA and biodegradation products unlike F1 and F2 (Figures 2 and 3).

Both the SFO and FOMC models were well adjusted to the experimental degradation curves from F1, F2, and F3 media, but only the SFO model was fitted to the experimental curve from Fmix. The goodness of fit for each model showed that FOMC was the best model to explain the DCA degradation by F1, F2, and F3 treatments (Table 2, Figure 3). The β values indicate a significantly lower degradation rate for DCA in the F3 culture with respect to the others. The DT90 values, which are more appropriate to estimate the compound persistence in biphasic models [22], indicate that DCA in the F3 culture was 3- and 8-fold more persistent with respect to F1 and F2. These differences in the degradation kinetics agree with the fungal-growth parameters measured (Table 1).

Table 2. Kinetic parameters for the biodegradtion of DCA by the fungal F1, F2, F3 and the fungal consortia (Fmix). Mean of duplicate samples ± standard deviation.

	F1	F2	F3	Fmix
Pseudo First Order Kinetic Model				
Ci (%)	99.94±2.95	99.98±1.97	98.32	100±1.15
k (d^{-1})	0.48±0.08	0.74±0.17	0.13±0.01	0.80±1.24
DT_{50} (d)	1.41	0.93	5.07	0.85
DT_{90} (d)	4.70	3.09	16.85	2.85
χ^2	5.2	7.1	14.3	3.3
t-test	<0.001	<0.05	<0.001	<0.05
First Order Multicompartment Kinetic Model				
Ci (%)	99.98 ±2.64	100 ±0.66	99.78±2.84	-
α	3.25±0.31	0.53±0.11	1.61±0.63	-
β	6.13 ± 0.72	0.05±0.04	7.22±4.41	-
DT_{50} (d)	1.45	0.13	3.81	-
DT_{90} (d)	6.30	3.57	22.82	-
χ^2	5.4	3.1	8.3	-

The degradation of DCA by the fungi generated various metabolites (Figure 4) which were not detected in the controls, pointing to biodegradation as the main degradation pathway. In the cultures with *F. oxysporum* (F1 and F2) at 7 days of incubation the metabolites TCAXB, TCAZ, DCQs, and DCAN were detected (Figure 4) and quantified. The DCQ isomers registered the highest amounts (17.7 % and 4.6 % of applied DCA, respectively) followed by TCAXB and TCAZB. The metabolite DCNB was quantified at day 15 in F1 and at day 7 in F2 (0.015 and 0.24 μg ml^{-1}, respectively). After 29 days of incubation, a new peak was determined at 0.004 and 0.011 μg ml^{-1} in F1 and F2, respectively (< 10% of applied DCA). This peak matched the DCAN structure. All the metabolites TCAXB, TCAZB, DCNB, and DCAN remained below the 10% of total applied DCA. Other peaks detected matched the 3,4-dichlorobenzene (DCB) structure (Figure 4). In the *A. niger* culture at the day 3 of incubation, only DCAN was detected at concentration higher than 3.93 μg ml^{-1} (25.8% of applied DCA).

In the Fmix culture, the first metabolite determined was DCAN, but in lower amounts with respect to F3 (0.2 μg ml^{-1} and 0.045 μg ml^{-1} for day 3 and 29, respectively). On day 7, 0.62 μg ml^{-1} of DCQ isomers was determined. TCAXB appeared in a small amount (0.16 μg m l^{-1}) and the TCAZB peak appeared but was below the detection limit.

Experimental declination curves of the metabolites showed a proper fit to the SFO model with a significant X^2 (Table 3). The declination constants (kd) in F1, followed the sequence: TCAXB > DCQ > TCAZB. By contrast, the sequence in F2 was: DCNB > TCAZB ≈ DCQ >TCAXB. The highest amount of the metabolite DCAN was determined in the F3 cultures but its persistence was also lowest (kd = 0.17 d^{-1}, DT50 = 4 days). In the Fmix the metabolite persistence was: DCQ > DCAN > TCAXB.

In general, the DT50 values indicate a high potential to degrade theses metabolites in the early stages, but the DT90 values reveal a slower degradation with time, perhaps due to the limited nutrients under these experimental conditions. Nevertheless, the fact that the persistence measured was relative should not be disregarded, since it depended on the amount of metabolite formed and the time when it occurred for each treatment. Detailed information of chromatograms (Table A.1) and mass spectra of the metabolites determined in the biodegradation of 3,4-DCA (Fig. A1 and A.2) are shown in Appendix A.

Table 3. Kinetic parameters for the biodegradation of DCA metabolites by fungal F1, F2, F3 and the fungal consortium (Fmix). Mean of duplicate samples ± standard deviation.

Metabolites	F1	F2	F3	Fmix
kd (d^{-1})				
3,4-DCNB	-	0.08±0.03	-	
3,3´,4,4´-TCAXB	0.08±0.013	0.04±0.02	-	0.03±0.012
3,3´4,4´-TCAZB	0.02±0.030	0.06±0.04	-	-
3,4-DCAN	-	-	0.17±0.045	0.07±0.028
DCQ	0.04±0.013	0.05±0.01		0.11±0.068
ffM* (%)				
3,4-DCNB	-	0.06±0.01		-
3,3´,4,4´-TCAXB	0.14±0.01	0.02±0.00		0.12±0.08
3,3´4,4´-TCAZB	0.005±0.02	0.04±0.03	-	-
3,4-DCAN	-	-	0.79±0.15	0.03±0.06
DCQ	0.28±0.06	0.07±0.01		0.19±0.03
DT_{50}				
3,4-DCNB	-	8.11	-	-
3,3,4,4´-TCAXB	8.32	14.54	-	21.47
3,3´4,4´-TCAZB	30.90	11.04	-	-
3,4-DCAN	-	-	4.0	9.62
DCQ	19.19	14.71		6.02
DT_{90}				
3,4-DCNB	-	26.96	-	-
3,3´,4,4´-TCAXB	27.64	48.30	-	71.32
3,3´4,4´-TCAZB	102.67	36.67	-	-
3,4-DCAN	-	-	13.4	31.96
DCQ	63.74	26.96		20.01
χ^2				
3,4-DCNB	-	8.7	-	-
3,3´,4,4´-TCAXB	0.4	7.9	-	0.46
3,3´4,4´-TCAZB	2.0		-	-
3,4-DCAN	-	-	4.9	10.10
DCQ	13.0			12.41
t-test				
3,4-DCNB	-	<0.05	-	-
3,3´,4,4´-TCAXB	<0.05	<0.05	-	<0.05
3,3´4,4´-TCAZB	<0.05	<0.1	-	-
3,4-DCAN	-	-	<0.001	<0.001
DCQ	<0.05	<0.05		<0.05

*FFM= Formation fraction of metabolite

3.4 Degradation pathway of DCA by fungi

The fungi clearly varied in the rate of degrading DCA. It was also clear that DCA affected the fungal growth. Furthermore, differences were found in the formation and decay of these metabolites. For all these reasons, various strategies in the metabolism of DCA were considered to propose the pathway illustrated in Figure 6. Thus, the presence of DCNB, DCB, TCAZB, TCAXB, and DCQs in *F. oxysporum* cultures and only of DCAN in the *A. niger* culture indicated different catabolic reactions. The metabolite DCNB determined came from the sequential oxidation of the aniline's amine group to nitro group. Peroxidases such as chloroperoxidases are fungal enzymes having a structure similar to cytochrome P450 and are able to convert the arylamine functional group to the nitrose oxidation state [27] by the introduction of an oxygen atom from hydrogen peroxide to the substrate, acting as monooxygenases catalysing the heteroatom oxidation (nitrogen in this case). Within this oxidation path, other intermediates may be formed, such as *N*-(3,4-dichlorophenyl)-hydroxylamine (DCPHA) and dichloronitrosobenzene (DCNOB) [28] (Figure 5). These intermediates were not detected, however, the detection of DCB (Table A1, Fig. A1) indicates a reduction by denitrification. Shoun et al. [29] reported that *F. oxyspurum* and *F. solani* have a peculiar denitrifying system where NO is reduced to N_2O by nitric oxide reductase cytochrome P450nor. This process is called co-denitrification, in which a hybrid N_2 or N_2O species is formed upon combination of nitrogen atoms from NO and other nitrogen compounds (nitrogen donor). According to Oshima et al. [30], the denitrifying system in *F. oxysporum* MT811 can produce N_2 as the co-denitrification product, but it cannot form N_2 by denitrification. Cytochrome P450nor involves a multifunctional detoxifying enzyme. Thus, the nitrite group from DCNB would be reduced to NO generating the metabolite DCNOB. Two DCNOB molecules by the codenitrification reaction by P450nor released N_2O and the DCB metabolite (Figure 5).

Azo and *azoxy* dimers (TCAZB and TCAXB) were present probably because DCA and hydroxylamine (DCPHA) can react chemically with the C-nitroso compound (DCNOB) [28]. These *azo* compounds have been found in *Filaboletus* sp., *P. chrysosporium* and *Geotrichum candidum* cultures growing in presence of DCA, where lignin peroxidase and aniline oxidase have been involved [31]. These *azo* dimers can

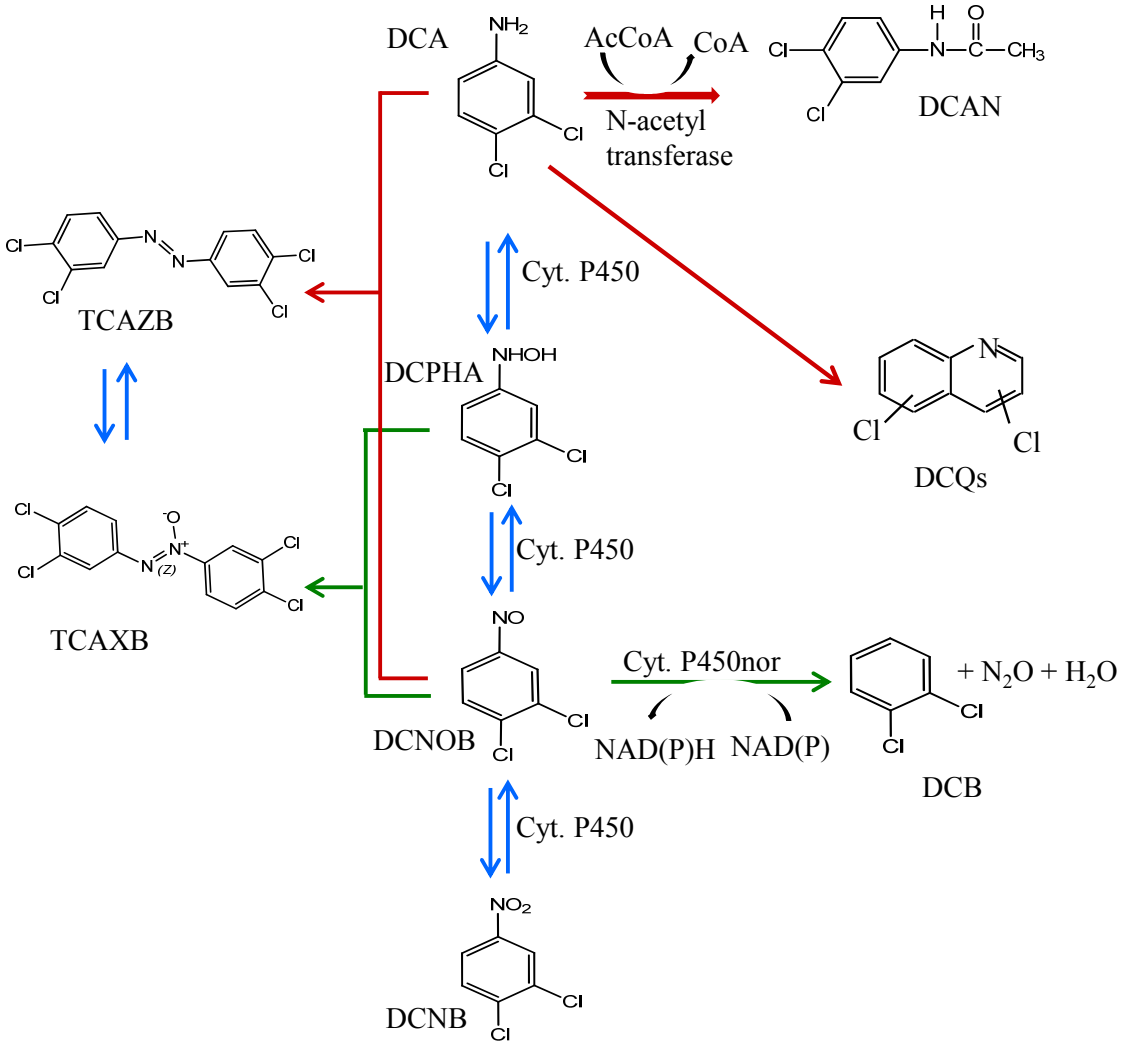

Figure 5. Biodegradation pathways for DCA by *F. oxysporum* strains (F1 and F2) and *A. niger* (F3)

undergo a metabolic activation where an electrophilic nitrenium ion can be produced and subsequently react with DNA and RNA, becoming a mutagenic compound [32]. The strategy of *F. oxysporum* strains may likely be the formation of harmless polymeric compounds. Polyphenoloxidase enzymes such as laccase catalyse the coupling of phenolic compounds by fungi under limited nitrogen conditions. Laccase can catalyse the four electron reduction of molecular oxygen to water, coupled with the concomitant oxidation of organic substrate, producing four radicals, which can react to give dimers, oligomers, and polymers [28]. Within these oligomers, the formation of DCQs can be a detoxification mechanism. Aromatic amines (AA) with ortho-position free as DCA can

be a substrate in different chemical reactions in the synthesis of quinolines. Furthermore, it is known that fungi can produce quinolines [33, 34].

In the case of the *A. niger* media the presence of DCAN as a major metabolite supports the idea that the N-acetylation reaction must be the main biodegradation path. This reaction leads to the detoxification of AA and is also involved in mechanisms of tolerance to xenobiotics [35] through the production of chemically stable arylacetamides. The N-acetyltransferase (NAT) catalyses the transfer of acetyl groups from the acetyl-Coenzyme A (AcCoA) to arylamines, where AcCoA acts as an acetyl donor, while the primary amine of DCA is the acceptor. Sandermann et al. [3] reported the biodegradation of DCA by *P. chrysosporium*, mediated by a coenzyme A transferase. Metobromuron was also acetylated to p-bromoacetanilide by *F. oxysporum* and *Talaromyces wortmanii* [36]. Martins et al. [35] identified and characterized a NAT2 enzyme that is required for the growth and survival of the filamentous ascomycete *P. anserina* in presence of toxic AA. The catalytic efficiency of *A. niger* to transform DCA may be related to the presence of 5 putative genes encoding the NAT enzymes [35]. This enzyme provides an alternative strategy to confer herbicide tolerance to microorganisms and a detoxification pathway, since the acetylated metabolite is less toxic for *Vibrio fischeri* than DCA [37]. This fact may be related to the peak biomass observed for *A. niger* after 15 d (Fig. 2), where the amount of DCA was even more than 2-fold higher with respect to *F. oxysporum* strains (Fig. 3) and support the contention that *A. niger* despite its slower development (Fig.1) was more tolerant to DCA.

4. CONCLUSIONS

The literature reveals that DCA is a persistent toxic metabolite from the phenylureas biodegradation by bacteria. An alternative to degradation of DCA is the use of fungi. *F oxysporum* and *A niger*, isolated during preconditioning period in the vineshoot vermicomposting, that had the ability to degrade DCA, alone or in consortium, across multiple degradation pathways. The presence of different metabolites such as dichlorobenzene, dichloroquinoline, dichloroacetanilide among others, indicated a high biodegradation potential of these fungi and a stronger tolerance to the DCA, contributing to the possible mineralization under environmental conditions. These results demonstrate

the biocatalytic potential of the microbiota involved in the vermicomposting of agroindustrial wastes with respect to the biodegradation of persistent metabolites such as the DCA. Thus, vermicomposting process can also be considered to be a microorganism source with a high catabolic potential that offers a new low-cost alternative in applying environmental biotechnologies in the DCA degradation. Similarly, this microbial potential can be applied under controlled conditions as a bioremediation system, although this requires further investigation.

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APPENDIX A. SUPPLEMENTARY DATA

This appendix gives information of the pre-processing period of winery wastes where fungi were isolated as well as data from analyse, chromatograms and mass spectra of the metabolites determined in the biodegradation of DCA (Table A1 and Figures A.1 and A.2).

Contents of supplementary data:

- Detailed information of the Preprocessing of the vermicomposting of vine-shoot where fungi were isolated
- Table A.1. Retention time, molecular weight and the electron impact mass of the metabolites determined in the biodegradation of 3,4-DCA by fungi sp.
- Fig. A.1. Presents the chromatograms obtained by using SPME with the polyacrylate (PA)-coated fibre as well as the mass spectra information of the metabolites determined in the biodegradation of 3,4-dichloroaniline (DCA) by the fungi (DCAN, DCB and DCNB).
- Figure A.2. Presents the chromatograms obtained by using SPME with the polydimethylsiloxane (PDMS)-coated fibre as well as and mass spectra information of the metabolites (DCAN, DCB determined in the biodegradation of 3,4-dichloroaniline (DCA) by the fungi.

Preprocessing of the vine-shoot vermicomposting

Often lignocellulosic wastes has high C/N ratio and in general, need to be the mixing with others organic substrates of low C/N ratio, and, optionally, a pre-composting or macerating period to make them acceptable to earthworms .

Vine shoots collected from a vineyard had 4% moisture and a C:N ratio of 82. This waste was air-dried and milled to 2-5 cm fragments and then was mixed at 4:1 (dw:dw) with biosolid vinasse (85% moisture, C:N = 6) collected from a vinasse wastewater-treatment plant. Of this mixture, 46 kg (dw) were placed in a rectangular wooden box and moistened to 60%. After 5 days, the temperature of the mixture increased to 50°C, and subsequently, after 10 days, the temperature returned to mesophilic conditions

(<25°C). At that time, the waste mixture was completely covered by fungal mycelia and it had a C:N ratio of 17, optimal value to be inoculated with earthworms. Four organic samples were collected at this preprocessed period of the winery vermicomposting. These samples were mixed thoroughly in order to have a representative organic sample for the isolation of the fungi.

Table A. 1. Retention time, molecular weight and the electron impact mass of the metabolites determined in the biodegradation of 3,4-DCA by fungi sp.

Metabolites	Rt (min)	Mw	m/z of fragment ions (relative intensity)
(1) 3,4-dichloroacetanilide (DCAN)	16.50	203	161(100), 163(63), 43 (55), 203 (24), 205 (15), 63(11), 165 (10), 162 (89), 90 (80), 62(77).
(2) 1,2-dichloronitrobenzene (DCNB)	14.05	191	109 (100), 191(92) 145(77), 161 (62), 133 (62), 193 (59), 74(59), 147(49), 75(46), 30(44)
(3) 3,3'4,4'-tetrachloroazoxibenzene (TCAXB)	22.29	336	145(100), 124(63), 109(55), 147(51), 172(42), 336(32), 236(30), 74(26), 75(24), 334(24), 336(32)
(4) 3,3'4,4'-tetrachloroazobenzene (TCAZB)	21.55	318	145(100), 124(63), 109(55), 147(51), 172(42), 336(32), 236(30), 74(26), 75(24), 334(24),
(5) 1,2-dichlorobenzene (DCB)	10.80	146	146(100), 148(65), 111(53), 75(41), 74(22), 113(16), 150(10),73(9), 50(7), 147(5.8)
(6) Dichloroquinolines isomers (DCQs)	17.57	198	170(100), 199(63), 162(52), 127(22), 164(17), 99(16), 198(14), 74(13), 126(12), 200(10)

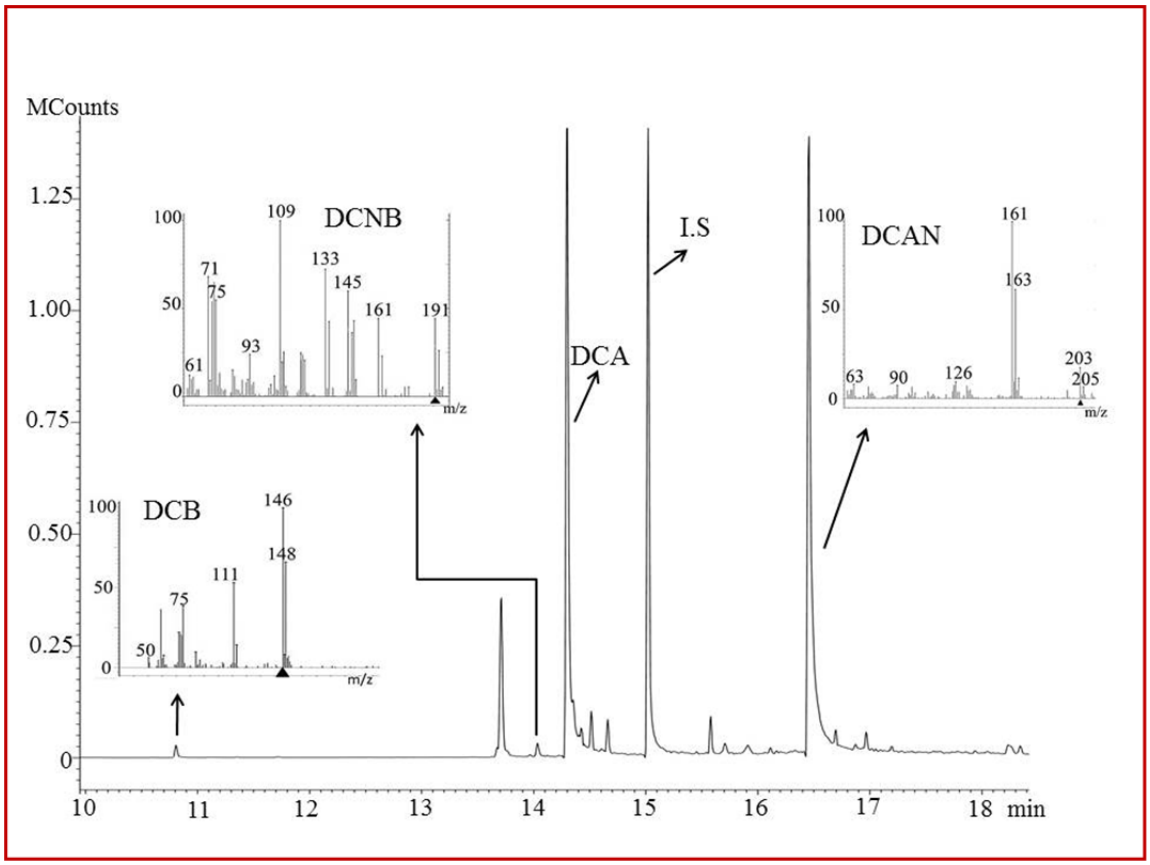


Figure A.1. Chromatograms obtained by using SPME with the polyacrylate (PA)-coated fibre as well as the mass spectra information of the metabolites determined in the biodegradation of 3,4-dichloroaniline (DCA) by the fungi (DCNB, DCB and DCAN).

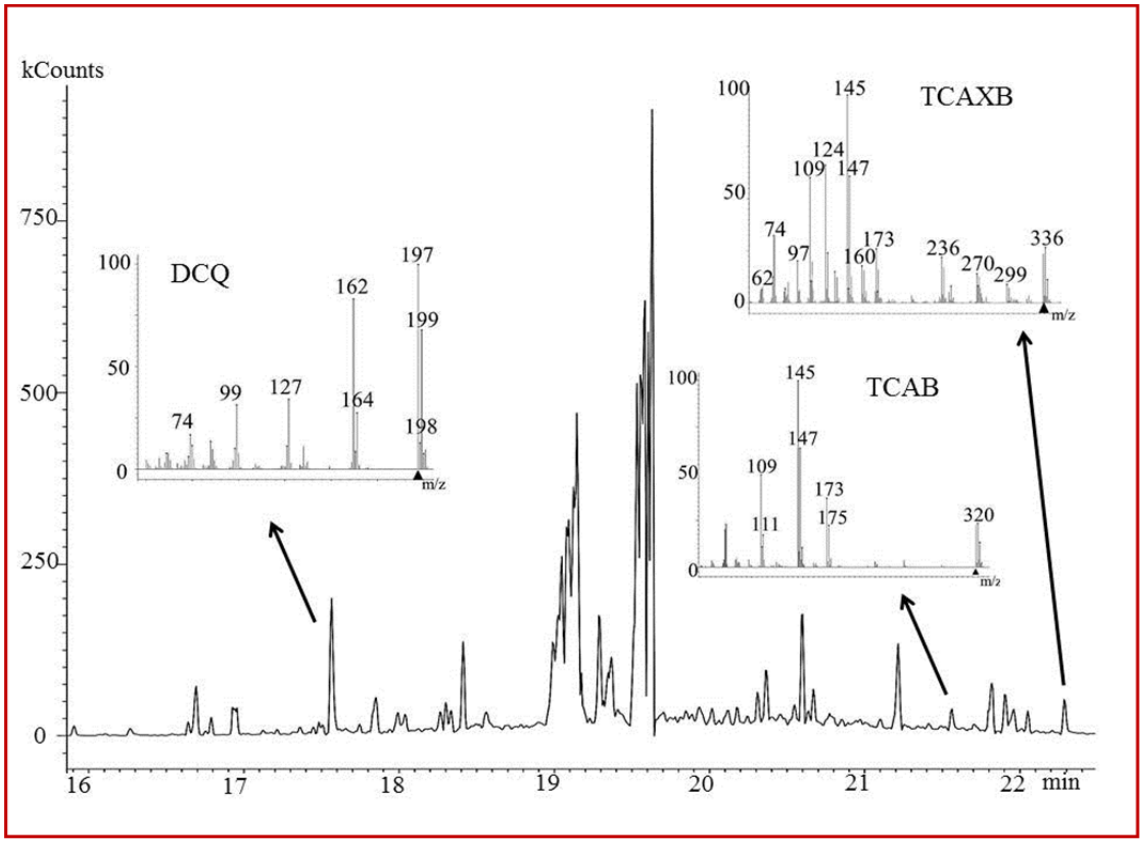


Figure A.2. Chromatograms obtained by using SPME with the polydimethylsiloxane (PDMS)-coated fibre as well as and mass spectra information of the metabolites determined in the biodegradation of 3,4-dichloroaniline (DCA) by the fungi (DCQ, TCAXB and (TCAB).

CAPÍTULO 3

Evaluación multidisciplinar de la reducción de los plaguicidas en suelo enmendados con vermicompost de residuos agroindustriales

Multidisciplinary assessment of pesticide mitigation in soil amended with vermicomposted agroindustrial wastes

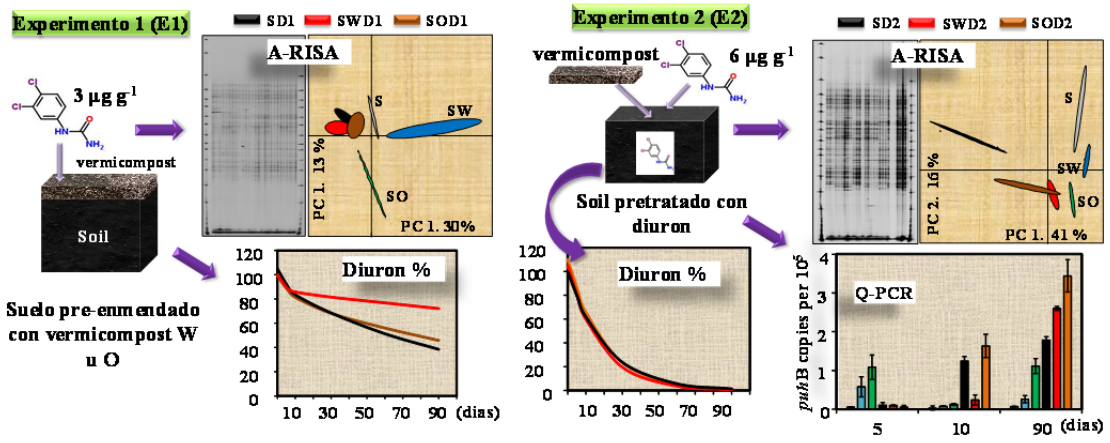
El capítulo 3 ha sido aceptado en la revista Journal of Hazardous Materials

Castillo-Diaz, J.M., Beguet, J., Martin-Laurent, F., Romero, E. 2015. Multidisciplinary assessment of pesticide mitigation in soil amended with vermicomposted agroindustrial wastes. Journal of Hazardous Materials. Aceptado. HAZMAT17201. DOI: 10.1016/j.jhazmat.2015.10.056

Resumen

La enmienda orgánica del suelo afecta a los procesos bióticos y abióticos que controlan el comportamiento de los plaguicidas, pero el historial de tratamiento a que es sometido el suelo también es apreciable. En el presente estudio multidisciplinar, ambos procesos fueron evaluados con el objetivo de minimizar el impacto de los plaguicidas en el suelo. Para ello microcosmos de suelo pretratado (E2) o no (E1) con Diuron fueron enmendados con vermicompost de residuos vitivinícolas (W) o de residuos de olivar (O). La disipación de Diuron siguió un modelo doble de primer orden en el microcosmos E1, mientras que en el E2 siguió un modelo simple de primer orden. Además, la persistencia de Diuron en el suelo fue mayor en E1 que en E2 (E1-DT_{50}> 200d^{-1}, E2-$DT5_0$ <16d^{-1}). La estructura genética de la comunidad bacteriana del suelo fue modificada tanto por la aplicación de Diuron como por la de la enmienda. vermicompost de alperujo (O) aumentó las actividades enzimáticas del suelo, pero el potencial genético degradador de Diuron (*puh*B) fue cuantificado únicamente en el microscomo E2, lo cual se relacionó con la reducción de la persistencia de ese herbicida. En base a ello, se concluyó que la enmienda con vermicompost de alperujo (O) favoreció la proliferación de microorganismos degradadores de Diuron, aumentando, por tal motivo, la capacidad del suelo para eliminar ese herbicida.

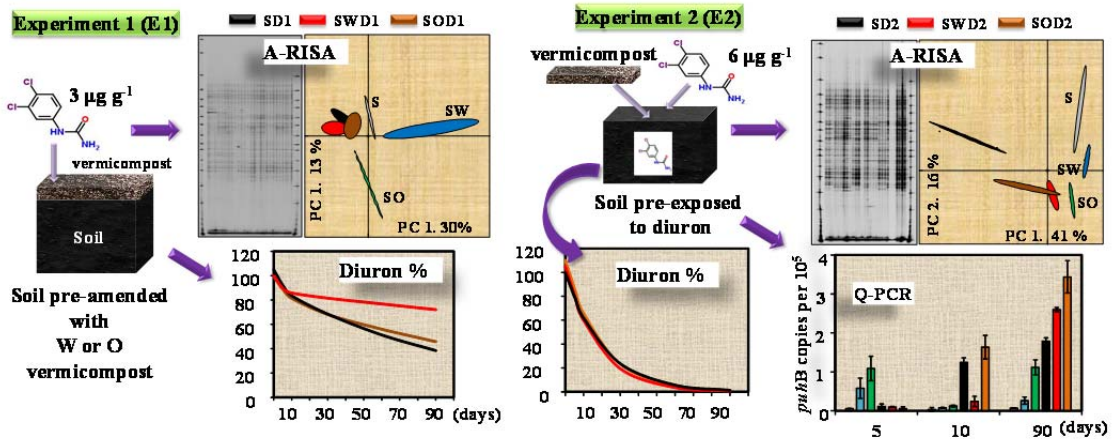
Palabras claves: Diuron, Vermicompost, Biodegradacion, ARISA, genes de hidrolasa fenilurea.



Abstract

Soil organic amendment affects biotic and abiotic processes that control the fate of pesticides, but the treatment history of the soil is also relevant. These processes were assessed in a multidisciplinary study with the aim of optimizing pesticide mitigation in soils. Soil microcosms pre-treated (E2) or not with diuron (E1) were amended with either winery (W) or olive waste (O) vermicomposts. Herbicide dissipation followed a double first-order model in E1 microcosms, but a single first-order model in E2. Also, diuron persistence was longer in E1 than in E2 (E1-DT_{50}>200d^{-1}, E2-DT_{50}<16d^{-1}). The genetic structure of the bacterial community was modified by both diuron exposure and amendment. O-vermicompost increased enzymatic activities in both experiments, but diuron-degrading genetic potential (*puh*B) was quantified only in E2 microcosms in accordance with reduced diuron persistence. Therefore, O-vermicompost addition favoured the proliferation of diuron degraders, increasing the soil diuron-depuration capability.

Keywords: Diuron, Vermicompost, Biodegradation, ARISA fingerprinting, Phenylurea hydrolase genes.



1. INTRODUCTION

To ensure the yield of crops, modern agriculture worldwide relies on the extensive use of pesticides such as the phenylurea herbicides [1]. Among these, diuron is a systemic herbicide widely applied in soils to control weeds in different agricultural crops [2]. In addition to be considered relatively persistent in soil [3], diuron has been detected in surface waters [4] and groundwater [5-7]. Consequently, diuron had been included in the list of priority pollutants by the EU Water Framework Directive (Directive 2000/60/EC).

As there is no alternative immediately available to remove pesticides entirely from agriculture, there is an urgent need to develop new techniques to ensure their environmentally safe use. Among innovative techniques, mitigation solutions to reduce the persistence and the risk of transfer of pesticides are of prime interest. The most widely known include the use of plant buffer strips [8, 9] and organic amendments [10, 11] such as the application of agro-industrial wastes, which could be previously stabilized by vermicomposting [12, 13] and composting [14], increasing the soil organic matter (SOM), microbial activity, and mineralization of recalcitrant material as well as the removal of xenobiotic residues by promoting biotic (biodegradation) and abiotic (adsorption) processes [15, 16]. This application promotes soil-purification capabilities and constitutes a low-cost, eco-friendly solution, offering an alternative to mitigate xenobiotic compounds such as pesticides. Nevertheless, developing and assessing the efficiency of the mitigation solutions remains a challenge. For this objective, biochemical approaches could be applied to estimate the activity of some oxidoreductases and hydrolytic enzymes (e.g. dehydrogenase and urease enzymes, respectively) in soil and used as proxies of the ability of its native microbiota to degrade several organic substrates and xenobiotics and as enzymatic indicators of soil microbiological activity as well [12, 17]. Similarly, molecular approaches can also be applied to estimate the abundance of specific pesticide-degrading gene sequences, providing insight into the pesticide-degrading potential of the soil microbial community [18]. Recently, Pesce et al. [19] have developed a qPCR assay targeting *puhA* and *puhB* genes coding for the enzyme phenylurea hydrolase (PUH) that has been found in *Arthrobacter globiformis* D47 [20] and *Mycobacterium brisbanense* [21]. This allows

the quantification of these genes in river sediments, demonstrating the value of this technique for monitoring genetic potential of diuron-degrading capacity. However, as underlined by Bers [22], these types of studies relating pesticide-degrading gene potential to pesticide-degrading activity remain scarce in the soil environment, and no studies available have evaluated the impact of mitigation techniques on the evolution of PUH-degrading genetic potential.

To fill this gap of knowledge, the present work seeks to evaluate the effect of mitigation approaches that use vermicomposts on PUH-degrading gene potential and degrading activity. Thus, microcosm experiments were established to evaluate the effect of two vermicomposts prepared from two-phase olive-oil waste (wet olive cake or *alperujo*) (O) and wine waste (vine shoots) (W) on diuron degradation. The fate of diuron (sorption, biodegradation, and metabolites formation) was monitored and interpreted in light of biochemical (dehydrogenase and urease activities) and molecular (genetic structure of the bacterial community and abundance of *puh*A and *puh*B genes) parameters, describing the evolution of the soil microbial community, including diuron-degrading populations.

2. MATERIALS AND METHODS

2.1. Soil amendments and chemicals

The soil was collected in an agricultural area under crop rotation in south-eastern Spain (37º 22´19.41´´N, 3º 36´5.54´´W). Soil samples from the uppermost 20 cm were air dried and sieved through a <2 mm mesh. The soil was a calcareous (43% $CaCO_3$) silty-clay loam (11% sand, 49% silt, and 40% clay). Soils were amended either with O or W-vermicompost obtained as described by Elvira et al. [23]. Both vermicomposts were ground and sieved through a < 2 mm mesh. Physico-chemical properties of both matrices were determined following established methodologies [12, 24] (Table 1).

Diuron (D) and its N-demethylated products, 3-(3,4-dichlorophenyl)-1-methylurea (DPMU) 99% purity, 3,4-dichlorophenylurea (DPU) 97.5 % purity and 3,4-dichloroanyline (DCA) with 99.5% purity, were supplied by Dr. Ehrenstorfer (Augsburg, Germany).

2.2. Sorption measurements

Sorption-desorption isotherms of diuron and its metabolites were determined following the classical batch-sorption method [24]. For sorption measurement, 5 g of dry soil sample, in triplicate, were placed in 50-ml glass tubes, and 20 ml of an aqueous standard solution containing diuron at five concentrations ranging from 1 to 5 mg L^{-1} were added. Desorption was measured using an herbicide concentration of 5 mg L^{-1} in 0.01 M $CaCl_2$ solution. The adsorption and desorption rate constants (K_{ads} and K_{des}) and apparent hysteresis index (H) were established fitting the data to a linear Freundlich model [24].

2.3. Experimental setup

Two different experiments were conducted to test the vermicompost effect on diuron degradation in soil. The first experiment, E1, involved the study of diuron dissipation in soil samples submitted to organic amendments with two types of vermicompost. Soil samples were amended twice with 5% either with O-vermicompost (SO) or W-vermicompost (SW) and aged after each vermicompost addition by a 3-month incubation at 20ºC in darkness and at 80% field capacity (i.e. in total 10% amendment). Thereafter, 40 g (dw) of soil (S), SO and SW soils were placed in triplicate in a 100 ml glass flask and spiked with diuron at 3 μg g^{-1} (equivalent to 7.8 kg ha^{-1} at a depth of 20 cm). Diuron treatments (SD1, SWD1, and SOD1), were left to evaporate overnight, homogenized, and finally incubated for 3 months under the conditions described above.

The second experiment, E2, was designed to evaluate the effect of the vermicompost amendment in a soil previously treated with diuron. Thus, the soil samples were firstly fortified with 3μg g^{-1} of diuron and aged by incubation for 3 months under the same condition as E1. Afterwards, soil fractions of 40 g (d.w.) in triplicate were amended with 10% of either W or O vermicompost and spiked with diuron to reach a final concentration of 6 μg g^{-1}± 0.42. The treatments of E2 (SD2, SWD2, and SOD2) were incubated as described for E1. Soil samples from E1 and E2 were analysed at

different incubation times (0, 7, 10, 30, 60, and 90 days) to monitor the dissipation of diuron.

2.4. Chemical Analysis

An Agilent series 1100 high-performance liquid chromatography (HPLC) (Agilent, Germany) equipped with a diode array detector and a Zorbax Rx-C8 2.1x150 mm analytical column packed with diisopropyl n-octyl (5 μm) and an Eclipse XDB-C8 guard cartridge (2.1 × 12.5 mm i.d.) filled with the same material were used. Operating conditions are described by Romero et al. [17]. Recovery values for diuron and its metabolites were close to 100%, except for DCA, which gave recoveries of 81% ±1.2 for S, 56% ±7.1 for SW and 54% ±0.9 for SO. The low DCA recoveries agree with the results of Polati et al. [25], suggesting that it is strongly retained in the soils fractions.

2.5. Enzyme activity analyses

Soil samples of 1 g of wet soil in triplicate were used to determine dehydrogenase (DHS) and urease activity. DHS was determined as reported by García et al. [26]. The iodonitrotetrazolium formazan (INTF) produced was measured in a spectrophotometer at 490 nm (Shimadzu UV Mod. 684). Urease activity was determined using urea as a substrate as described by Kandeler and Gerber [27] and samples were read at 690 nm. Samples without soil were used as control to DHS activity and samples without urea were run simultaneously as control to urease activity.

2.6. DNA Extraction and quantification of 16S rRNA, *puhA* and *puhB* gene sequences in soil DNA samples

Nucleic acids were extracted in triplicate from 250 mg of soil sampled at different incubation times. The procedure deriving from the initial protocol [28] is described in the ISO standard 11063 [29]. DNA extracts were purified by using polyvinyl-polypyrrolydone columns (PVPP, Sigma-Aldrich, USA). Bacterial abundance (BA) was estimated by qPCR according to ISO/DIS 17601, and qPCR assays were performed with

a StepOnePlus qPCR machine (Applied Biosystems, USA) using the universal bacterial primers 341f and 534r, as described by López-Gutiérrez et al. [30]. The abundance of *puh*A and *puh*B genes were estimated by qPCR with puHAf/puHBr and puHBf/puhBr primers, respectively, as described by Pesce et al. [19]. Functions that described the relationship between Ct (threshold cycle) and the number of 16s rRNA, *puh*A and *puh*B sequences (log copy number) for each DNA sample (x) were Ct16S rRNA = -3.28 x + 39.26 (R^2=0.996), Ct *puh*A= -3.36 x + 35.91 (R^2= 0.991), and Ct *puh*B = -3.30 x + 35.25 (R^2= 0.999). The efficiency values of the qPCR assays were 101%, 98%, and 100%, respectively. Three replicates were run for each qPCR assay. Three non-template controls (NTC) were also run for each gene target.

2.7. A-RISA fingerprinting

The genetic structure of the bacterial community was analysed by automated ribosomal intergenic spacer analysis (A-RISA) [18]. The 16S–23S intergenic spacer of the bacterial rRNA was amplified with primers 38r/72f and PCR conditions was as described by Martin-Laurent et al [15]. The primer 72f was labelled at position 5' with IRD 800day fluorochromo (MWG SA Biotech, Ebersberg, Germany). The PCR products were loaded onto a 3.7% polyacrylamide gel (66 cm in length) and run on a LiCor 4300 DNA Analysis System (Biosciences, USA) for 15 h at 1500 V/80 W. The gels were further analysed using 1-D Scan (ScienceTec, France), which converts fluorescence data into electropherograms. The size of the bands (in base pairs) were calculated using a standard DNA ladder with 15 bands ranging from 200 to 1200 bp.

2.8. Data analysis

Modelmaker 4.0 was used for dissipation data analysis. Experimental data were fitted to Double First Order in Parallel (DFOP) and Single First Order (SFO) models. Values of dissipation time DT_{50} and DT_{90} in DFOP were calculated using an iterative procedure [19]. Spearman correlations and analysis of variance (ANOVA) of experimental data were made with Statistic software SPSS® 15(Chicago, Illinois, USA). The A-RISA results were converted into a distance matrix based on band presence (i.e.

peak at a given position) and intensity (i.e. height of peak) using PrepRISA (http://pbil.univ-lyon1.fr/ADE-4/microb/). The A-RISA matrix was then used to perform the principal component analysis (PCA) using ADE-4 software (http://pbil.univ-lyon1.fr/ADE-4/home.php). The correlations between microbiological and biochemical variables as diuron degradation were assessed using Mantel's multivariate test. Distance matrices for A-RISA profiles of 16S rRNA were determined using a Jaccard distance transformation and for the rest of variables, the Euclidian distances were inferred.

3. RESULTS AND DISCUSSION

After 6 months of incubation, the effect of double amendments done either with W or O vermicompost led to the increment of the soil properties in the amended soils SW and SO (Table 1). The increase in soil organic matter (SOM) was two-fold higher in the amended soils than for the control. This gain in SOM was positively correlated with the increase in the lignin and fulvic acid (FA) concentration in the soils amended with vermicompost ($r = 0.82$ and 0.81, $p<0.01$ respectively). On the other hand, SOM was negatively correlated with the fall in pH by the amendments ($r = -0.91$, $p<0.01$), these results being consistent with those of Fernández-Bayo et al. [24].

3.1 Sorption-desorption of diuron

The sorption-desorption Freundlich coefficients determined for diuron and its main metabolites were affected by the vermicompost amendment (Table 2, Figure A.1). The Kfads increased in SW and SO by 2.3 and 1.7 fold, respectively, as compared to the control (S). The Freundlich exponent (1/nads) was close to 1, attesting for a good linearity in the amended soils, especially in SO, which is consistent with a partition-sorption process. In addition, as a result of vermicompost amendment, the sorption of the metabolites DPMU and DPU increased 1.6 fold in SO and 1.9-fold in SW as compared to S (Table 2). Similarly, sorption of DCA increased 2.2 fold in SW and SO with respect to S. The k_{fdes} were also higher in the amended soils than in control soil (Table 2). However, the H index revealed that metabolites in S, SW, and SO were retained at more irreversible sorption sites. The lowest reversibility was observed for DCA in the amended soils (Figure A.1d).

Table 1. Physico-chemical properties of W and O-vermicomposts, unamended (S) and amended soil twice with 5% of each vermicompost (SW and SO), and aged after each vermicompost addition by a 3-month incubation.

Properties	S	SW	SO	O	W
TOC (%)	1.40±0.01	3.66±0.09	3.31±0.12	29.2±1.4	29.5±0.8
OM (%)	2.42±0.01	6.30±0.12	5.71±0.21	51±2.4	59±1.6
Lignin (g kg^{-1})	4.70±0.20	20.7±0.20	19.2±0.20	200±4	255±9
HA (g kg^{-1})	4.96±0.02	6.70±0.26	6.90±0.17	152±6	146±7
FA (g kg^{-1})	1.19±0.42	3.78±0.12	3.71±0.10	100±4	101±3
WSC (g kg^{-1})	0.04±0.00	0.13±0.01	0.49±0.30	8.8±0.3	3.4±0.4
pH	8.6	8.4	8.2	8.6	7.3

The k_{fads} increment for diuron and its metabolites in the amended soils (SW and SO) correlated with the increase in SOM, FA, and lignin ($r > 0.75$, $p < 0.05$).The SOM is heterogeneous and composed mainly of an expanded component such as lignin, humic and fulvic acids (HA, FA); and a condensed component such as humin. The proportion and structure of these components affect sorption and linearity of the sorption isotherms [33]. Although the increment of lignin and humic substances (HA and FA) in the amended soil were similar, the k_{fads} differences with diuron and metabolites could be associated to structure and nature of SOM. It is known that different humic fractions from the same soil can have different sorption coefficients [34]. Therefore, in SO the content of these fractions might be involved in the near linearity of sorption isotherm (1/n close to 1), but the structure and composition of these fractions in SW counteracted this effect, showing a nonlinear sorption isotherm (1/n= 0.85). It is known that humic fractions in W vermicompost have a low $E_4{:}E_6$ ratio, indicating a higher level of polymerization and condensation of aromatic constituents, with a dominance of humic acids of higher molecular weight [35]. Therefore, a change in SOM can affect the adsorption process [36].

Table 2. Adsorption and desorption Freundlich coefficients (Kf and 1/n) for diuron and its metabolites DPMU, DPU, and 3,4-DCA in the unamended (S) and 10%-amended soils by two consecutive additions of 5% with O or W vermicompost (SW and SO).

	k_{fads} ±ES	$1/n_{ads}$±SE	R^2_{ads}	k_{fdes} ±SE	$1/n_{des}$±SE	R^2_{des}	k_{oc}±SE	H
Diuron								
S	7.45±0.72c	0.77±0.01b	0.909	10.29±0.10c	0.40±0.01b	0.997	530±52a	0.52
SW	16.77±1.05a	0.85±0.01a	0.973	17.13±0.01a	0.47±0.01a	0.993	459±39ab	0.56
SO	13.14±0.08b	0.92±0.07a	0.989	14.39±0.52b	0.46±0.01a	0.991	397±16b	0.51
DPMU								
S	11.68±0.11c	0.66±0.01a	0.996	13.95±0.10c	0.19±0.01a	0.970	153±0.6b	0.29
SW	22.95±0.97a	0.68±0.09a	0.963	19.20±0.20a	0.12±0.01c	0.910	272±12a	0.18
SO	18.78±0.33b	0.70±0.01a	0.996	17.98±0.07b	0.15±0.01b	0.935	261±4.6a	0.22
DPU								
S	12.21±0.07c	0.65±0.01a	0.996	13.81±0.04c	0.24±0.01a	0.961	160±0.9b	0.37
SW	23.15±1.55a	0.72±0.11a	0.956	17.94±0.16a	0.09±0.15c	0.792	274±19a	0.13
SO	19.12±0.41b	0.71±0.01a	0.997	17.58±0.06b	0.16±0.02b	0.919	266±5.7a	0.23
DCA								
S	9.48±0.09c	0.69±0.01a	0.997	13.09±0.04c	0.11±0.00a	0.984	124±0.8c	0.16
SW	23.07±0.96a	0.69±0.04a	0.979	19.11±0.11b	0.04±0.04b	0.911	278±15b	0.06
SO	21.26±0.33b	0.69±0.01a	0.998	18.72±0.06a	0.02±0.00c	0.872	296±4.1a	0.03

kf unit: $\mu g^{1-1/n}\, g^{-1} mL^{1/n}$; SE standard error.

In relation to Kfdes values, these were also higher in SW and SO than in S. The H index with values of less than 1 reflects that the molecules diffuse throughout the micropores of the organic matter, these being less accessible [10]. Consequently, by increasing the sorption capability of soil, the vermicompost amendments may contribute to the accumulation of diuron metabolites, especially DCA, if the degrading ability of the soil microbial community is low.

3.2. Dissipation of diuron

Significant differences in the dissipation kinetics of diuron were found between the different treatments (Table 4) when diuron was applied in the pre-amended soil (E1). The experimental data from SD1, SWD1, and SOD1 were fitted to DFOP kinetic with a

fast initial decrease of diuron the first 10 days of incubation, followed by a slower decline (Figure 1). Significant differences in the degradation constants k_1 (F= 9.43 p= 0.014) and k_2 (F= 21.42 p= 0.002) were found (Table 3), with a higher degradation rate (k_1) in the amended soils (SWD1 and SOD1), but lower values in the second phase (k_2). The high DT_{90} value reflected the diuron persistence in the soils amended with vermicompost (Table 3).This was supported by Pearson's correlation between DT_{90} and SOM (r = 0.92, p<0.01) and negative correlation between k_2 and lignin concentration after amendment (r = -0.97 p<0.01). The DFOP kinetic for diuron in E1 might have been due to the relative increase in the diuron sorption and decline in microbial activity [37]. In fact, in k_2 the slow diuron degradation constant was significantly correlated with the K_{fads} in the amended soils (r = 0.82 p<0.01) and was consistent with the longer dissipation time registered in SWD1. According to Wauchope et al. [38] a bi-exponential decline in pesticide can be accepted as sufficient evidence for long-term sorption kinetics.

Table 3. Kinetic parameters estimated from diuron degradation in soil samples from E1 and E2 based on DFOP and SFO models, respectively. Different letters indicate significant differences (α=0.05).

	Treatment	Ci (%)	g	k_1 (d^{-1})	$k_{2\ X\ 10^{-2}}$ (d^{-1})	DT_{50} (d)	DT_{90} (d)	R^2	$X^{2\gamma}$	*t-test*
					***DFOP* model**					
	SD1	104.98	0.12	0.224^{c}	0.95^{a}	62	210	0.99	1.1	< 0.05
E1	SWD1	108.99	0.18	0.514^{a}	0.22^{c}	200	830	0.99	0.9	< 0.05
	SOD1	108.90	0.19	0.439^{b}	0.75^{b}	58	260	0.99	0.8	< 0.05
					***SFO* model**					
	SD2	104.92	n.d	0.051^{a}	n.d	13.41	44.60	0.98	2.1	< 0.05
E2	SWD2	99.62	n.d	0.042^{a}	n.d	16.34	54.30	0.98	1.7	< 0.001
	SOD2	110.17	n.d	0.046^{a}	n.d	15.15	50.34	0.97	1.0	< 0.001

$^{\gamma}$ Error level of X^2, n.d: No data

In relation to E2, the diuron dissipation results were fitted to SFO model, showing rapid dissipation in all treatments (Figure. 1, Table 3) and lower DT_{50} values than

observed in E1 treatments. In this case, 50% of the diuron added disappeared within 2 weeks. However, no significant differences (F= 0.336 p= 0.728) were found between the k values in the different treatments (Table 3). Unlike diuron degradation in E1, the accelerated disappearance of diuron in E2 could be attributed to the repeated application of diuron, which led to the adaptation of microorganisms to diuron degradation [39, 40]. This deduction was supported by the detection of diuron metabolites. Indeed, the metabolite DPMU in the soil managed in E1 was detected in the SD1 and SOD1treatments and quantified at concentration levels of 0.64 and 0.74 μg g^{-1}, respectively, which corresponded to 20.41% and 22.82% of the diuron applied (Figure. 1a and c). The metabolite DPU was detected in SWD1 within the first 5 days (10.85%) and in SOD1 after 90 days at the highest concentration (26.07%). However, in E2, the concentrations of DPMU were lower than 5% of the diuron applied, but the DPU metabolite was not detected.

The detection of DPU and DPMU indicated that the metabolism of diuron started with N-demethylation [1, 2]. In E1, the slow formation of DPMU in SWD1 may be the consequence of the slow diuron degradation, a hypothesis supported by k_2 values (Table 3). In E2, the slight increase in DPMU production in all treatments may be associated with the faster diuron degradation. These results are consistent with previous studies by Fernández-Bayo et al. [24] in which diuron degradation followed an SFO kinetic with a relatively short DT50 (DT_{50} = 22 days) and a low accumulation of the metabolite DPMU. The DCA metabolite formed by hydrolysis reaction was not detected, probably because of its (i) strong and less reversible sorption to soil components (Table 2) or (ii) low recovery from soil (< 81%).

3.3. Soil enzyme activities

As expected, the addition of vermicompost stimulated the soil microbial activity, which was stable in S, but reached its highest values at 30 and 60 days for SW and SO, respectively (Figure 2a).

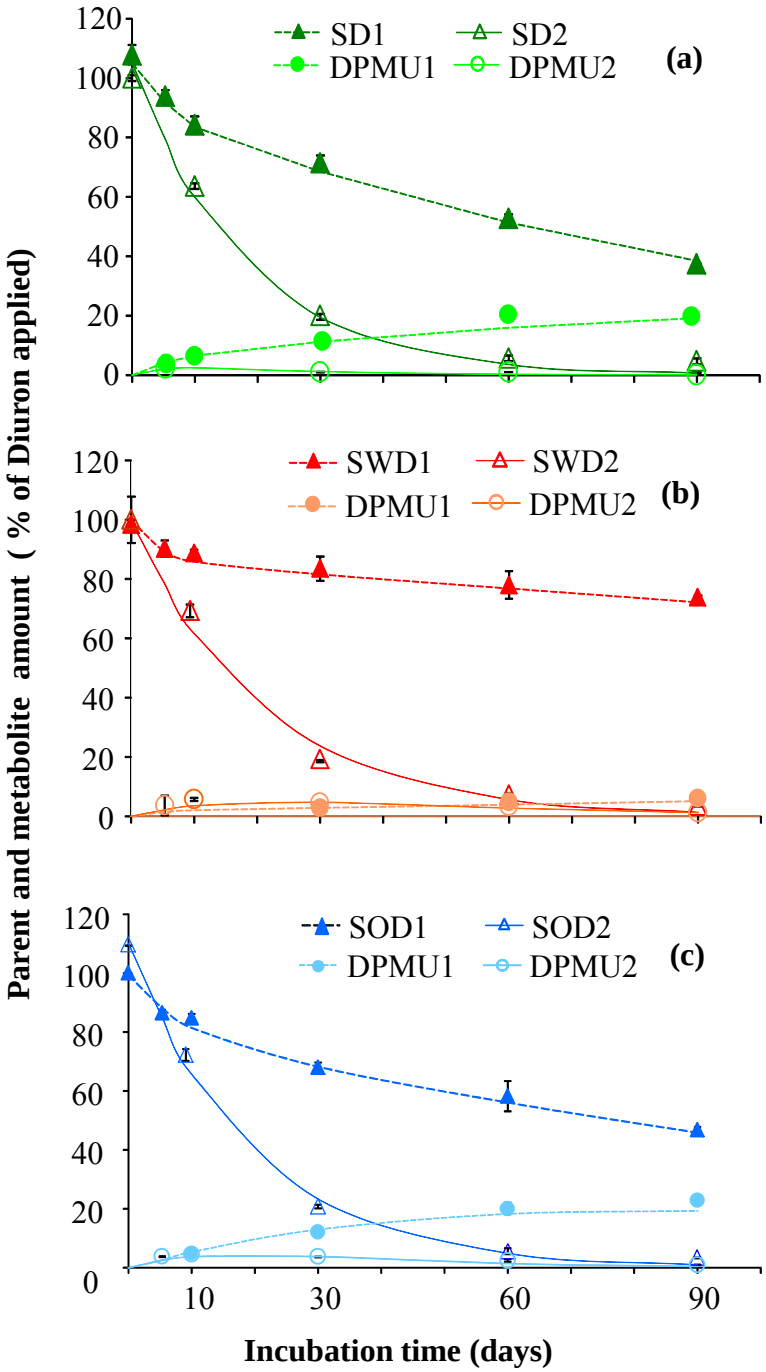


Figure 1. Degradation kinetics of diuron and DPMU in unamended soils SD1 and SD2 (A), amended soils with W-vermicompost SWD1 and SWD2 (B) and amended soils with O-vermicompost SOD1 and SOD2 (C) from the experiments E1 (no pre-treated with diuron) and E2 (pre-treated with diuron). The bars represent the standard deviations

For SWD1 and SOD1 in E1, the application of diuron depressed DHS activity, especially in SWD1 (Figure 2b). However, in SOD1 the disturbance was weaker than

that in SD1 and SWD1. In relation to E2, SD2 showed lower DHS activity than SWD2 and SOD2 at the end of the incubation time, this latter showing a significant enhancement (F= 249.77 p<0.05). This great surge of DHS in SOD2 despite the lower sorption of diuron in SO with respect to SW (Table 2) point to a possible adaptation ability of microbial community in SOD2. Furthermore, in both experiments, E1 and E2, greater soil microbial activity resulted from the addition of the O-vermicompost, which could be attributed to the vermicompost quality.

The urease activity in S declined over the first 30 days, but then increased again but without reaching initial values (Figure 2d). The SW treatment stimulated the urease activity during the first 30 days, which then remained constant until 90 days. At the end of the incubation time, only SO showed significantly higher urease activity than S. With diuron application in the SD1 and SWD1 treatments (Figure 2e), the urease activity rose until 60 days and then fell at 90 days. Conversely, SOD1 showed a fluctuating rise, reaching an activity level similar to that of SD1 and SWD1 at 90 days. On the other hand, in the E2 treatments the urease activity slightly increased, but the values remained below of 300µg NH_4^+ g^{-1} h^{-1} over the incubation time.

The performance of DHS and urease activities might be used as proxies to estimate the resistance and the resilience of the soil microbial community, reflecting its ability to cope with environmental stress. Within this context, vermicompost amendment caused only minor disturbance and prompted the recovery (resilience) of enzymatic activity. Notably, the E1 treatments had a marked impact on the recovery of DHS activity. Perhaps due to physico-chemical factors such as the pesticide sorption in organic and inorganic matrices, this has major implications for the competition between immobilized enzymes and pesticides, with the subsequent release of free enzymatic molecules from matrices. Conversely, in E2 the recovery of DHS was immediate, especially in SOD2, probably because of the addition of O vermicompost, which can counterbalance the toxic effect of diuron. The urease activity in E1 was high but unstable over time. This might be explained by biological factors such as the cell-wall permeability or cell lysis, increasing the accessibility of the substrate to intracellular urease [41]. In E2, the high DHS activity and low urease activity can be explained by the inhibition of urease activity by the important high diuron concentration in soils.

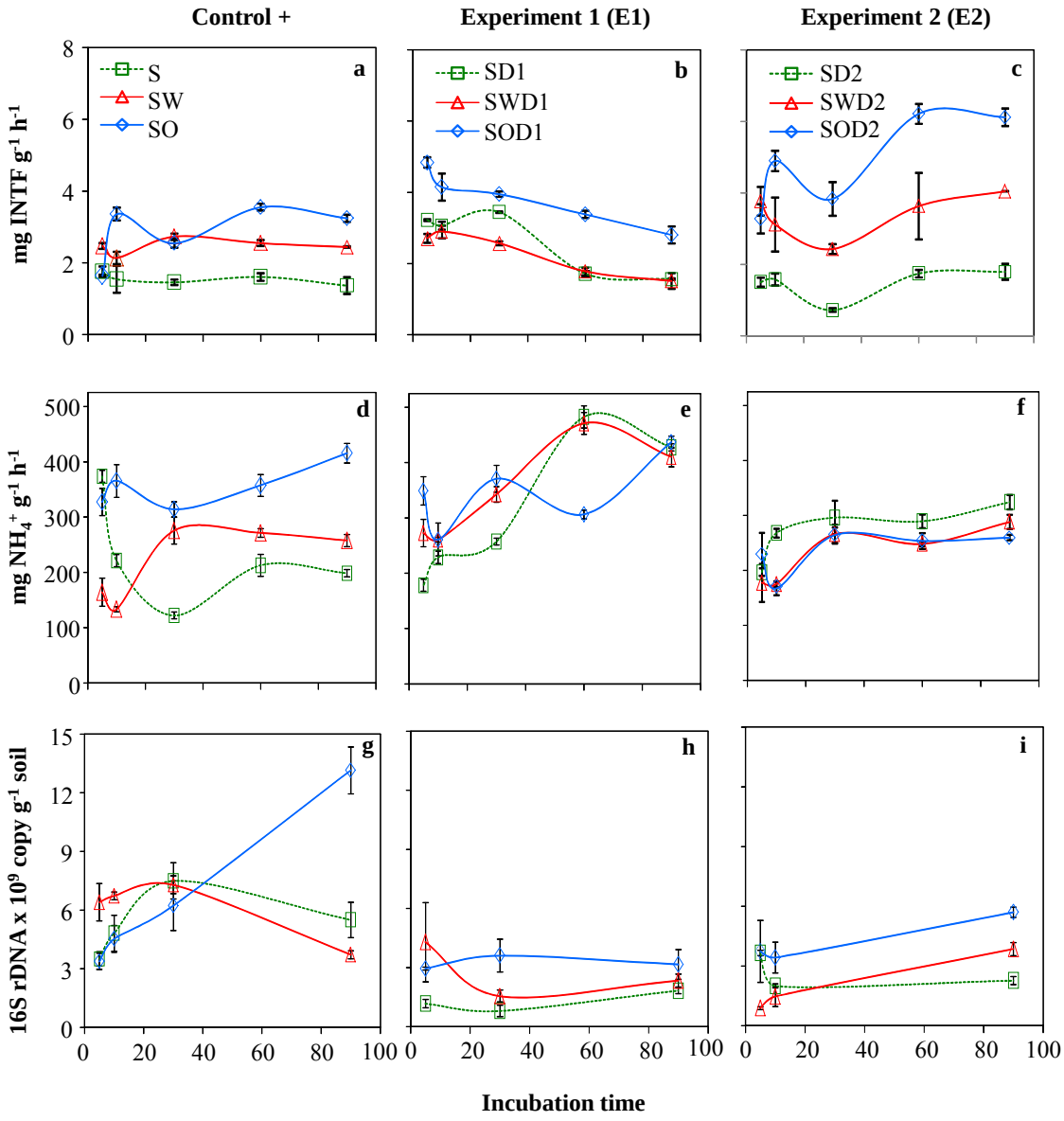


Figure 2. Enzyme activities, dehydrogenase (a, b, and c), urease (d, e, and f), and copy number of 16S rRNA gene (g, h and i), determined in unamended (S) and amended (SW and SO) soils as well as in corresponding soils treated with diuron (unamended: SD1, SD2; amended with O-vermicompost, SOD1, SOD2) or with W-vermicompost (SWD1, SWD2) from E1and E2 experiments.

3.4. Bacterial abundance and structure

Given the differences found in the diuron-degradation kinetics between the E1 and E2, a subset of soil samples showing marked differences were chosen (i.e. 5, 30, and 90

days for E1 and 5, 10, and 90 days for E2) to extract soil DNA. In S and SW, 16S rRNA gene abundance reached its maximum after 30 days of incubation and then declined (Figure 2g, h, and i), while values in SO progressively rose to 12.8 x 10^9 copies g^{-1} soil after 90 days (Figure. 2g). In E1, at 5 days, the BA in SD1 was significantly lower than in the amended soils SWD1 and SOD1 (F = 7.34 p<0.05). However, after 90 days of incubation, the BA was similar in all treatments, suggesting that the bacterial community had recovered from the stress induced by diuron (Figure 2h). In E2, at the initial time, the lowest abundance was determined in SWD2, but after 90 days the BA in SWD2 and SOD2 were significantly higher (F = 22.47; p<0.05) than that in SD2, which showed no significant variations (Figure 2i).

As well as the enzyme activity, changes in BA can be interpreted in the ecological concept of resistance and resilience [42]. In E1 and E2, BA was reduced by the effect of diuron and vermicompost, as observed in SD1, SD2, and SWD1. In these treatments the BA had weak resistance and poor resilience at the end of the experiment. SOD1 in E1 showed no significant differences in the BA over time, while SOD2 in E2 registered a recovery of BA. The resilience observed in SWD2 and largely in SOD2 may be a consequence of repeated exposure of the soil to a lower herbicide level (3µg g^{-1}), which promotes the development of a bacterial community adapted to pesticide, as well as to the changes in the physico-chemical properties of the soil and nutrients available in the amendment.

BA changes in both E1 and E2 can be attributed to alterations in the genetic structure of the bacterial community. In an effort to test this hypothesis, the evolution of genetic structure of the bacterial community was monitored by A-RISA (Figure A.2), and its fingerprints were compared by PCA (Figure 3). In both experiments, the addition of vermicompost and the treatment with diuron altered the soil bacterial structure. In E1, 5 days after treatment, the factorial map showed that the ordination on PC1 explained 53% of the variance in the data, allowing the discrimination of the microbial communities in S and soil treated with 3µg g^{-1} of diuron (SD1). Meanwhile the PC2 explained 22% with a differentiation of S from SO and SOD1 (Figure 3a), revealing the impact caused by vermicompost and diuron application, respectively. Thirty days after treatment, only the microbial communities of SO could be discriminated from all other treatments throughout PC1 (Figure 3b). Ninety days after treatment, the PC1 (29%),

discriminated the microbial communities of SW from all treatments. In addition, ordination on PC2 (13%) discriminated the SO bacterial community from all the other treatments (Figure 3c).

In E2, 5 days after treatment, the factorial map showed that ordination on PC1 (33%) did not enable the discrimination of any microbial communities, but PC2 (20%) discriminated the microbial communities of S from SD2, SOD2 and SWD2, which could not be distinguished from each other (Figure 3d). After 10 days of incubation, similar trends were found, except SD2, which was discriminated from SOD2 and SWD2 throughout PC2 (16%) (Figure 3e). This revealed that the diuron treatment had an impact on the bacterial genetic structure of soils amended or not with vermicompost. After 90 days of incubation, the bacterial structure of the soils treated with diuron could be discriminated from its respective controls throughout PC1 (40%). Nonetheless, the bacterial community of SD2 could be discriminated from that of SWD2 and SOD2 throughout PC2 (16%) (Figure 3f).

In summary, the A-RISA analyses revealed that, in both experiments, only O-vermicompost modified the bacterial community structure as compared to the control soil. This suggests that the nature of the vermicompost determines the impact provoked on the bacterial structure of the soil. This agrees with a previous report holding that the nature of the organic amendment determines the shifts caused in the microbial genetic structure in agricultural soils [43] with different organic-matter management. The impact induced by the addition of organic matter was counterbalanced by diuron treatment in both experiments. This effect was more evident in E1 than in E2. It should be borne in mind that fingerprinting techniques allow the dominant group to be studied and therefore does not enable the detection of new specific phylotypes involved in diuron degradation that is a small fraction of the total microbiota [18]. It could be hypothesised that some of the dominant groups were altered by the diuron treatment, in particular those favoured by organic amendment (Figure 3c and f). However, under diuron selective pressure (i.e. pre-treatment applied in E2), organic amendment was shown to contribute to the bacterial survival and adaptation of new microbial communities capable of degrading this pesticide, as was found in E2.

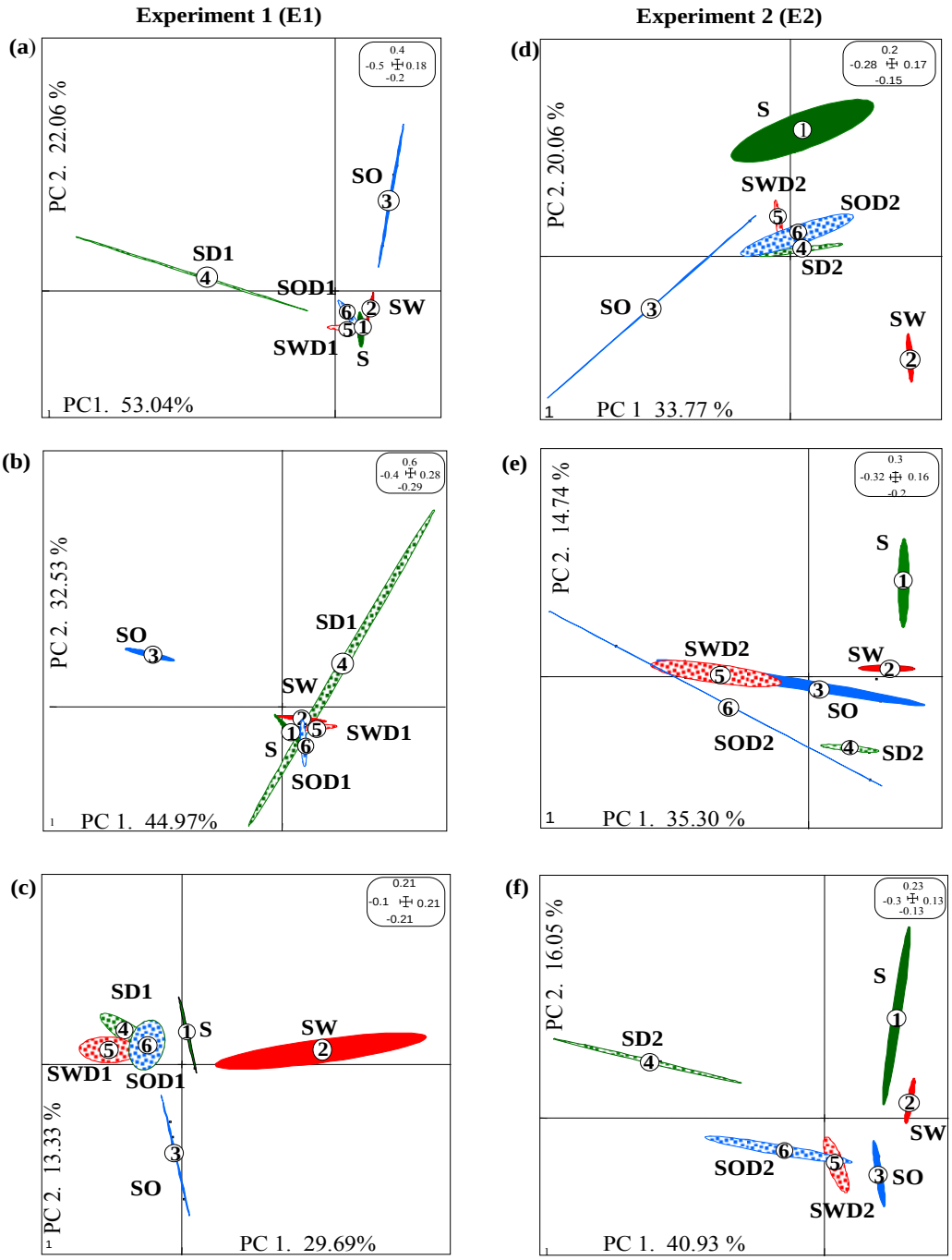


Figure 3. PCA ordination at the initial (a, d), intermediate (b, e), and final time (c, f) from E1 and E2 experiments, based on the analysis of the total bacterial communities, analysed by ARISA fingerprints of PCR products from DNA extracted directly from unamended (S=1) or amended soils (SW=3 and SO=2) without pre-treatment or pre-treated with diuron (SD1 and SD2=4, SWD1 and SWD2=5, SOD1 and SOD2=6).

Therefore, our results suggest that repeated diuron exposure promotes the development of microbial communities that utilize the pesticide as a carbon or nitrogen source [4]. This hypothesis was tested by monitoring the abundance of the bacterial community able to mineralize diuron, followed by qPCR assays targeting *puh*A and *puh*B genes coding for phenylurease enzymes PuhA and PuhB, respectively.

3.4. Quantification of *puh*A and *puh*B genes

The *puh*A and *puh*B gene sequences in E1 were detected in low copy number close to the detection limit (10 copies, C_T =33.52), which may be related to the low diuron degradation (Figure 1). In soil samples of E2, only the *puh*B gene sequence was detected in high abundance in the microcosms pre-exposed to diuron (SD2, SWD2 and SOD2; Figure 4a), confirming that this treatment exerted selection pressure, favouring the growth of diuron degraders.

In the soils not treated with diuron (S, SW and SO), the copy number of the *puh*B gene determined at day 5 in SW and SO was 9- and 18-fold greater than in S, respectively. This suggests that this enzyme has promiscuous hydrolysis activity with the ability to catalyse the amide bond (C-N) or ester bond (P-O). This was probably because the PuhB enzyme has a Michaelis constant (Km) for diuron lower (7.6 μM) than for similar molecules and has a substrate-binding cavity more efficient in the hydrolysis of *N*- dimethylphenylurea compounds [21]. However, it was evident that the largest copy number of *puhB* sequences was quantified in the soils pre-exposed to diuron (E2). The *puh*B sequences in SOD2 and SWD2 after 90 days was similar but significantly higher than in SD2 (1.8×10^5 copy number g^{-1}). The relative abundance of diuron degraders within the total bacterial community was estimated as the *puh*B/16S rRNA gene ratio (Figure 4b). The *puh*B ratio throughout the incubation period was higher in the diuron treatments than in its respective controls, although SO showed a relatively greater *puh*B ratio, which also altered the genetic structure of the bacterial community (Figure 4b).

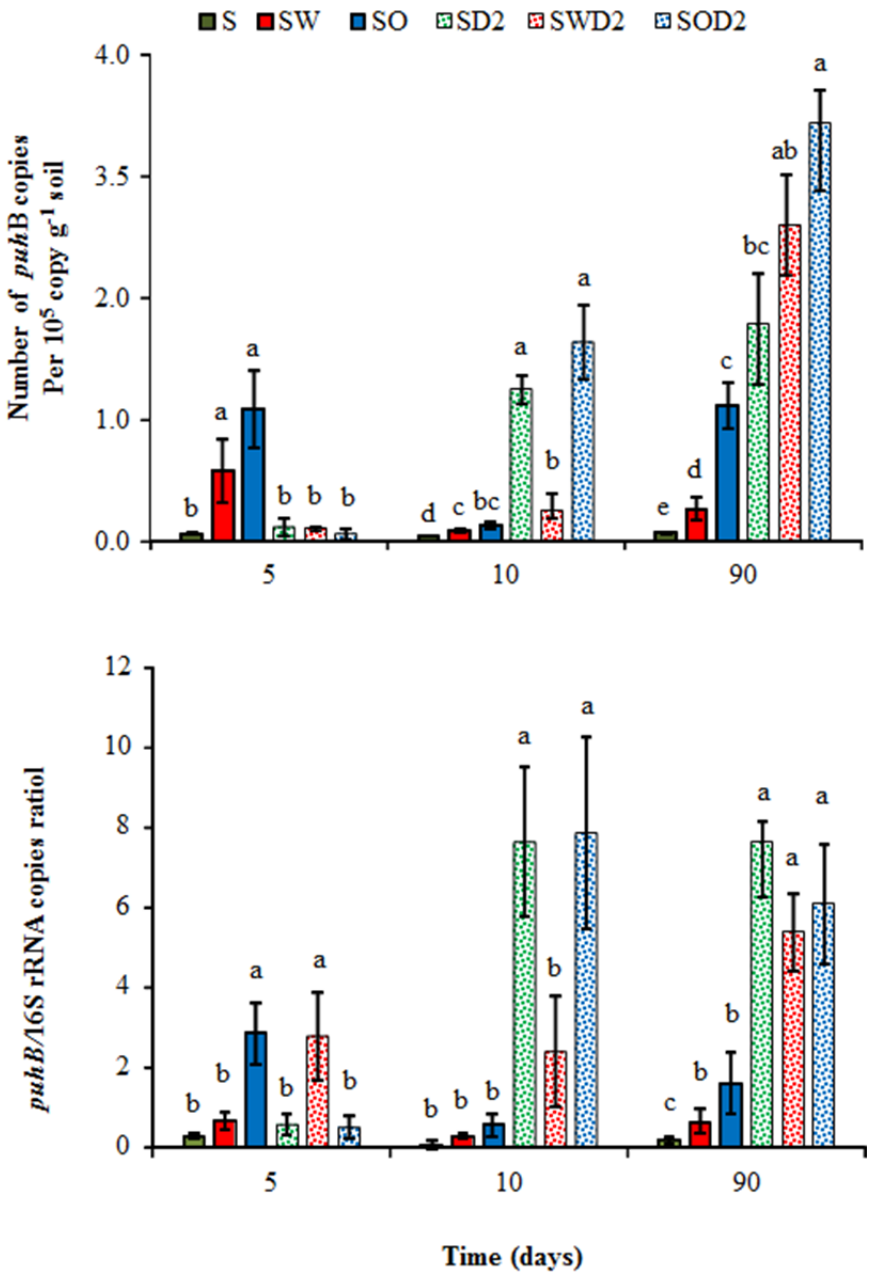


Figure 4. Quantification of diuron-degrading community (*puhB* gene sequence) (a). *puhB* ratio of community in the total bacterial abundance, it was estimated by calculating the puhB/16S rRNA ratio (given as a percentage) (b). The qPCR assays were carried out from DNA extracted directly from soil (S) or amended soils (SW and SO) and soil pre-treated with diuron (SD2, SWD2, and SOD2) from experiment 2. The bars represent the standard deviations calculated from the triplicates. Data with the same letter are not significantly different ($p < 0.05$).

The Mantel correlation test confirmed that increases in the *puh*B gene-copy number positively correlated with a faster rate of diuron degradation ($r = 0.64$ $p<0.01$) and bacterial structure and BA ($r >0.40$; $p<0.05$). These results support the importance of quantifying the abundance of functional groups responsible for key steps of pesticide degradation [44, 45] and confirm the importance of agricultural practices such as organic amendment to regulate the microbial degradation of pesticide which is the key driver of the biofiltration, an important ecosystemic service supported by arable soils.

4. CONCLUSIONS

This multidisciplinary study reports the positive impact on pesticide dissipation exerted by soil amendment with agro-industrial vermicomposts. These results demonstrate that the nature and composition of vermicompost affect the fate of diuron as well as of the microbial community, promoting the development of diuron-degrading populations. The history of diuron application counterbalanced the vermicompost effect. O-vermicompost increases bacterial and *puh*B abundance, corroborating its effectiveness as a tool to enhance the diuron biodegradation in soil. Further work is needed to understand the mechanisms by which vermicomposts promote pesticide biodegradation in order to define the best management options to mitigate environmental pesticide risk.

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APPENDIX A. SUPPLEMENTARY DATA

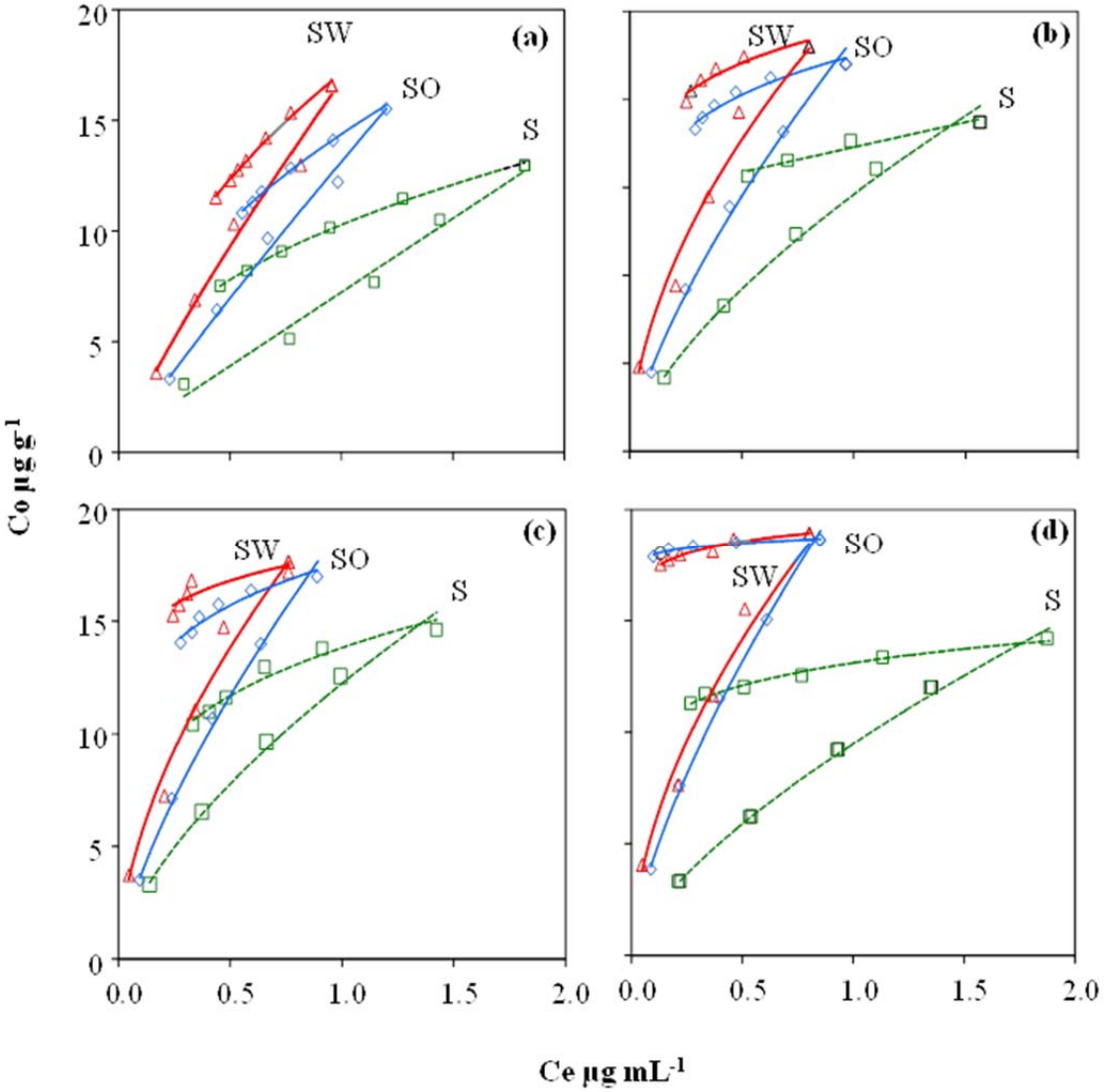

Figure A. 1. Adsorption-desorption Freundlich isotherms for diuron (a) and the metabolites DPMU (b), DPU (c), and DCA (d) in the ageing unamended soil (S) and 10%-amended soil (SW and SO). Experimental data are the symbols while lines represent the theoretical calculation.

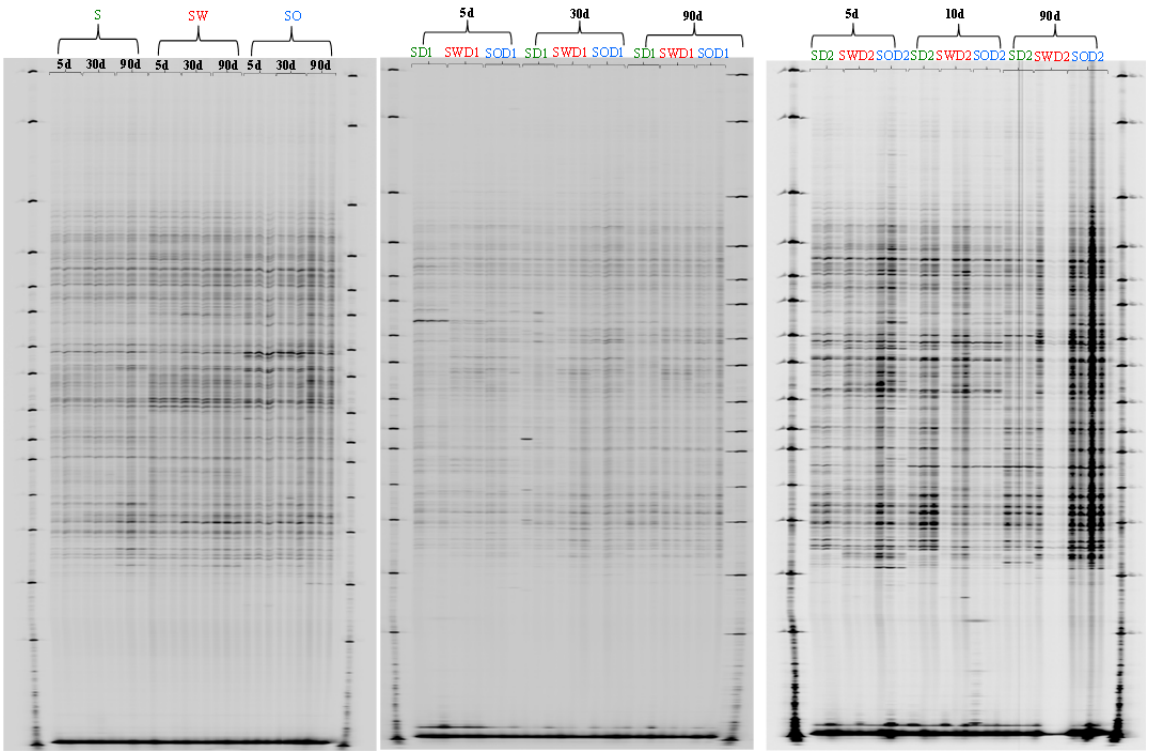

Figure A. 2. ARISA fingerprint gel of PCR products from DNA extracted at the initial, intermediate, and final time of the incubation period, in the control soils S, SW, and SO and in the soils treated with diuron from E1 and E2 experiments.

CAPÍTULO 4

Efecto de los vermicomposts sobre el comportamiento de Imidacloprid en suelo y riesgos asociados en la funcionalidad del suelo

Vermicompost effects on the soil imidacloprid fate and risk associated to soil function

El capítulo 4 será enviado a la revista Frontiers in Microbiology

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Resumen

El objetivo de este estudio fue evaluar el comportamiento de Imidacloprid (IMI) en suelos vermicompostados y contaminados con IMI. La distribución y disponibilidad fue determinada en suelos enmendados con vermicompost de residuos vitivinícolas (W) o con residuos oleícolas (O) y tratados con ^{14}C-IMI. La dinámica de IMI fue analizada en dos microcosmos para determinar su impacto en la estructura de la comunidad bacteriana del suelo, en la actividad enzimática del suelo así como en la resistencia del suelo a los tratamientos con IMI cuando el suelo está bajo condiciones diferentes. El microcosmos 1 contenía suelos enmendados envejecidos, SI1, SWI1 and SOI1, y el microcosmos 2, suelos envejecidos pre-expuestos a IMI, SI2, SWI2 y SOI2. El ^{14}C-IMI fue distribuido principalmente en la fracción de tamaño arena. El proceso de sorción fue significativamente mayor en SWI (42.5 mL g^{-1}) que en SI o SOI. La vida media más baja (DT_{50}) fue determinada en el suelo SOI2 (67 días). El IMI afectó las actividades deshidrogenasa y ureasa en las muestras de suelo del microcosmos 1. Los datos cinéticos en SOI2 estuvieron relacionados con el aumento de la actividad enzimática del suelo y con los cambios en la estructura de la comunidad bacteriana. Por lo tanto, los vermicomposts pueden minimizar la disponibilidad de IMI y su distribución, y puede ser una alternativa para mitigar el impacto de IMI y promover su degradación en suelos contaminados.

Palabras claves: Imidacloprid, Vermicompost, Biodegradación, ARISA, fingerprinting, bacteria.

Abstract

The aim of this study was to evaluate the Imidacloprid (IMI) behaviour in vermicomposted soils and contaminated with IMI. The distribution and availability was determined in soils amended with vermicompost from winery (W) and olive (O) wastes and treated with ^{14}C-IMI. The fate of IMI was analysed in two microcosms to determine its impact on the structure of soil bacterial community, on soil enzymes activity as well as the soil resistance to IMI treatments when the soil is under dissimilar conditions. The microcosms 1 contained aged amended soils, SI_1, SWI_1 and SOI_1, and microcosms 2 aged soils pre-exposed to IMI SI_2, SWI_2 and SOI_2. The ^{14}C-IMI was mainly distributed in sand-size fraction. The sorption process was significantly greater in SWI (42.5 mL g^{-1}) than in SI or SOI. The lowest half-life (DT_{50}) was determined in the soil SOI_2 (67days). The IMI affected dehydrogenase and urease activities in soil samples from microcosm1. Kinetic data in SOI_2 are in accordance with increments in the activities of soil enzymes and shifts in the bacterial community structure. Therefore, vermicomposts can minimize the availability of IMI and its spread in the environment, and can be an alternative to mitigate the IMI impact and to promote its degradation in polluted soils.

Keywords: Imidacloprid, Vermicompost, Biodegradation, ARISA fingerprinting, bacteria.

1. INTRODUCTION

Imidacloprid (IMI) is a neonicotinoid insecticide used worldwide due to its low mammalian toxicity and high effectiveness in target insects (Goulson, 2013; Phugare et al., 2013). It is applied to seeds, plant and soils (Rouchaud et al., 1994). However, its possible relation to the decline of crop pollinators like *Apis mellifera,* has worldwide concern and its application has been regulated by the European Commission (EU Regulation No 485/2013).

Despite the wide use of IMI, factors leading to its dissipation in soil are not completely understood. IMI retention in soil depends on organic carbon (O.C) content (Liu et al., 2006). However, other soil properties as clay minerals, moisture, pH, temperature and cation exchange capacity contribute also when the O.C content is low (Fernández-Bayo et al., 2008). In sandy soils, IMI was weakly sorbed (K_{OC} = 163–230) with a half-life of 1.0–2.6 years (Leiva et al., 2015). Other process as biodegradation is also involved in the dissipation of pesticides. In relation to IMI, the co-metabolism is the dominant process (Anhalt et al., 2007). It is known that some microorganisms of soil as *Bacillus aerophilus* and *Bacillus alkalinitrilicus* isolated from sugarcane soils can contribute to the soil IMI dissipation (Sharma et al., 2014). Nevertheless, few microorganisms have the ability to mineralize this molecule (Kandil et al., 2015).

Consequently, the persistence of IMI leads to potential risk of soil microbiota (Deborah et al., 2013; Cycoń and Piotrowska-Seget, 2015), decreasing the nitrification rate while stimulated the ammonification process. Considering the above, it is clear that IMI is relatively stable in the top soil (Goulson, 2013; Leiva et al., 2015), and due to its polar character it is displaced to lower horizons (Fernández-Bayo et al., 2015). Thus, the IMI use should be strictly controlled, especially in soils with low organic matter content where the risk of soil and water pollution is much higher. The addition of organic amendments improves the soil quality and the O.C content, increasing the sorption of IMI in the ploughed layer, minimizing its transport to water reservoirs (Fernández-Bayo et al., 2007). In this sense, the use of organic amendment can be an effective alternative to minimize adverse IMI effect. However, factors that lead to its dissipation in soil are not clearly understood.

Agro-industrial wastes of olive oil and wine are used as organic amendment to improve crops yield when soil are submitted to intensive production or when O.C content is low. This practice also increases the sorption and degradation process of pesticide in soils (Gámiz et al., 2013). However, due to their high C:N ratio and the presence of biocides or phytotoxic compounds, they require to be previously bioprocessed in order to turn these wastes into valuable fertilizers (Castillo et al., 2013). The vermicomposting is a sustainable eco-friendly biotechnology to stabilize these wastes. Vermicompost possesses greater microbial biodiversity than compost (Pathma and Sakthivel, 2012) and it promotes an increment on the metabolic capacities of soil microorganisms to degrade organic pollutants (Castillo et al., 2014) and increases their tolerance towards pesticides (Fernández-Gómez et al., 2011). The application of vermicompost as soil amendment for pesticide removal has been scarcely studied (Fernández-Bayo et al., 2007). In this sense, the vermicompost amendment could be an effective alternative to avoid the IMI spread in the environment, improving the IMI degradation in soil and minimizing the microbial changes and the metabolic state of soil microbiota. Despite of that these aspects have been barely studied, this information is needed to assess the risk of IMI when it is applied to soils submitted to organic amendments, which is a common agricultural practice.

The present study was carried out in order to evaluate the vermicompost effect on imidacloprid fate (sorption and dissipation) in soil under dissimilar conditions. To reach this objective, two experiments under microcosm conditions were established. The first one (M1) was to assay the imidacloprid fate in a soil previously amended with vermicompost. The second one (M2), was to evaluate the vermicompost efficiency to mitigate IMI adverse effect in a soil previously exposed to the pesticide. Two vermicomposts from wet olive cake “alperujo” (O) and vine shoots (W) were used. In both microcosm systems, the efficiency of these vermicomposts on the fate of IMI as well as on biochemical (enzyme activities) and molecular parameters (bacterial structure and bacterial abundance), were also monitored.

2. MATERIALS AND METHODS

2.1 Soils, amendments and chemicals.

The soil, a calcareous (43% $CaCO_3$), silty clay loam soil, containing 40.05 % clay, 11.07% sand and 48.88% silt was sampled at the first 20 cm in a farm located in the south-eastern of Spain. Samples were air dried, ground and sieved through a <2 mm mesh. Vermicompost from wet olive cake waste (*alperujo*) (O) and from wine waste (vine shoots and vinasse sludge) (W) were obtained as described by Elvira et al. (1998), these were assayed as organic amenments. Soil preconditions were: (i) Soil previously amended. The soil was twice 5% amended with either W or O vermicompost (SO and SW) and aged by incubation in darkness for 3 months at 20ºC±1ºC and 80% of field capacity. The total amendment in the aged amended soils was equivalent to 10%, the soil S was incubated in parallel. (ii) Soil previously exposed to IMI. One kg of unamended soil was spiked with IMI (3μg g^{-1}) and aged by incubation for 3 months under the same condition described above.

Imidacloprid (1-[(6-cloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine) was supplied by Dr. Ehrenstorfer (Augsburg, Germany). Imidazolidine ring-^{14}C-Imidacloprid, with 3.7 MBq activity (98% purity) was supplied by IZOTOP (Hungary). Imidacloprid has a water solubility of 510 mg L^{-1} and an octanol-water partition constant (k_{ow}) of 3.72 (Tomlin, 2011). HPLC-grade acetonitrile from Scharlau Chemie, S.A (Barcelona, Spain) and Milli-Q water purified in a Millipore system were used to IMI extraction.

2.2 Distribution of imidacloprid in soil fractionation

Samples of 110g of unamended and amended aged soils were treated with 1.5 μg g^{-1} imidacloprid with 0.195 μg g^{-1} (719 Bq g^{-1}) of ^{14}C-IMI (SI, SWI and SOI) and incubated at 80% of field capacity. At initial time (1 day after treatment) and final time (90 days of incubation), a soil fractionation was carried out following the methodology proposed by Séquaris and Lewandowski (2003) and described by Fernández-Bayo et al. (2008). The sand-sized (SF> 20μm) and silt-size (SiF, 2-20 μm) fractions were separated by

sedimentation, respectively. The supernatant was centrifuged (9000 x g 90 min) and clay-sized and dissolved organic matter (DOM, <0.05 μm) fractions were separated. The fractions were frozen at -20ºC and lyophilized (Thermo Electron Corp. SAVANT Modulyo). The labelled IMI in these fractions was extracted for 3 h with acetronitrile-water (60:40), and centrifuged at 8000 x 10 g for 30 min. The solid fractions were dried and subjected to combustion in a biological Oxidizer OX-600 (Harvey Instruments Corp.). The radioactivity of DOM fraction, extracted and no-extracted samples were measure in a scintillation coulter system (LS 6500 Beckmand Coulter, USA).

2.3 Sorption-desorption measurements

Sorption isotherms of imidacloprid were carried out following the classical batch-sorption method. Unamended and amended aged soil samples (5 g dry weight), were placed in triplicate into 50-mL glass tubes. A volume of 20 ml of 0.01 M $CaCl_2$ aqueous solution of IMI at 1, 2, 3, 4 and 5 mg L^{-1} was added. The tubes were shaken at 20ºC ± 1ºC for 24 h. Desorption process was studied at 5 mg L^{-1} of IMI. Sorption and desorption rate constant (K_{ads} and K_{des}) and apparent hysteresis index (H) were calculated as described by (Fernández-Bayo et al., 2009), fitting data to linear Freundlich model.

2.4 Dissipation of imidacloprid in soil under dissimilar conditions.

The IMI dissipation in soils was evaluated under dissimilar microcosm conditions (M1 and M2). The objective of M1 was to evaluate the IMI dissipation in a pre-amended and aged soil. Thereafter, 40 g (d.w) of each aged soil were placed in 100 mL glass flask and spiked with 2.33 mL of an aqueous solution to reach up a final concentration of 3 μg g^{-1}soil. The treatments (SI_1, SWI_1 and SOI_1), were incubated for 3 months under the conditions described above. The aim of the second microcosm (M2) was to study the vermicompost effect on the soil pre-exposed to IMI. For this proposal, soil fractions were spiked with 6.6 mL of IMI-aqueous solution to reach up a final concentration of 3 μg g^{-1}. After that, the samples were left overnight and subsequently were amended with 10% of either W or O vermicompost. The soil treatments from this microcosm (SI_2,

SWI_2 and SOI_2) were incubated for 3 months under the condition settled. The samples from M1 and M2 were analysed in triplicate at 0, 5, 10, 30, 60 and 90 days.

2.5 Chemicals Analysis

Soil samples of 2.5 g (d.w. equivalent) in 25 mL flasks were extracted with 5 ml of acetonitrile: water (60:40), shaken for 24 h, centrifuged (1800 x g) for 10 min, and filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters for high-performance liquid chromatography (HPLC) analysis. An Agilent series 1100 liquid chromatograph equipped with a diode array detector was used. A Zorbax Rx-C8 2.1x150 mm analytical column packed with diisopropyl n-octyl (5 μm) and an Eclipse XDB-C8 guard cartridge (2.1 × 12.5 mm i.d.) filled with the same material was used. The operating conditions were as follow: injection volume, 10 μL; flow rate, 0.2 mL min-1; and column temperature, 40 ºC, mobile phase acetonitrile/MilliQ water at pH 3. The detection wavelength was 270 nm. The recoveries were greater than 95%.

2.6 Enzyme activity analysis

Dehydrogenase (DHS) activity was determined as reported by (García et al., 2003). Samples of 1 g of wet soil were incubated in triplicate for 20 h at 25 °C with 0.2 mL of 0.4% 2-p-iodophenyl-3-p-nitrophenyl-5-tetrazolium chloride (INT). The iodonitrotetrazolium formazan (INTF) produced after INT reduction was extracted with acetone: tetrachloroethene (1.5:1) and measured in a spectrophotometer at 490 nm. Samples without soil and samples without INT were run simultaneously for control purposes.

Urease activity (U) was determined as described by Kandeler and Gerber (1988). Soil samples of 1 g wet soil in triplicate were treated with 0.5 mL of 0.48% urea substrate and incubated for 2h at 35ºC, then 2.5 mL of a sodium salicylate: NaOH solution (1:1) and 1 ml of sodium dichloroisocyanurate were added to 0.5 ml of extract and allowed to stand 30 min in darkness and read by spectrophotometry at 690 nm. Blanks without soil and samples without urea were run simultaneously for control purposes.

2.7 Extraction of DNA of soil.

DNA extraction at 5, 30 and 90 days in M1 and M2 was made following the methodology proposed by Martin-Laurent et al. (2001) and described in the ISO standard 11063 by Petric et al. (2011). The quality of purified DNA was estimated by comparison with known concentrations of calf thymus DNA (Biorad) included in each gel as a calibration standard (1.25, 2.5, 5.0, 10 and 20 ng μL^{-1}).

2.8 Quantification of 16S rRNA

The bacterial abundance was estimated by qPCR according to ISO/DIS 17601. It was carried out with a StepOnePlus qPCR machine (Applied Biosystems, USA) using the universal bacterial primers 341f (5′-CCT ACG GGA GGC AGC AG-3′) and 534r (5′-ATT ACC GCG GCT GCT GGC A-3′) according to López-Gutiérrez et al. (2004). Standard curve was obtained using tenfold serial dilution of plasmid pGEM-T Easy Vector containing the respective 16S rRNA sequence. The relation between threshold cycle (Ct) and the number of 16s rDNA sequences (log copy number) was Ct16S rRNA = -3.16x + 42.15 (R^2 = 0.995).The efficiency of the qPCR assay was 105 %.

2.9 A-RISA fingerprinting

The structure of the bacterial community was analysed by automated ribosomal intergenic spacer analysis (Ranjard et al., 2003). The 16S–23S intergenic space was amplified in a final volume of 25 µL with 50 ng of soil DNA as template and 0.5 µM of universal primers 38r (5'-CCG GGT TTC CCC ATT CGG-3') and 72f (5'-TGC GGC TGG ATC TCCTT-3') plus 2.5 U of Taq DNA polymerase (Appligene Oncor, France). The primer 72f was labelled at position 5' with IRD 800day fluorochromo (MWG SA Biotech, Ebersberg, Germany). The PCR products were diluted at the same concentration (40 ng μL^{-1}) and denatured at 92ºC for 3 min. These dilutions of PCR products were loaded on a 3.7% polyacrylamide gel (66 cm in length) and run on a LiCor 4300 DNA Analysis System (Biosciences, USA) for 15 h at 1500 V/80 W. The gels were further analysed using 1-D Scan (ScienceTec, France), which converts

fluorescence data into electropherograms. The size of the bands (in base pairs) were calculated using a standard DNA ladder with 15 bands ranging from 200 to 1200 bp.

2.10 Data analysis.

The distribution of the radiolabelled residue in the different soil compartments (extractable, no extractable, dissolved and mineralized) were calculated using the equations described by Fernández-Bayo et al. (2008). Two-phases partitioning model was used to determine the distribution constant (K_d) of IMI in the different solid fractions. K_{oc} values were calculated with the total organic carbon of each treatment.

The IMI dissipation of IMI in M1 and M2 were analysed by using Modelmaker 4.0 program. Values of DT_{50} and DT_{90} for DFOP were calculated using an iterative procedure (Boesten et al., 2005). The relationship between physicochemical properties and sorption parameters were evaluated by Spearman correlation. Differences between treatments were analysed by ANOVA and Tukey test with a risk level of $\alpha = 0.05$ (SPSS® 15 IBM, Chicago, IL, USA). The A-RISA fingerprinted were converted into a distance matrix based on band presence (i.e. peak at a given position) and intensity (i.e. height of peak) using PrepRISA (http://pbil.univ-lyon1.fr/ADE-4/microb/). The A-RISA matrix was then used to perform principal component analysis (PCA) using ADE-4 software (http://pbil.univ-lyon1.fr/ADE-4/home.php). Mantel's multivariate test, were used to find correlation between bacterial structure shifts and IMI dissipation. Distance matrices for A-RISA profiles of 16S rRNA were determined using a Jaccard transformation. For the rest of variables, Euclidian transformation was inferred.

3. RESULTS

The addition of either W or O vermicompost changed the properties of soil (Table 1). The total organic carbon (TOC) increased by more than 2 fold in the amended soils SW and SO. The lignin content, humic and fulvic acids and water soluble carbon of the soil were also significantly increased due to the characteristics of these lignocelulosic vermicomposts. Nevertheless, the soil pH decreased slightly to neutral values with the addition of the vermicomposts. As expected, the rise of total organic carbon (TOC) in

the amended soil showed a correlation coefficient above of 0.81 ($p<0.01$) with an increment in the organic matter (OM), lignin and fulvic acid (FA). In the same way, water soluble carbon (WSC) was correlated with humic acid content ($r = 0.83$ $p<0.01$).

Table 1. Physico-chemical properties of the aged soils S, SW and SO. The soil was amended with vermicomposts from olive-oil wastes (O) and from wine wastes (W). The soils were twice 5%-amended and aged after each addition by incubation 3 months.

Properties	S	SW	SO
TOC (%)	1.40±0.01	3.66±0.09	3.31±0.12
OM (%)	2.42±0.01	6.30±0.12	5.71±0.21
Lignin (g kg^{-1})	4.70±0.20	20.7±0.20	19.2±0.20
HA (g kg^{-1})	4.96±0.02	6.70±0.26	6.90±0.17
FA (g kg^{-1})	1.19±0.42	3.78±0.12	3.71±0.10
WSC (g kg^{-1})	0.04±0.00	0.13±0.01	0.49±0.30
pH	8.6	8.4	8.2

3.1 Distribution and sorption-desorption process of Imidacloprid

At initial incubation time (0 d), the highest total amount of ^{14}C-IMI distributed in solid soil fraction (sand size SF + silt size SiF) was found in SW (59 %) followed by SI (54 %) and SOI (48 %). Particularly, in the amended soil SWI the ^{14}C-activity was significantly higher in SF fraction (Table 2). However, for SI soil the highest activity was found in SiF. At the end of the incubation time (90 d), T-test showed that ^{14}C-activity increment in SF was significant for SOI and SI with respect to its initial time. For SWI sample, although the ^{14}C-activity was higher than in the other treatments, this was similar to the initial time (Table 2). In relation to ^{14}C-activity in SiF at 90 days, it was significantly greater to SI than SWI and SOI, but no significant changes were found with respect to initial time (Table 2).

The enrichment factor (EF) data, which is the relation between percentages of radiolabelled residues in the no-extractable compartment and their corresponding mass, reveals that according to T-test, the ^{14}C-activity in the SF fraction increased significantly

in all treatments between 0 and 90 days (Table 2). However, the ANOVA analysis did not show differences among treatments at 90 days. In contrast to SF fraction, in the SiF fraction was shown that the EF was significantly higher in SOI than in SWI after 90 days, indicating a different distribution of IMI in soil particles of these amended soils.

Table 2. ^{14}C-IMI distribution in the soil fractions (SF and SiF) and 14C-IMI enrichment in each soil fractions in the soil amended with W or O vermicompost (SWI and SOI) and unamended soil (SI) at 0 and 90 days. Data with the same letter are not significantly different ($p < 0.05$).

	14CIMI- distribution							
	Sand fraction (SF)				Silt Fraction (SiF)			
	0d	90d	t	p	0d	90d	t	p
SI	21.49±1.23c	35.07±1.39b	17	0.04	32.02±1.51a	28.36±1.56a	2.1	0.17
SWI	37.40±4.09a	41.20±2.47a	1.0	0.41	21.93±0.38b	22.82±2.86b	0.6	0.61
SOI	26.88±2.86b	36.97±1.82ab	7.6	0.02	21.47±0.70b	22.32±1.76b	0.9	0.48
	14C- Enrichment factor							
SI	0.09±0.02a	0.24±0.02a	27	0.01	0.19±0.02b	0.28±0.00ab	8.6	0.01
SWI	0.12±0.05a	0.34±0.10a	3.8	0.03	0.23±0.02a	0.26±0.00b	3.7	0.06
SOI	0.13±0.01a	0.29±0.08a	4.2	0.03	0.23±0.01a	0.31±0.02a	12	0.00

t = paired-sample T-test, p= Significance

The distributed amount of ^{14}C-IMI in the different portions revealed that it changed along the incubation time (Figure 1a). At initial time, the desorbed amount of ^{14}C-activity was significantly lower in SWI (35 %) than in SI (42 %) and SOI (45 %) (F= 12.7 $p<0.01$). After 90 days, these values decreased significantly in SI and SOI (29 % and 31 %, respectively, $p<0.05$). This reduction could be associated to the mineralization process which was significantly higher in SOI (10 %) than in SI (7 %) and SWI (6 %) (F=121 $p<0.001$).

The amount of extracted ^{14}C-IMI at initial time from the solid fraction was similar in SI and SWI (40 %) but significantly higher than SOI (30 %) (F=20 $p < 0.02$).The no-extracted portion in the amended soils SWI (20 %) and SOI (19 %) were slightly higher than that in SI (15 %). After the incubation time, the extractable fraction decreased in SI

(37 %) and SWI (34 %) and remains constant in SOI (29 %). However, no significant differences were found among these (F=1.8 p = 0.25).

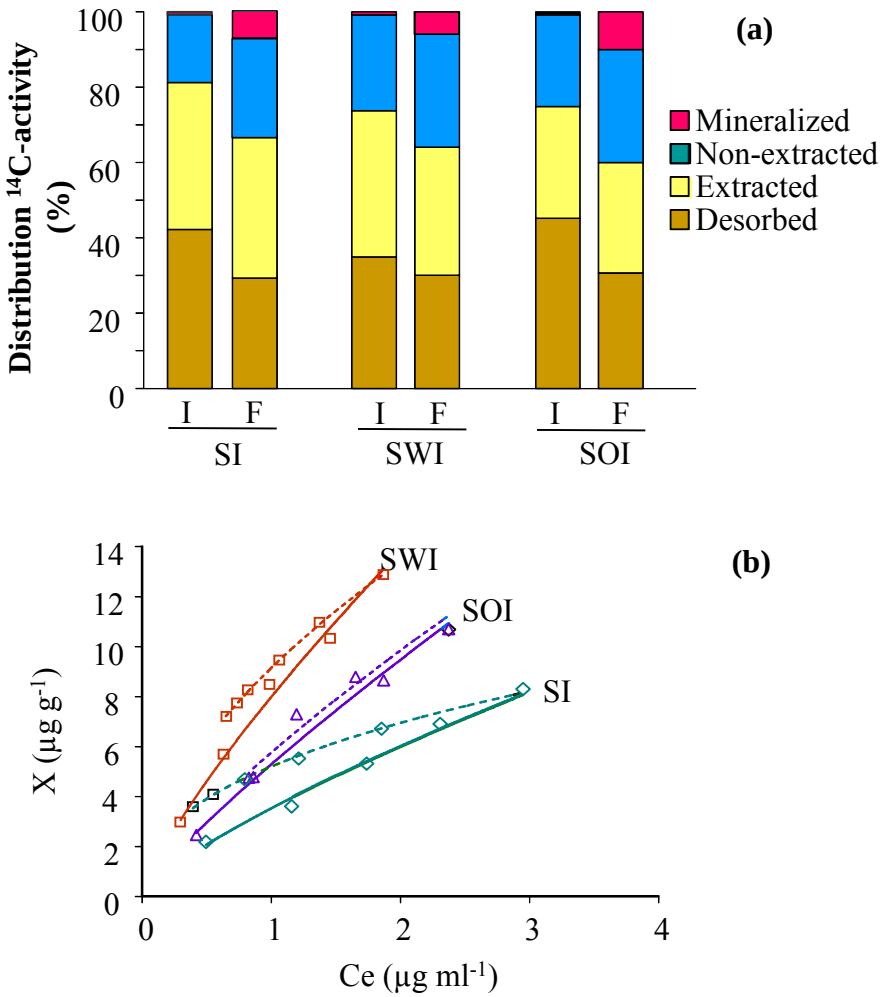

Figure 1. Distribution of ^{14}C-imidacloprid on the different soil compartments at initial and final time (a). Adsorption-desorption Freundlich isotherms for imidacloprid in the ageing unamended soil (SI) and 10%-amended soil (SWI and SOI). Experimental data are the symbols while lines represent the theoretical calculation (b).

The availability of ^{14}C-IMI was related to its distribution constant (K_d-total) in the different soil fractions (SF and SiF). At initial time, the K_d-total values (Table 3) were statistically greater in SWI in relation to SI and SOI (F=9.80 p<0.05). These results are in line with those obtained from sorption isotherms (Figure 1b, Table 3), where experimental data followed no-linear sorption with Freundlich exponents 1/n <1 (Table

3), increasing the sorption constant 2.26 and 1.5 times in SWI and SOI, respectively, with regard to SI. When K_d total and K_{ads} were normalized to the organic carbon content (K^d_{oc} and K_{oc}), the values in SWI and SOI were lower than in SI, indicating that imidacloprid was mainly present in the dissolved phase. The distribution constant in the SF fraction (K_dSF) was significantly highest in SWI (F=11.75 p<0.01). However, the highest distribution of ^{14}C-IMI in the SiF fraction (K_dSiF) was found in SI sample ($F=24.41$ p<0.01).

Table 3. Imidacloprid distribution constants (Kd) in the unamended (SI) and amended soil with W and O vermicompost (SWI and SOI), as well as in their corresponding fractions, sand size fraction (SF) and silt size fraction (SiF). Freundlich adsorption-desorption parameters (k_f and 1/n) and normalized values to the total organic carbon content (Koc). Data with the same lowercase are not significantly different.

Sorption constants	SI	SWI	SOI
K_d $^{\Psi}$ total ±SE	12.90 ± 0.49^{ab}	16.60 ±1.60^{a}	10.24 ± 0.57^{b}
K_d SF±SE	14.44 ± 0.50^{b}	26.17 ± 3.12^{a}	15.20 ± 0.99^{b}
K_d SiF±SE	12.08 ± 0.63^{a}	10.05 ± 0.60^{a}	6.99 ± 0.23^{b}
K^d_{oc} $^{\Psi}$	921.2 ± 34.3^{a}	453.7 ± 43.8^{b}	309.5 ± 17.1^{c}
K_f $^{\S}_{ads}$ ±SE	3.52 ± 0.18^{c}	7.98±0.23^{a}	5.28±0.06^{b}
$1/n_{ads}$±SE	0.76 ± 0.00^{b}	0.78±0.00^{b}	0.84±0.16^{a}
K_{oc} $^{\Psi}$	251.8 ± 7.8^{a}	218.1 ± 6.3^{b}	159.7 ± 1.8^{c}
K_f $^{\S}_{des}$ § ±SE	5.20 ± 0.09^{c}	9.14±0.06^{a}	5.68±0.05^{b}
$1/n_{des}$±SE	0.42 ± 0.00^{c}	0.55±0.00^{b}	0.80±0.02^{a}
H	0.55± 0.01^{c}	0.69 ± 0.00 ^{b}	0.95 ± 0.01^{a}

$^{\Psi}$ K_d units (mL g^{-1}), § K_f units ($\mu g^{1-1/n} g^{-1} mL^{-1}$), SE= Standard error.

The lowest K_dSF and K_dSiF values were obtained in the SOI soil. In relation to the desorption constant (K_{fdes}), it was 1.8 and 1.6 times higher in SWI than SI and SOI, respectively (Table 3). In agreement with experimental data (Figure1b), the hysteresis index (H) calculated for SI and SWI (Table 3) revealed values lower than 1, indicating the IMI irreversibility in the sorption process. However, for SOI the sorption and

desorption coefficients were closely similar (H ≈ 1) indicating a reversible sorption for IMI.

According to Papiernik et al. (2006), the sorption constants of imidacloprid K_{fads} in soils could be related to organic matter content. In this study, significant correlations were found with the soil organic matter (SOM), lignin and humic acid content ($r > 0.70$, $p < 0.05$). In the same way, the Freundlich desorption exponents ($1/n_{des}$) and hysteresis indexes (H) were correlated with the water soluble carbon (WSC) and the humic acid content of the soils ($r > 0.78$; $p < 0.05$).

3.2 Dissipation of Imidacloprid.

The experimental data of IMI-dissipation in microcosm 1 (M1) was fitted to SFO model (Table 4, Figure 2), because X^2 error level showed a good fit and t-test indicating reliability in the estimation of dissipation rate constant (k) (Table 3). The k parameter in all treatments in M1 was low, except for SOI_1, whose k value was 1.6 and 2.8 higher than in SI_1 and SWI_1, respectively. This increase was also reflected with a lower dissipation time (DT_{50}) than in SI_1 and SWI_1. According to Spearman correlation test, the k increment in SOI_1 was correlated with the increment of desorption coefficient ($1/n_{des}$), H index ($r = 0.93$ and 0.81, $p < 0.05$ respectively) and negative correlation with K_{oc} values ($r = -0.92$, $p < 0.05$). The IMI degraded concentration at the end of incubation time for SI_1 was 23% while for SOI_1 and SWI_1 were a 32% and 14% respectively.

In M2, where the soils were pre-exposed to the insecticide before the addition of the vermicomposts, the initial concentration of IMI was 5.84 $\mu g\ g^{-1} \pm 0.31$. A double first order in Parallel (DFOP) model was fitted to the degradation process in all treatments (Table 3, figure 2). The insecticide was instantaneously split between two independent compartments with a fast and slow k. The fast dissipation rate constant k_1 in SI_2 and SWI_2 was greater than in SOI_2. However, IMI fraction (g value in Table 3) in the compartment 1 and the k_2 constant were higher in SOI_2 than in SWI_2 and SI_2 which contributed to the lowest persistence of IMI. At the end of the incubation time, the amount of degraded IMI in SI_2 was 19 % of the initial amount, in SWI_2 was 10 % and in SOI_2 was 49 %. The sterile controls, in both experiments, showed degraded amounts much lower than 10%.

Table 4. Imidacloprid dissipation parameters for M1 and M2 microcosms obtained by fitting experimental data to a single first order kinetic model (SFO) and the double first order in parallel (DFOP), respectively. Different letters indicate significant differences (α=0.05).

Treatment	% Ci	g	k_1[a]	k_2[b]	DT_{50}	DT_{90}	R^2	X^{2c}	*t-test*
				***SFO* model**					
SI_1	100.11		0.29[b]	-	231.5	769	0.97	0.89	> 0.001
SWI_1	100.50	-	0.17[c]	-	401.1	1332	0.91	0.84	> 0.001
SOI_1	102.91	-	0.48[a]	-	154	512	0.99	0.74	> 0.001
				***DFOP* model**					
SI_2	101.92	0.15	7.78[a]	0.083[b]	585	< 1500	0.97	0.66	> 0.01
SWI_2	102.28	0.10	5.74[b]	0.057[b]	650	< 1500	0.91	0.69	> 0.05
SOI_2	102.33	0.21	3.17[c]	0.690[a]	67	265	0.99	1.11	> 0.01

[a] k_1 x $10^{-2}day^{-1}$, [b] k_2 x $10^{-2}day^{-1}$. [c] Error level of X^2

In M2, where the soils were pre-exposed to the insecticide before the addition of the vermicomposts, the initial concentration of IMI was 5.84 μg g^{-1} ± 0.31. A double first order in Parallel (DFOP) model was fitted to the degradation process in all treatments (Table 3, figure 2). The insecticide was instantaneously split between two independent compartments with a fast and slow *k*. The fast dissipation rate constant k_1 in SI_2 and SWI_2 was greater than in SOI_2. However, IMI fraction (g value in Table 3) in the compartment 1 and the k_2 constant were higher in SOI_2 than in SWI_2 and SI_2 which contributed to the lowest persistence of IMI. At the end of the incubation time, the amount of degraded IMI in SI_2 was 19 % of the initial amount, in SWI_2 was 10 % and in SOI_2 was 49 %. The sterile controls, in both experiments, showed degraded amounts much lower than 10%.

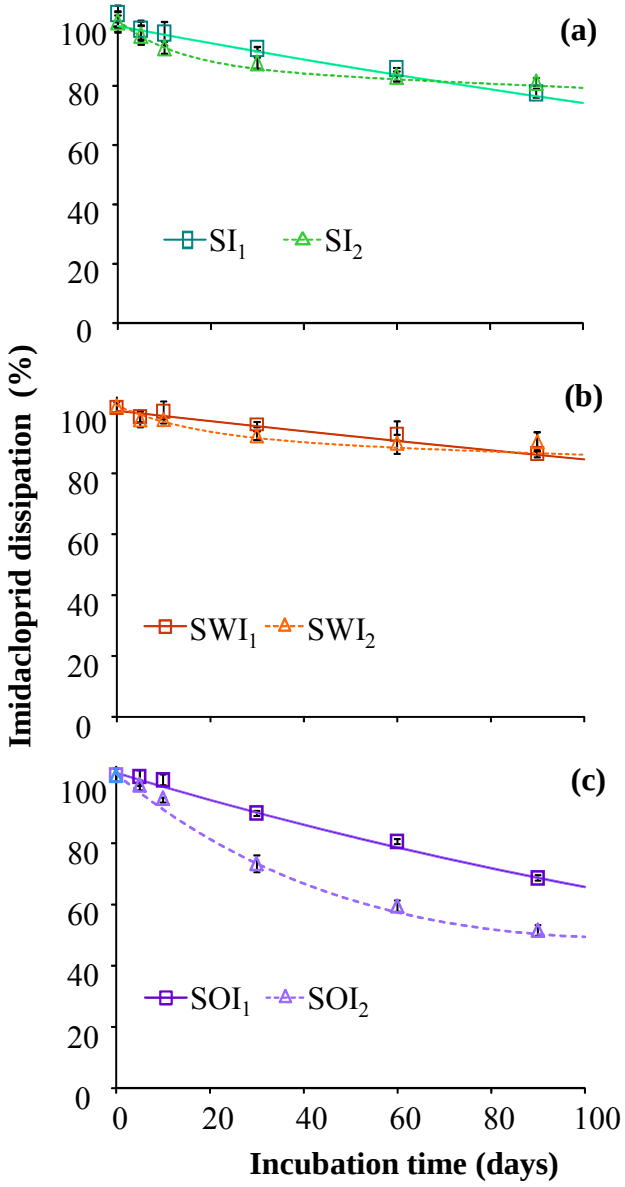

Figure 2. Comparison of the degradation kinetics of imidacloprid in unamended soils SI1 and SI2 (a), amended soils with W-vermicompost SWI1 and SWI2 (b) and amended soils with O-vermicompost SOI1 and SOI2 (c) from the microcosms 1 (no pre-treated with Imidacloprid) and microcosms 2 (pre-treated with Imidacloprid. The bars represent the standard deviations calculated from the triplicates. Symbols are experimental data and curves correspond to SFO and DFOP kinetic models.

3.3 Soil enzyme activity

The DHS and Urease activity (U) are an indirect method to measure the metabolic state of soil microbiota, which is affected by imidacloprid and can be attenuated by vermicompost amendment. The W or O vermicompost addition to the soil increased the DHS activity along the incubation period especially for SO (Figure 3a). Nevertheless, the addition of IMI to pre-amended soil from M1 (Figure 3b), exhibited only a significant increment in DHS activity until the first 30 days of incubation, especially in SOI_1 (F= 26.81; p = 0.01), but thereafter it was depressed in all treatments. Conversely, the addition of vermicompost to the pre-exposed soil to IMI in M2 (Figure 3c), increased the DHS activity at 10 days and then it was declined at 30 days. After that, the DHS incremented significantly 2 and 4 fold in SWI_2 and SOI_2 with respect to SI_2 (F = 110.2; p < 0.001).

The urease activity in the control soils, despite an initial fluctuation during the first 30 days, decreased abruptly in S and increased in SO. After 90 days, the U values inSW and SO were higher with respect to S (figure 3d). The addition of IMI to the pre-amended soil in M1 (figure 3e), increased slightly the urease activity in SI_1 and SOI_1 at 10 days, and then these were maintained along the incubation time. The M2 conditions declined the U activity at the initial time (figures 3e and f), then a significant increment was observed the first 30 days (F= 27.16 p < 0.05). Nevertheless, no-significant differences were found between amended soils SWI_2 and SOI_2 and unamended soil SI_2 at the end of the incubation time.

3.4 Microbial abundance and structure.

The total bacterial abundance (TBA) in the control samples increased in SO (Figure 3g). However, in S and SW no significant differences were observed along the incubation time, probably due to the high lignin composition in SW (mostly vine-shoot). In the microcosm-1, SWI_1 showed an increment the first 30 days, but unlike SI_1 and SOI_1, the TBA was then reduced to its initial values. SI_1 and SOI_1 exhibited a significant increment until 90 days (F=16.03, 55.47; p < 0.05), however, the TBA was similar to its control treatments.

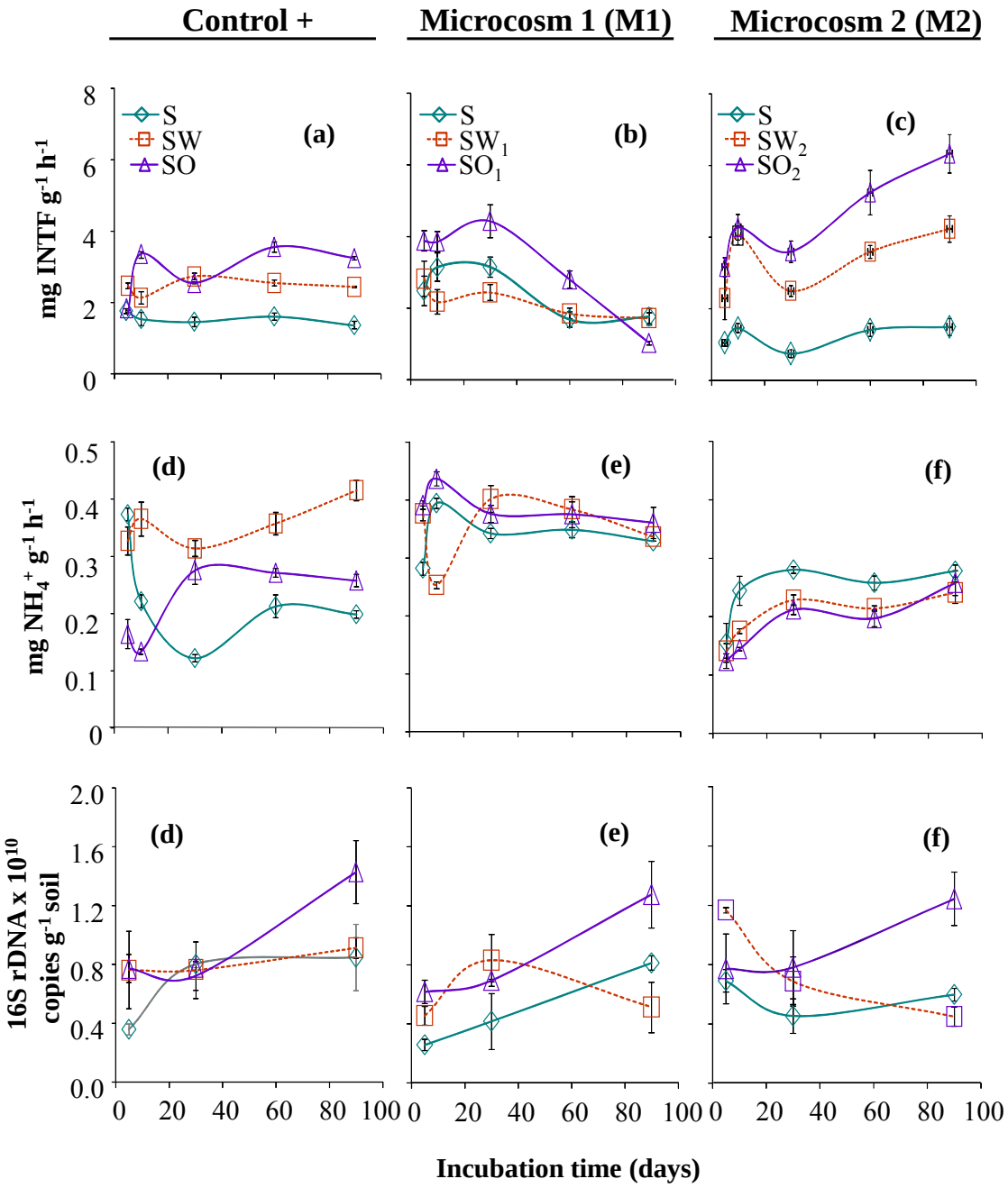

Figure 3. Enzyme activities, dehydrogenase (a, b, and c), urease (d, e, and f), and copy number of 16S rRNA gene (g, h and i), determined in unamended (S) and amended (SW and SO) soils as well as in corresponding soils treated with imidacloprid (unamended: SI1, SI2; amended with O-vermicompost, SOI1, SOI2) or with W-vermicompost (SWI1, SWD2) from M1and M2 experiments.

In the microcosm-2, SOI_2 showed a significant rise (F= 10.45 p = 0.01), however, this was similar to SO. In SI_2 as well as in SWI_2, the TBA decreased significantly ($p < 0.05$), indicating a low resilience of bacterial community to the insecticide concentration.

The observed changes in bacterial abundance could be related to changes in its bacterial structure. In an effort to test this hypothesis, bacterial structures were distinguished by comparison of their fingerprints. The electropherograms of A-RISA gel showed between 110 and 140 bands per profile and ranged from 200 bp to 930 bp (Figure A.1). The PCAs analysis showed that (Figure 4) in M1 as well as in M2, the vermicompost addition and the insecticide changed the soil bacterial structure. At 5 days in M1 (Figure 4a), PC1 and PC2 with 40.2% and 18.3% of the variance in the data, indicated an overlapping of statistical ellipses. It means that the soil bacterial community did not showed significant differences. Then, the shifts in the genetic structure were gradually changing with the incubation period. Thirty days after treatment, along of PC1 and PC2 with 24% and 20 % respectively, the bacterial community structure of soil (S) was discriminated from the other treatments, including SI_1 (Figure 4b). Ninety days after treatment, the factorial map showed on PC1 with 35% of the variance in the data that soil microbial structure (S) was distinguished from SI_1, as well as SO and SOI_1, except SW, whose bacterial structure was discriminated from SWI_1 (figure 4c).

The soil pre-exposed to IMI in M2 revealed differences at five days between SOI_2 and SO as was indicated on PC1with 42% of the data variance (Figure 4d). On the other hand, the PC2 with 13% allowed discriminating of S from SI_2 and SW from SWI_2. Thirty days after treatment, the microbial structure of soil was different by effect of vermicompost (SW and SO) as it was indicated by the factorial map for PC1 and PC2 with 65% and 9% of variance of data (figure 4e). Similarly, the treatments pre-exposed to IMI (SI_2, SWI_2 and SOI_2) showed a similar structure. This effect was maintained up to 90 days of incubation time. However, the changes in the bacterial community structure of SOI_2 only was correlated according to Mantel test with the second dissipation constant (k_2) (r = 0.76; p<0.01).

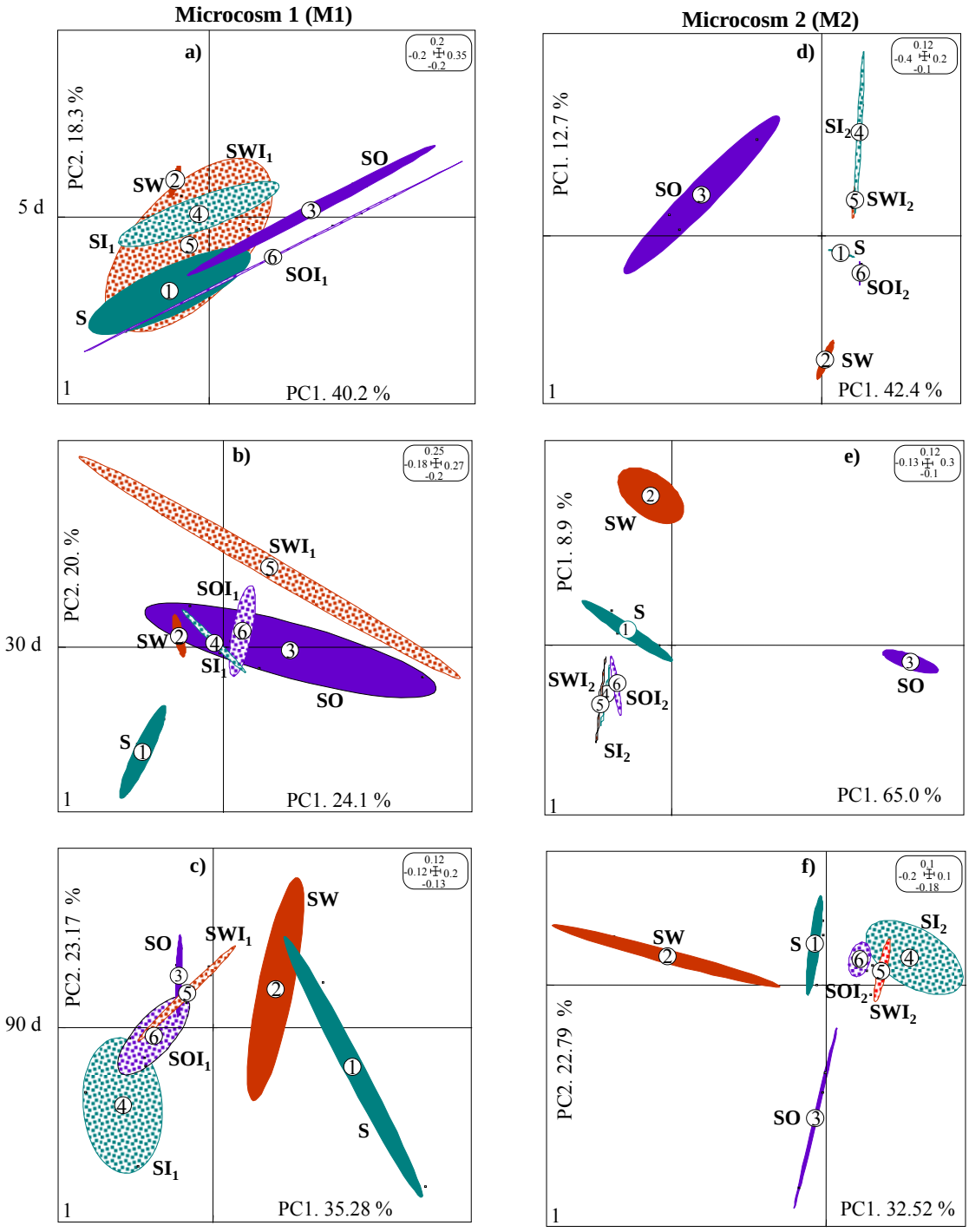

Figure 4. Principal component analysis at 5 days (a, d), 30 days (b, e), and 90 days (c, f), from M1 and M2 experiments, based on the analysis of the total bacterial communities, analysed by ARISA fingerprints of PCR products from DNA extracted directly unamended and amended soils control, S=1, SW=2 and SO=3, in aged amended soils not pre-treated or pre-treated with IMI (SI_1 and SI_2=4, SWI_1 and SWI_2=5, SOI_1 and SOI_2=6).

4. DISCUSSION

The differences in the ^{14}C-IMI distribution in the SF fraction of soil by effect of vermicompost amendment indicated that it could be related to the abundance of coarse size particles (lignin) and possible interaction of IMI with humic substances provided by both vermicompost (Table 1). Although according to studies by Romero et al. (2007) and Plaza et al. (2008) with similar vermicompost, indicated that both amendments showed a similar composition of carboxylic group contents, humification parameters and E4/E6 values, the differences in the insecticide distribution may be related to chemical composition of these humic substances. For example, W-vermicompost has a relative abundance of fulvic acids larger than humic acids and a slight acidity larger than in the O vermicompost. These differences could affect the IMI sorption. This is in agreement with the studies of Cox et al. (1998a) and Papiernik et al. (2006) where described that SOM could determine the IMI sorption in soils. It is known that diversity and structure of the organic matter can give a variation in the sorptivity of xenobiotic. This ability to sorb organic pesticides is related to the number of oxygen-containing functional groups. Lignin, despite of having a notable lack of carboxylic group (making it less hydrophilic), has the ability to sorb polar pesticides as IMI (Delle Site, 2001). This is due to its polycondensation degree, producing a major surface area (Romero et al., 2006). On the other hand, the variety of humic fractions provided by the vermicompost, can have different K_{oc} values (Ahangar et al., 2008). Flores-Cespedes et al. (2002) found that in calcareous soil the addition of dissolved organic matter (DOM) can reduce the sorption of IMI by the soil. Similarly, Barriuso et al. (2011) reported that IMI sorption can be reduced due to its competition with the DOM (where humic and fulvic acids are involved) by sorption sites on the soil surface, allowing a moderate IMI mobilization. Thus, the organic carbon increment in the amended soils with vermicompost can contribute to the retention and accumulation of insecticide for longer period (Papiernik et al., 2006). This is supported by a decrease of the extracted portion of 14C-IMI, probably due to the ageing process (Gevao et al., 2005).

The low imidacloprid dissipation for the treatments in M1, could be conditioned to the pesticide sorption-desorption, which in turn is conditioned by physicochemical composition of the organic matter supplied by each vermicompost. In the case of SWI, it

was shown a major distribution constant and adsorption of IMI. However, for SOI, these parameters were low but with a hysteresis index near to 1, indicating a rise of IMI in the desorbed compartment, allowing a higher availability and dissipation in soil. The biphasic decrease observed in the M2 treatments was described by Cox et al. (1998b) and Capri et al. (2001) in sorption studies and IMI dissipation in soils. This dissipation model assumes that exist two sites of adsorption: fast and slow equilibrating sorption sites, located on the external and internal surfaces of soil matrix, increasing the sorption and residence time in soil. These effects may be mainly found in soils with high organic carbon and clay content, such as the amended soil in this study. However, the increment of IMI dissipation in SOI2 could indicate the resilience and resistance capability of soil microorganisms.

The declined of the DHS and U activities in soil revealed that imidacloprid affect the soil microbiota as was observed recently by Wang et al. (2014). Although it is known that some microorganisms isolated from agricultural soil exposed to imidacloprid as *Pseudomonas sp*, can use it as carbon sources (Pandey et al., 2009) and others as *Klebsiella pneumonia* (Phugare et al., 2013) and *Ochrobactrum sp* (Hu et al., 2013) can biodegrade IMI to minor metabolites under oxidative conditions with a degradation to 6-chloronicotinoic acid, that is dechlorinated to 6- hydroxynicotinic acid (Shettigar et al., 2012) and then cleaved to urea derivatives. When imidacloprid is added to soil, decreases the nitrification rate while stimulate the ammonification process as described by Cycoń and Piotrowska-Seget (2015)**.** This might increase the urea level in the soil which in part explains the urease activity fluctuations observed (figure 3d,e,f). The increment of DHS activity in SOI_2 might be associated to the resilience capability and adaptation of the microbial community by effect of repeated pesticide applications (Monard et al., 2010) and bioestimulation of the organic amendment. This effect was small in SI_2 and SWI_2.

The observed changes in the TBA showed that imidacloprid have a negative effect on bacterial community when the soil was amended with W-vermicompost. Conversely, O-vermicompost in SOI1 and SOI2 under M1 and M2 conditions maintained a similar TBA to unfortified treatment SO. This indicates that O-vermicompost mitigates the negative effect on bacterial community of soil. These results were confirmed with changes in the bacterial community structure. The vermicompost amendments affect the

bacterial structure. However, the insecticide counteracts this effect and exerts a selective pressure on bacterial community of soil, maintaining the bacterial population with the ability to resist the contaminant. Therefore, this suggests that the nature of organic amendment can determine the impact on the soil genetic structure as was exposed by Lejon et al. (2007). The fingerprinting techniques favors the study of dominant groups in relation to new specific phylotypes involved in the pesticide degradation (Martin-Laurent et al., 2004), it could be hypothesized that under IMI selection pressure, O-vermicompost contributes to the bacterial survival of this dominant groups and increases the resilience and adaptation of new microbial communities with the capability to tolerate and degrade this insecticide.

5. CONCLUSIONS

This is the first study that evaluates simultaneously the persistence of imidacloprid in soil under different conditions, using vermicompost from agro-industrial wastes as mitigation technique. Our results indicated that the distribution, sorption and dissipation of IMI in soil can be affected by the vermicompost composition, decreasing its availability and minimizing the potential environmental risk. Furthermore it is clear that IMI altered the soil structure and bacterial community activity resulting in a different dissipation kinetic. The use of 10% of W and O vermicompost as amendment changes the soil bacterial community. However, when unamended and amended soils are pre-exposed to imidacloprid, the bacterial structure changes are counterbalanced. In relation to imidacloprid dissipation, the pre-treated soil with imidacloprid and amended with O-vermicompost had the higher dissipation rate and was correlated with changes in the bacterial structure, indicating that this vermicompost better support the development of microbial community with the ability to degrade imidacloprid. All these findings indicate that O-vermicompost can be used in management strategies for the depuration of soils contaminated with imidacloprid.

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APPENDIX A. SUPPLEMENTARY DATA

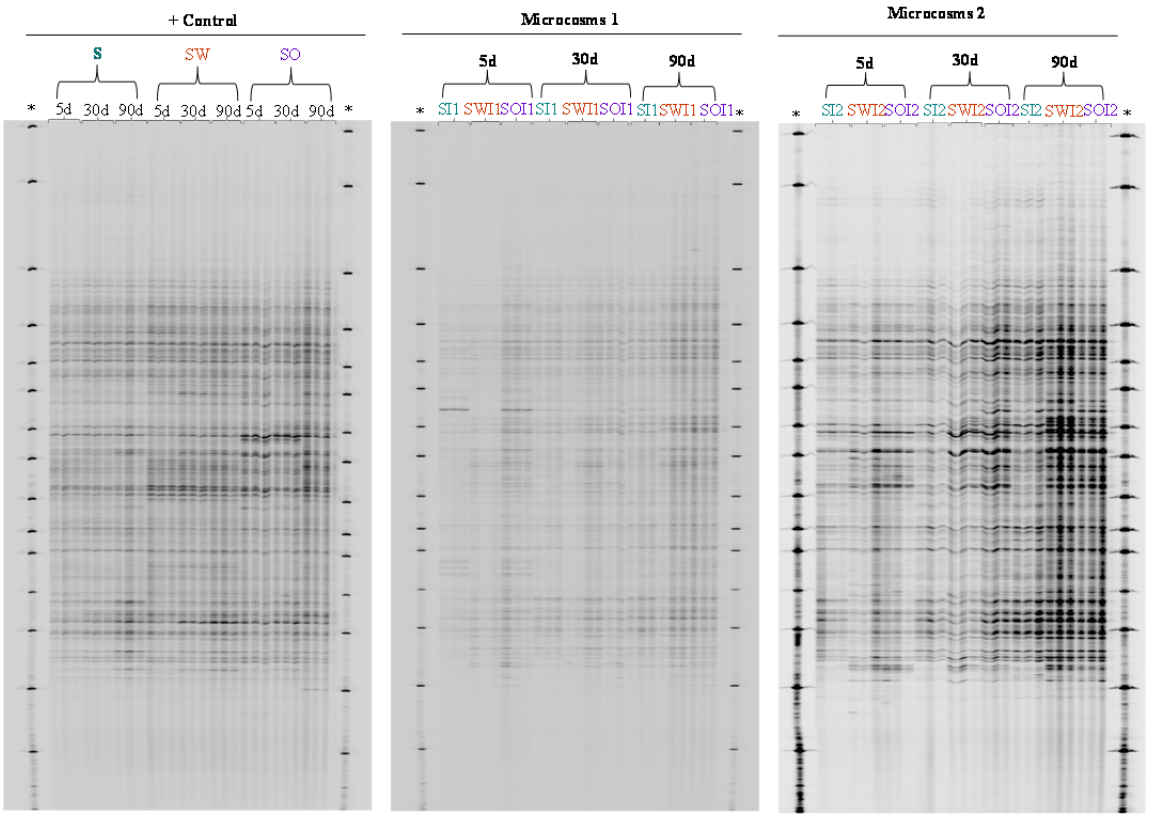

Figure A. 1. ARISA fingerprint gel of PCR products from DNA extracted at the initial, intermediate (30 d), and final time (90 d) of the incubation period, in the control soils S, SW, and SO and in the microcosm 1 (soils pre-amended before the IMI application) and microcosms 2 soils pre-exposed to IMI before a new application of IMI).

DISCUSIÓN GENERAL

Como se ha venido mencionando en la presente Tesis Doctoral, la generación y manejo de los residuos agroindustriales y el desarrollo de nuevas estrategias de mitigación de plaguicidas en suelos son de vital importancia en la protección del medioambiente. Tanto es así que se han convertido en dos de las siete principales estrategias adoptadas por el Consejo y el Parlamento Europeo, los cuales fueron definidos en el VI Programa de Medio Ambiente (Comisión Europea, 2006).

La gestión de los residuos agroindustriales es quizás una de las más relevantes debido al valor agregado, social, económico y medioambiental, que puede llegar a tener después de ser transformados y utilizados dentro de la misma cadena industrial o como subproducto. Dentro de los residuos disponibles, los residuos vitivinícolas (sarmientos y lodos de vinaza) y oleícolas (alperujo) son los de mayor relevancia para su aplicación en suelos mediterráneos, dado que es en estas áreas climáticas donde se cultiva extensivamente el olivo y la vid y donde se generan frecuentemente este tipo de residuos derivados de su agroindustria. Por lo tanto, muchos estudios se han enfocado al desarrollo de estrategias, que permitan transformar estos residuos mediante el compostaje (Alburquerque et al., 2009; Bustamante et al., 2009) y el vermicompostaje (Benítez et al., 2002; Romero et al., 2007) en productos estables para su directa aplicación al campo. La mayoría de los trabajos de vermicompostaje publicados se han enfocado principalmente en el control de los cambios de parámetros físico-químicos y bioquímicos (actividad enzimática) que rigen este proceso. Sin embargo, existe una información limitada sobre los cambios microbiológicos y las posibles interacciones con los demás parámetros. Por esta razón, el primer capítulo de la presente tesis se orientó en el estudio, mediante procesos de vermicompostaje, de la dinámica microbiana y su relación con los parámetros físico-químicos y bioquímicos, con la finalidad de tener un conocimiento más amplio del proceso de vermicompostaje y del producto final.

Durante el proceso de vermicompostaje de sarmientos de vid con lodos de vinaza (W), se observó una rápida colonización de las lombrices (*E. fetida*), probablemente debido a que los sarmientos fueron cortados en pequeños fragmentos (< 5 cm) permitiendo una rápida colonización de hongos filamentosos en la etapa de pre-acondicionamiento para que la lombriz pudiera entrar en el substrato (Capítulo 1, Figura 1). Estos resultados fueron relacionados con un incremento de la población fúngica que fue cuantificada por PCR cuantitativa durante esta etapa. Según Wal et al. (2007), el

hecho de tener fragmentos pequeños de madera, en este caso de sarmientos, hace que se genere una área superficial más accesible para los microorganismos reduciendo el tiempo de pre-descomposición. Por otro lado, la alta abundancia de hongos filamentosos permite la translocación de nutrientes y estimula la colonización de la lombriz al constituirse los hongos en su fuente nutritiva (Chigineva et al., 2011).

Un proceso distinto ocurrió en el vermicompostaje de alperujo mezclado con estiércol caprino (O) (Capítulo 1, Figura 1). En este caso la colonización de la lombriz empezó en la semana 3 y la mayor biomasa se obtuvo después de 15 semanas. La lenta colonización de las lombrices en este substrato puede deberse al alto contenido de grasas y compuestos aceitosos del alperujo donde la lombriz no puede desarrollarse con facilidad. Por tanto, es de esperar que cuando se utilizan *alperujos* procedentes de una extracción más exhaustiva y donde además se separa el hueso como otro subproducto, el desarrollo del proceso de vermicompostaje sea más eficaz.

En relación a las propiedades físico-químicas, los compuestos orgánicos como lignina, celulosa, hemicelulosa, así como también el carbono hidrosoluble y el carbono orgánico total, disminuyeron en ambos procesos (W y O). Sin embargo, la reducción del carbono orgánico fue mayor en O, probablemente debido al alto contenido de azúcares (Alburquerque et al., 2009) con un mayor contenido de fracciones húmicas a causa de una mayor mineralización.

Respecto a los parámetros bioquímicos, las actividades deshidrogenasa, O-diphenol oxidasa (o-DPO), ureasa, β-glucosidasa y fosfatasa ácida fueron mayores durante la primera semana del proceso de vermicompostaje de W (Capítulo 1, Figura 2), ya que éste tenía un alto contenido de carbono y nitrógeno suministrado tanto por el sarmiento como por los lodos de vinaza, lo que podría inducir la producción de urea provocando una alta actividad ureasa que luego es inactivada por exceso del amonio producido, decreciendo la actividad de esta enzima (Castaldi et al., 2008). Un resultado bastante significativo con respecto a la actividad enzimática en ambos procesos, fue la correlación entre el desarrollo de la lombriz con la actividad β-glucosidasa y o-DPO registrada (Capítulo1, Tabla 3). Esta relación puede ser debido a la alta actividad de β-glucosidasa encontrada en el intestino de la lombriz y por ende en los residuos excretados (denominado "cast"). Estos resultados indican que esta actividad dependerá del estado de desarrollo de la lombriz, coincidiendo una mayor actividad con la mayor

cantidad de biomasa de lombriz en la litera de vermicompostaje. Resultados similares fueron observados en procesos de vermicompostaje de purines de cerdo (Aira et al., 2007) y residuos de tomate (Fernández-Gómez et al., 2010). Por tanto, la enzima β-glucosidasa podría ser utilizada como un buen biomarcador para evaluar el estado del proceso de vermicompostaje. En el caso de la actividad o-DPO, ésta puede estar relacionada con la degradación de compuestos fenólicos por los microorganismos que están en el tracto intestinal de la lombriz (Sabrina et al., 2012) o también por la presencia de estas enzimas en el tejido de la lombriz donde están involucradas en la formación de melanina para defensa de patógenos (Procházková et al., 2006).

En relación a la estructura microbiana, tanto de bacterias como de hongos, ésta fue cambiando durante el proceso de vermicompostaje y de maduración como fue evidente en el análisis de la migración de fragmentos de ADN en un gel de electroforesis mediante DGGE (Capitulo 1, Figura 4). Estos cambios se correlacionaron con el desarrollo de la lombriz en el proceso de vermicompostaje de ambos substratos (O y W). Esta relación indica que la ingesta por parte de la lombriz modifica la estructura, como fue observado por la reducción de la abundancia bacteriana y fúngica. Estas diferencias fueron también observadas en la disminución de las clases taxonómicas *Alpha-* y *Gamma-proteobacteria* (Capitulo 1, Figura 3) las cuales fueron dominantes en el inicio del proceso de vermicompostaje. En el caso del proceso de vermicompostaje de W, estos dos grupos fueron correlacionados con la actividad ureasa, la cual fue alta en la etapa de pre-acondicionamiento. En este caso, el aumento de la abundancia de hongos y de estos dos grupos bacterianos en esta primera etapa, podrían ser los responsables de la degradación de los residuos ligninolíticos como los sarmientos (DeAngelis et al., 2011). Los grupos taxonómicos *Beta-proteobacteria* y *Actinobacteria* fueron aumentando su abundancia a través del tiempo en el vermicompostaje (Capítulo 1, Figura 3), siendo la clase *Actinobacteria* el grupo más abundante al final del periodo de este proceso. Este grupo bacteriano, tuvo una correlación positiva con la actividad o-DPO, enzima que se ha descrito en varias especies de *Actinobacterias* y que suelen encontrarse en el tracto intestinal y en desechos producidos por la lombriz, indicando una alta habilidad de colonización debido a su alto potencial enzimático y producción de antibióticos, controlando la población de hongos (Yasir et al., 2009) y la de patógenos (Monroy et al.,

2009). Por lo tanto, la alta abundancia de este grupo bacteriano podría ser un indicativo de la seguridad y calidad del producto vermicompostado.

Esta nueva información suministrada con respecto a la dinámica microbiana y sus relaciones con el desarrollo de la lombriz, las propiedades físico-químicas y bioquímicas en el proceso de vermicompostaje de residuos lignocelulósicos, nos condujo a explorar la alta capacidad de transformación de la microbiota que habita en estos procesos frente a otro problema medioambiental como es la persistencia y toxicidad de plaguicidas o de sus metabolitos que pueden llegar a ser más tóxicos que el compuesto parental.

En este sentido, los capítulos 2, 3 y 4, se focalizaron en evaluar de manera multidisciplinar el efecto tanto físico-químico como microbiológico que pueden tener los vermicomposts en la dinámica de los plaguicidas diuron e imidacloprid y metabolitos tóxicos como 3,4-dichloroanilina.

El Diuron es un herbicida fenilureico considerado toxicológicamente como clase III según la EPA, debido a la presencia de residuos de diuron en aguas subterráneas o superficiales. Dentro de estos residuos, el metabolito 3,4-dicloroanilina (3,4-DCA) es considerado más toxico que diuron debido a que es más apolar y se adsorbe fuertemente, incrementando así su permanencia en suelos (Giacomassi y Cochet, 2004). Por esta razón, en el capítulo 2 de la presente Tesis Doctoral se evaluó el potencial biodegradador de hongos que proliferaban de forma muy abundante durante la etapa de preacondicionamiento del vermicompost de residuos de sarmiento y lodos de vinaza (W) frente a contaminantes orgánicos.

En este estudio, se aislaron diferentes hongos, pero solo tres (F1, F2 y F3) lograron crecer en medio de cultivo con 3,4-DCA como fuente de nitrógeno. Dos de ellos pertenecen al género *Fusarium* presentando una homología del 99% con la especie *Fusarium oxysporum*. El tercer hongo fue identificado como *Aspergillus niger* (Capítulo 2, Figura 1) La mayor degradación de 3,4-DCA se registró entre los días 7-15 de incubación. La detección de varios metabolitos de degradación en las 2 cepas de *F. oxisporum* (F1 y F2, Capítulo 2, Tabla 3 y Figura 5) indican el potencial enzimático de éstas cepas. Dentro de estos metabolitos, la detección de diclorobenceno (DCB) es quizás una de las más relevantes ya que 3,4-DCA es transformado a dicloronitrobenceno (DCNB) que luego pasa a Dicloronitrosobenceno (DCNOB) mediante reacciones oxidativas, probablemente catalizadas por cloroperoxidasas con estructura similar a las

que conforman el citocromo P450. El grupo NO de DCNOB es reducido a N_2O más DCB, probablemente por la óxido-reductasa citocromo P450nor, enzima muy peculiar descrita en cepas de *Fusarium* en el proceso llamado co-denitrificación. Otros metabolitos identificados como tetracloroazobenceno (TCAZB), tetracloroazoxibenceno (TCAXB) y dicloroquinolinas (DCQs), pueden estar asociados a un mecanismo de detoxificación por parte de las cepas de *Fusarium* mediante la formación de oligomeros a partir de estos compuestos (Doerge et al., 1991).

En el cultivo de *A. niger* la presencia de dicloroacetanilida (DCAN) podría ser debido a una N-acetilación, mediante una N-acetiltransferasa (NAT). En este tipo de cepa, se ha descrito la presencia de 5 supuestos genes que codifican para estas enzimas, las cuales están involucradas en la tolerancia y detoxificación de xenobióticos (Tixier et al., 2002). Esta estrategia por parte de *A. niger* puede estar asociado con una mayor producción de biomasa como fue observado después de 15 días de incubación (Capítulo 2, Figura 2, Tabla 1). Estos resultados demuestran el potencial biocatalítico que tienen los microorganismos encontrados en el proceso de vermicompostaje W frente a compuestos xenobióticos, como es el caso de 3,4-DCA. Por lo tanto, este potencial catabólico es un valor agregado más que tienen los vermicomposts de residuos agroindustriales para ser usados como alternativa en la mitigación de residuos persistentes de plaguicidas, ya que es de bajo coste y respetuoso con el medio ambiente.

El uso de enmiendas orgánicas como el vermicompost en el control de plaguicidas en suelos, especialmente en los procesos de sorción de plaguicidas como diuron e imidacloprid, ha sido evaluado por Fernández-Bayo et al., (2007, 2008, 2009). Sin embargo, el potencial microbiano de estas enmiendas en la degradación de estos plaguicidas no ha sido evaluado, como tampoco las interacciones que pueden tener con otros factores como son los físico-químicos y bioquímicos presentes en el suelo bajo diferentes condiciones de aplicación de los plaguicidas.

Como ha sido observado en otros estudios, la adición de enmiendas orgánicas afecta los procesos de sorción de diuron, especialmente en suelos con bajo contenido en carbono. En el caso estudiado en el capítulo 3, el incremento de la materia orgánica del suelo (SOM) así como de sus componentes (lignina y sustancias húmicas como los ácidos húmicos (HA) y fúlvicos (FA)) en suelos enmendados ya sea con vermicompost W u O (Capítulo 3, Tabla 1), aumentó la constante de adsorción de diuron, la cual fue

significativamente mayor en el suelo enmendado con W (SW) (Capítulo 3, Tabla 2). Este incremento de la adsorción tanto en SW como SO está correlacionado con la estructura y composición de la materia orgánica, aunque la concentración de SOM, HA, FA y lignina hayan sido prácticamente similares. En el caso de SW, la concentración de lignina se correlacionó con el alto coeficiente de adsorción de diuron (K_{fads}) presente en SW. Por el contrario, la K_{fads} en SO fue correlacionada con el contenido de ácidos húmicos. Esto indica que aunque la concentración de los componentes de la materia orgánica sean similares, la composición de éstos pueden tener diferente capacidad de adsorción como fue descrito por Ahangar et al., 2008.

Cuando se evaluó la disipación de diuron bajo condiciones agronómicas distintas, en el suelo previamente enmendado antes de la aplicación de diuron correspondiente al primer experimento (E1), se observó una cinética de disipación bifásica, con una lenta desaparición del plaguicida y una mayor vida media (DT_{50}) (Capítulo 3, Tabla 3). Sin embargo, en el segundo ensayo (E2), cuando el suelo fue previamente fortificado con diuron e incubado por 3 meses, para luego ser contaminado nuevamente con una dosis alta de diuron y enmendado posteriormente con vermicompost (W u O), la disipación de diuron fue más rápida tanto en los suelos enmendados como en los sin enmendar (Capítulo 3, Tabla 3). Estos resultados reflejan la diferente capacidad de resistencia y resiliencia que tiene la microbiota del suelo dependiendo de la práctica agrícola que se ejerza. En el caso de E1, los microorganismos tienen una baja resiliencia frente a la presencia del plaguicida, sin embargo, cuando se repite la aplicación con una dosis más alta, la capacidad de resistencia y resiliencia aumenta, lo que indica la capacidad de adaptación de la microbiota del suelo. Esta capacidad de adaptación está estrechamente relacionada con el potencial genético y por ende enzimático que tienen las poblaciones microbianas.

En este sentido la evaluación de la abundancia del gen *puh*B, el cual expresa la enzima hidrolasa fenilurea, involucrada en la degradación de diuron por bacterias, mostró una mayor abundancia en E2, principalmente en los suelos enmendados con vermicompost y significativamente en el suelo enmendado con vermicompost de alperujo (SOD2). Este resultado nos lleva a hipotetizar que el vermicompost, como enmienda orgánica en el suelo, contribuye a la supervivencia y adaptación de las nuevas comunidades microbianas con capacidad de degradar diuron. Estos resultados fueron

reflejados en los cambios de la estructura bacteriana analizados por ARISA (Capítulo 3, Figura 3). En este caso, la enmienda orgánica cambió la estructura bacteriana del suelo, sin embargo, con la aplicación de diuron en el caso del suelo pre-enmendado (E1) estos cambios en la estructura fueron contrarrestados por la presencia del herbicida, no encontrándose diferencias entre los tratamientos enmendados (SWD1 y SOD1) y sin enmendar (SD1). En el caso de E2, el efecto del herbicida fue contrarrestado en los suelos enmendados, sin embargo, en el suelo sin enmendar (SD2) la estructura bacteriana fue totalmente diferente. La abundancia de *puh*B, en los tratamientos de E2 a los 90 días de incubación, correlacionó con los cambios observados en la estructura bacteriana y la rápida degradación de diuron, corroborando de este modo la hipótesis del efecto del vermicompost en la supervivencia y adaptación de la microbiota del suelo al herbicida diuron.

Con respecto al insecticida Imidacloprid (Capítulo 4), el efecto del vermicompost sobre la dinámica de este insecticida en suelos ha sido poco estudiada. El Imidacloprid (IMI) es un insecticida neonicotinoide que ha sido ampliamente utilizado para el control de plagas de insectos (Goulson, 2013). Sin embargo, su uso ha sido cuestionado por su efecto negativo en las abejas polinizadoras. Esto condujo a que la Comisión Europea regulara su aplicación. La bibliografía consultada sobre la dinámica de IMI en suelos, describe estudios mayormente enfocados a conocer su retención en suelos y su relación con el contenido de carbono orgánico (CO). Asimismo, se ha observado que otros factores, como el contenido de arcilla, también contribuyen a la retención de este insecticida en suelos cuando el contenido de CO es bajo (Cox et al., 1998; Fernández-Bayo et al, 2008). No obstante, la relación de los factores físico-químicos y microbiológicos que condicionan la disipación de IMI en suelos, no ha sido descrita en profundidad. Por lo tanto, este estudio fue otro objetivo a abordar en esta tesis y está desarrollado en el capítulo 4 de esta memoria.

Los resultados del fraccionamiento físico para conocer la distribución de ^{14}C-IMI en las fracciones de arena y de limo (SF y SiF, respectivamente) de los suelos enmendados con vermicompost, indicaron que la composición del carbono orgánico añadido por efecto de la enmienda, incrementa la distribución de ^{14}C-IMI en estas fracciones (Capítulo 4, Tabla 2). Siendo significativamente mayor en SWI para la fracción SF. Este aumento puede estar relacionado con la capacidad de adsorción, la cual

está asociada a la composición y estructura de la materia orgánica. Se sabe que las sustancias húmicas pueden tener diferente coeficiente de adsorción, lo que explica la mayor adsorción por SWI.

La porción adsorbida de IMI en el suelo puede incrementar con en el tiempo, esto fue corroborado por el incremento del Factor de Enriquecimiento (EF) en las fracciones del suelo (Capítulo 4, Tabla 2). En este caso, el valor de EF en la fracción SF fue mayor en SWI. Por el contrario, en la fracción SiF, EF fue mayor para SOI. Estos resultados demuestran que el vermicompost incrementa la retención y acumulación de imidacloprid por largos periodos, lo que se conoce como proceso de envejecimiento. Esto explica la disminución de ^{14}C-IMI en la porción extractable del suelo y el aumento en la no-extractable, como también en la fracción mineralizada, la cual fue mayor en SOI (10%).

Respecto a los ensayos de incubación realizados para conocer la disipación del imidacloprid bajo diferentes condiciones agrícolas (M1 y M2), la lenta disipación de IMI en los suelos previamente enmendados (M1) como fue en el caso de SWI-1 con una DT50 de 401 días, puede estar asociada a la alta adsorción y mayor distribución de IMI en las fracciones del suelo tal como se observó en SWI (Capítulo 4, Tabla 4). Por el contrario, el leve incremento de la disipación de IMI en SOI-1 (DT50= 154 d) puede estar relacionado con el hecho que los coeficientes de adsorción y desorción sean similares con un índice de histéresis cercano a 1, lo que podría significar una mayor disponibilidad de IMI para ser degradado.

En el caso de los suelos procedentes del tratamiento M2, previamente fortificados con IMI, y nuevamente contaminados y enmendados con vermicompost de W y O, la disipación de IMI en el suelo enmendado SWI-2 y sin enmendar SI-2 fue similar y presentaron valores de DT50 mayores a los registrados en M1 (Capítulo 4, Tabla 4). Sin embargo, nuevamente el suelo enmendado con vermicompost de *alperujo* SOI-2 mostró una disipación más rápida (DT50= 67 días), lo que probablemente podría indicar un proceso de adaptación de la microbiota del suelo al compuesto favorecido por la enmienda. Esto fue corroborado por un incremento de la actividad deshidrogenasa y la abundancia bacteriana (Capítulo 4, Figura 3) y por los cambios observados en la estructura bacteriana (Capítulo 4, Figura 4). En este caso y como se observó con diuron, los cambios en la estructura por efecto de la enmienda fueron contrarrestados en ambos microcosmos (M1 y M2) por la presencia de IMI.

En resumen, el uso de vermicompost como enmienda orgánica puede ser utilizado como alternativa para la mitigación de diuron e imidacloprid. El uso de vermicompost de residuos de sarmiento (W) en el caso de diuron e imidacloprid, aumenta significativamente la adsorción de estos plaguicidas, por otro lado, el uso de vermicompost de alperujo (O), previo o posteriormente a la aplicación de estos plaguicidas en suelo, no solamente incrementa la adsorción sino que funciona como soporte de la microbiota con capacidad de degradar tanto diuron como imidacloprid. Estos resultados nos permiten concluir que los vermicomposts de residuos agroindustriales, son materias activas que por sus características cuando se aplican como enmiendas orgánicas al suelo favorecen la retención y degradación de contaminantes orgánicos, minimizan su impacto sobre la población microbiana del suelo y fomentan la estabilización del medio edáfico. Asimismo, es importante investigar más sobre los mecanismos por los cuales los vermicomposts promueven la degradación de plaguicidas con el fin de definir nuevas estrategias de manejo en la mitigación del riesgo de los plaguicidas en el medioambiente.

CONCLUSIONES

1. En el proceso de vermicompostaje de sarmientos, una reducción en el tamaño de los fragmentos de este residuo orgánico (<5 cm) promovió una rápida colonización de los hongos durante la etapa de pre-acondicionamiento, acelerando la entrada y desarrollo de la lombriz en el sustrato y reduciendo el tiempo de vermicompostaje.

2. La evolución de la actividad β-glucosidasa en los dos procesos de vermicompostaje, del alperujo y del sarmiento, fueron correlacionados con el desarrollo de la lombriz, las bacterias totales y los cambios en la estructura microbiana. Por lo tanto, se puede asumir la utilización de esta enzima como bioindicador del desarrollo del proceso de vermicompostaje.

3. Los cambios en la estructura de la comunidad bacteriana y fúngica durante el proceso de vermicompostaje y maduración podrían estar ligados a biotransformación realizada por la lombriz durante la ingesta y deposición del sustrato orgánico durante su desarrollo. En ambos procesos de vermicompostaje, los grupos taxonómicos Alfa- y Gamma-proteobacteria disminuyeron a medida que Beta-proteobacteria y Actinobacteria aumentaron.

4. Los hongos filamentosos *Fusarium oxysporum* y *Aspergillus niger* aislados durante el periodo de pre-acondicionamiento del vermicompostaje de sarmiento, fueron capaces de crecer con 3,4-dichloroanilina como fuente de nitrógeno.

5. Las estrategias metabólicas utilizadas por las cepas de *Fusarium* y *A. niger* fueron diferentes generando varios metabolitos que fueron identificados y cuantificados por SPME-GC/MS.

6. La obtención de nitrógeno a partir de 3,4-DCA por las cepas de *Fusarium* fue llevado a cabo a través de reacciones oxidativas hasta dicloronitrobenceno, para posteriormente ser reducido a diclorobenceno y óxido nitroso. Por otro lado, *A. niger* a través de transferasas realiza una N-acetilación de la 3,4-dicloroanilina transformándola en dicloroacetanilida, metabolito que es menos toxico para el desarrollo del hongo.

7. La aplicación de vermicompost al suelo, antes o después del tratamiento con diuron o imidacloprid, modifica tanto la abundancia como la estructura de la comunidad bacteriana.

8. La capacidad de adsorción de diuron y de imidacloprid por el suelo fue significativamente aumentada por la aplicación del vermicompost de sarmiento. Sin embargo, la mayor disipación de estos plaguicidas tuvo lugar cuando el suelo fue enmendado con vermicompost de alperujo.

9. La abundancia del gen *puh*B que codifica la enzima hidrolasa fenilurea, responsable de la degradación de diuron, fue comparativamente mayor en el suelo pretratado con diuron. Sin embargo, la mayor abundancia del gen *puh*B fue registrada en el suelo enmendado con vermicompost de alperujo. Estos resultados nos permiten asumir que el vermicompost de alperujo soporta eficientemente la microbiota degradadora de este herbicida.

10. El estudio de fraccionamiento físico y distribución de imidacloprid marcado en diferentes fracciones del suelo, indicaron un aumento significativo del insecticida en las fracciones del suelo enmendado con los vermicompots.

11. El factor de enriquecimiento de imidacloprid en las fracciones del suelo indicó que la adsorción aumenta con el tiempo, lo que demuestra que los vermicompost evaluados incrementa la retención del insecticida. La mayor tasa de mineralización registrada fue para el suelo enmendado con vermicompost de alperujo, y que concuerda con un mayor coeficiente de disipación de este insecticida, un aumento de la abundancia bacteriana y de la actividad deshidrogenasa y ureasa.

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ANEXO

ARTÍCULOS CIENTÍFICOS PUBLICADOS E INCLUIDOS EN LA TESIS DOCTORAL

Resultados. Capítulo 1. Castillo-Diaz, J.M., Romero, E., Nogales, R. 2013. Dynamics of microbial communities related to biochemical parameters during vermicomposting and maturation of agroindustrial lignocellulose wastes. Bioresource Technology, 46, 345-353. DOI: 10.1016/j.biortech.2013.07.093

Resultados. Capítulo 2. Castillo-Diaz, J.M., Nogales, R., Romero, E. 2014. Biodegradation of 3,4 dichloroaniline by fungal isolated from the preconditioning phase of winery wastes subjected to vermicomposting. Journal of Hazardous Materials, 267, 119-127. DOI: /10.1016/j.jhazmat.2013.12.052

Resultados. Capítulo 3. Castillo-Diaz, J.M., Beguet, J., Martin-Laurent, F., Romero, E. 2015. Multidisciplinary assessment of pesticide mitigation in soil amended with vermicomposted agroindustrial wastes. Journal of Hazardous Materials. Aceptado. HAZMAT17201. DOI: 10.1016/j.jhazmat.2015.10.056

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Dynamics of microbial communities related to biochemical parameters during vermicomposting and maturation of agroindustrial lignocellulose wastes

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HIGHLIGHTS

- β-Glucosidase is a suitable biomarker to assess worm biomass in vermicomposting.
- Changes in bacterial and fungal structure depend on worm biomass.
- Actinobacteria increased throughout the vermicomposting period.
- Total fungal abundance increased throughout the maturation period.
- Enzyme activities abated after the maturation period.

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Mantel test

ABSTRACT

Scarce information is available on the changes in abundance of microbial taxa during vermicomposting. Quantitative PCR and DGGE analysis were used to monitor variations in the microbial structure, relative abundance of four bacterial classes and fungi over the vermicomposting and maturation period of wet olive cake (O) and vine shoots (W). Multivariate correlation analysis between microbial structure and abundance, earthworm biomass and enzyme activities revealed similar and divergent interactions in both processes. Although *Eisenia fetida* development was different, significant correlations were found with β-glucosidase activity and with bacterial and fungal structure. In the vermicomposting period of O and W, a decline was found in bacteria (94% and 77%), fungi (93% and 94%), and Gammaproteobacteria (56% and 71%) but an increase in Betaproteobacteria and Actinobacteria (62–79%). Alphaproteobacteria increased only in O (26%). Despite the different initial lignocellulose wastes, the mature vermicomposts were similar in microbial and biochemical properties.

1. Introduction

Vermicomposting is an eco-biotechnological mesophilic process that transforms complex organic wastes into a stabilized humus-like product, consists of two different periods related to the earthworm activity: (i) a vermicomposting period when earthworms process the waste for growth, modifying its physical and chemical properties as well as its microbial composition; and afterwards (ii) a maturation period, following the removal of the earthworms, when microorganisms take over decomposition of the waste processed up to then by the earthworms. Also, organic wastes with a high C:N ratio require a preprocessing or preconditioning period (before vermicomposting) to make them acceptable to earthworms. Such preliminary treatments can involve mixing with other organic wastes with low C:N ratio and/or predecomposition with microorganisms naturally present in the wastes or inoculated (Ndegwa and Thompson, 2000 and Kumar and Shweta, 2011).

Lignocellulosic wastes are composed of complex heteropolymers that confer recalcitrant characteristics, becoming a challenge for microorganisms due to the inhibition of cellulosic enzymes (DeAngelis et al., 2011). Composting and vermicomposting are two of the best-known processes for the biological degradation and stabilization of these wastes. Most studies concerning these processes have focused on changes of physical–chemical properties and biochemical (enzymatic) parameters (Singh and Suthar, 2012). These parameters reflect the earthworm and microbial activity. Hydrolytic enzymes such as dehydrogenase, β-glucosidase, urease, and phosphatase, involved in the C, N, and P cycle, as well as phenol oxidases involved in lignin degradation, have been studied elsewhere but their relationship with different microbial taxa over the vermicomposting process has not been extensively studied (Sen and Chandra, 2009).

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Limited information is available on changes in abundance and structure of microbial taxa and the dynamics of each process. Thermophilic composting and mesophilic vermicomposting can determine the microbial communities and thus the decomposition of the organic matter (Lazcano et al., 2008). In regard to lignin decomposition, Proteobacteria and Actinobacteria are two major taxa involved in this process, where Alpha- and Gammaproteobacteria classes are two of the most important degraders (DeAngelis et al., 2011). Bacteria from the class Actinobacteria are fundamental in lignin and polyphenol degradation (Kirby, 2005) as well as in the production of antibiotics and enzymes such as chitinases, which are able to degrade the fungal-cell membranes (Jayasinghe and Parkinson, 2009). Recently, molecular techniques such as denatured gel gradient electrophoresis (DGGE) and single-strand conformational polymorphism (SSCP), based on the PCR-amplification of small ribosomal subunit, have been used to analyse of microbial community structure during vermicomposting (Sen and Chandra, 2009). The analysis of phospholipid fatty acid (PLFA) has revealed that earthworms have a diverse pool of digestive enzymes that can digest the microbiota, reducing the microbial populations (Gómez-Brandón et al., 2012). Real-time quantitative PCR (Q-PCR) has recently become a valuable molecular tool for quantifying indigenous organisms in environmental samples directly from environmental DNA extracts. This method is powerful, accurate, and culture independent (Prevost-Boure et al., 2011).

The use of agroindustrial wastes to produce organic fertilizers is growing as a profitable and sustainable solution to the high prices of fertilizers, which also cause soil organic-matter loss. Some of the most abundant agroindustrial wastes in the world come from the olive-oil and wine production. Spain has more than 25% of the world's olive growing surface area, with an average production of 0.7–1 $\times 10^6$ t year^{-1} of olive oil, generating 3–4 $\times 10^6$ t year^{-1} of wet olive cake or *alperujo*. In addition, Spain has a 14% of the total world surface area of vineyards, being the world's third wine producer (34–37 $\times 10^6$ Hl), resulting also in abundant waste of grapevine shoots (3 $\times 10^6$ t year^{-1}). Both wastes contain recalcitrant lignocellulose materials with high C:N ratios that need to be mixed with other organic wastes with low C:N ratios and pre-processed in order to optimise the biological transformation and to produce an adequate organic fertilizer.

Therefore, the present study firstly evaluates the changes in earthworm biomass, chemical properties, and biochemical functions (dehydrogenase, β-glucosidase, acid phosphatase, urease, and ortho-diphenol oxidase). Secondly the changes in the bacterial and fungal structure are assessed during vermicomposting and after a maturation period. In addition, the total abundance of bacteria and fungi as well as microbial taxa (Alpha-, Beta- and Gammaproteobacteria and Actinobacteria) are quantified, using taxa-specific real-time PCR assay. The final aim is to elucidate the interactions among those parameters in relation to the microbial dynamics during the biotransformation of those complex organic wastes.

2. Methods

2.1. Organic-waste collection and earthworms

Vine shoots collected from a vineyard had 4% moisture, 490 g kg^{-1} organic carbon (o.c.) and 6 g kg^{-1} total Kjeldahl nitrogen (TKN). This waste was air-dried and chipped into pieces of 2–5 cm. Biosolid vinasse (85% moisture, 440 g kg^{-1} o.c. and 75 g kg^{-1} TKN) was collected from a vinasse wastewater-treatment plant (Tomelloso, Spain). The wet olive cake from a commercial olive-oil manufacturer (ROMEROLIVA, S.L. Deifontes, Spain), contained 65% moisture, 540 g kg^{-1} o.c. and 10 g kg^{-1} TKN. Goat manure had 71% moisture, 394 g kg^{-1} o.c. and 22 g kg^{-1} TKN. Earthworms (*Eisenia fetida*) from Humus-Fertil (La Roda, Spain) were used.

2.2. Experimental setup

The vermicomposting process was conducted at a pilot scale using wooden boxes (1.7 m × 1.4 m × 0.25 m) sloped 5% for drainage under semi-controlled field conditions, as reported previously (Melgar et al., 2009). The winery substrate (W) consisted of 46 kg of vine shoots mixed at 4:1 (dw:dw) with biosolid vinasse, while the olive-mill substrate (O) was composed of 49 kg of the wet olive cake mixed with goat manure at 4:1 ratio (dw:dw). Two wooden boxes were used for each substrate, which underwent an initial pre-processing time of two weeks (initial periods, I). Subsequently, two narrow rows of vermicomposted manure were placed on both sides of each mixture to serve as an initial habitat for the earthworms and also as a source of microbial inoculum. An earthworm biomass equivalent to 3% of the dry organic material was inoculated.

During the vermicomposting period, no new substrate was added and the moisture content was kept at 70–80% by irrigation. From each box, four samples were collected by using a sampler of 20 × 20 cm and 25 cm deep. The sampling timetable was: at 1, 3, 5, 7, 11, and 15 weeks to follow the earthworm biomass; when the vermicomposting period was finished (FV = 15 wks in the W substrate and 18 wks in the O substrate) the earthworms were removed by hand, and the vermicomposted compounds were matured for 2 months without water addition. Finally, these samples were stored in plastic bags at 4 °C for enzyme-activity analysis or were frozen in liquid nitrogen at −80 °C until DNA extraction. Representative samples were also collected at the initial time (I), and after the maturation period (M) they were air-dried and finely ground to determine the changes in the chemical properties of these substrates.

2.3. Chemical and enzyme analysis

The pH and electrical conductivity (EC), total organic carbon (TOC), total Kjeldhal nitrogen (TKN), total extractable carbon (TEC), carbon contents in humic and fulvic acids (HAC, FAC) and water-soluble carbon (WSC) were determined according to established methods (Fernández-Gómez et al., 2011). Hemicellulose, cellulose, lignin, and total phenolic compounds were determined according to validated methods described by Romero et al. (2006). The activities of certain enzymes (dehydrogenase, β-glucosidase, acid phosphatase, and urease) were analysed in triplicate using 0.2 g of organic samples, as described elsewhere (Fernández-Gómez et al., 2011). Ortho-diphenol oxidase (o-DPO) activity was determined using catechol as the substrate (Perucci et al., 2000).

2.4. Genomic DNA extraction

Total DNA was extracted from 250 mg of each of three representative organic samples collected following the ISO-11063 procedure described by Petric et al. (2011). For purification, aliquots (100 μL) of crude DNA extracts were loaded onto PVPP (polyvinyl polypyrrolidone) minicolumns (BIORAD) and centrifuged for 4 min at 1000*g*. This step was repeated to ensure the complete removal of PCR inhibitors when necessary. The quality of the DNA extracts was checked by electrophoresis on 1% agarose gel and quantified at 260 nm using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA), and later were stored at −20 °C until used.

2.5. PCR-DGGE analysis of bacterial and fungal communities

The 16S rRNA and 18S rRNA genes were amplified from 25 ng of DNA in a final volume of 25 μL using 0.5 μM of each primer (341F-GC/518R and FR1GC/FF390) for bacteria and fungi, respectively (Table 1), 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 1 μL of T4 gen 32 (Qbiogene, France) and 1 U of Taq DNA polymerase (Eurotaq). Denaturing gradient gel electrophoresis (DGGE) was conducted by loading 200 ng of PCR products onto 9% and 8% (w/v) polyacrylamide containing 45–70% and 30–60% of denaturant gradients for bacteria and fungi, respectively [100% denaturant contained 7 M urea and 40% (vol/vo) formamide]. The banding patterns of DGGE gels were analysed using the GelCompar II software (Applied Maths, Ghent, Belgium). The band profiles were converted into a binary matrix representing the occurrence of DGGE bands (band presence/absence).

2.6. Real-Time PCR Quantification (Q-PCR) of bacteria and fungi

The total abundance of bacteria and fungi as well as Alpha-, Beta-, Gammaproteobacteria, and Actinobacteria classes in the organic samples were quantified using the taxon-specific 16S rRNA or 18S rRNA, respectively. For this purpose, a set of primers were selected (Table 1). Standard curves were drawn for plasmids containing cloned the16S rRNA gene from *Sinorhizobium meliloti*, *Burkholderia* sp., *Pseudomonas putida* KT2440, and *Arthrobacter* sp., which represent these bacterial taxa, as well as the 18S rRNA from a pure culture of *Fusarium oxysporum* (EEZ-CSIC strain collection). The bacterial and fungal Q-PCR conditions were according to Philippot et al. (2011) and Prevost-Boure et al. (2011), respectively, with some modifications (Table 1). The PCR reactions were conducted in an iCycler MyiQ™ Systems (Bio-rad, USA) using Sybr-Green® as the detection system. The reaction was performed in a final volume of 15 μl containing 7.5 μl of iQ™ SYBR® Green Supermix, 0.5 μM of each primer, 0.5 μl of T4 gen 32 (Qbiogene, France) and 2 ng of template DNA. The function that described the relationship between Ct (threshold cycle) and the number of sequences of each bacterial and fungal taxon, ranged between −3.40 and −3.27 with a PCR efficiency of 95.6–99.9%, respectively. Controls without templates gave null or negligible values.

2.7. Statistical analysis

Earthworm biomass, biochemical and microbiological data during the vermicomposting process were analysed by repeated-measures analysis of variance (RM-ANOVA). Violation of the sphericity assumption (Maulchy's test) was amended by application of the Huynh-Feldt correction to the significance level. The Least Significant Difference test (LSD) was used to compare the different means in each treatment (W and O) and each period. Physico–chemical properties were analysed using Student's *t*-test between the final vermicompost (FV) and mature vermicompost (M) periods. All these analyses were made using the SPSS version 15.0 (IBM, Chicago, IL, USA). Bacterial and fungal-DGGE profiles were analysed by principal components analysis (PCA) on the covariance matrix that was performed on a data matrix (sampling time of the vermicomposting periods as rows and presence/absence bands as columns) created by using the GelCompar II software. This ordination method provided a factorial map of the genetic structure of the microbial community based on similarities. The PCAs (Canoco, Windows 4.5) and a multivariate analysis of Variances (MANOVA) were employed to test significant differences among the PC scores closely associated. MANOVA is a sensitive analysis for the inequality of variances represented by Box's test of equality of covariance matrices. In cases where more than two sampling times were compared, Tukey's *post hoc* test was employed. The correlation between microbiological and biochemical variables as well as *E. fetida* biomass during the vermicomposting period was assessed using Mantel's multivariate test and corroborated using Pearson's correlation (PCORD 5). With Mantel's test, distance matrices for DGGE profiles of 16S rRNA and 18S rRNA were determined using a Jaccard distance transformation, and for the rest of variables, the Euclidian distance were inferred.

3. Results and discussion

3.1. Evolution of total earthworm biomass during the vermicomposting period

The colonization of earthworms in the mixture of vine shoot and biosolid vinasse (W) was very fast (Fig. 1). Three weeks after the inoculation, the earthworm biomass reached a maximum value

Table 1
Primers and thermal cycling conditions used for PCR-DGGE and quantification by real-time Q-PCR of the different classes.

Primers	Sequences (5′–3′)	Thermal conditions
Total bacteria		
341F*	CCTACGGGAGGCAGCAG	95 °C, 15 min, one cycle. 95 °C for 15 s, 50 °C for 30 s, 70 °C for 30 s,72 °Cfor 60 s, 35 cycles
518R	ATTACCGCGGCTGCTGG	
Total fungi		
FR1*	AXCCATTCAATCGGTAXT	95 °C, 15 min, one cycle. 95 °C for 15 s, 57 °C for 30 s, 72 °C for 30 s,75 °C for 30 s, 35 cycles
FF390	CGATAACGAACGAGACCT	
Alphaproteobacteria		
E338F	ACTCCTACGGGAGGCAG CAG	95 °C, 15 min, one cycle. 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s,75 °C for 30 s, 35 cycles
Alfa- 685R	TCTACGRATTTCACCYCTAC	
Betaproteobacteria		
E338F	ACTCCTACGGGAGGCAG CAG	95 °C, 15 min, one cycle. 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s,75 °C for 30 s, 35 cycles
Beta-680R	TCACTGCTACACGYG	
Gammaproteobacteria		
Gamma 395f	CMATGCCGCGTGTGTGAA	95 °C, 15 min, one cycle. 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s,75 °C for 30 s, 35 cycles
Gamma 871r	ACTCCCCAGGCGGTCDACTTA	
Actinobacteria		
Actino 235f	CMATGCCGCGTGTGTGAA	95 °C, 15 min, one cycle. 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30s,75 °C for 30 s, 35 cycles
E518r	ACTCCCCAGGCGGTCDACTTA	

* Primers with a GC clamp at the 5′ end for DGGE.

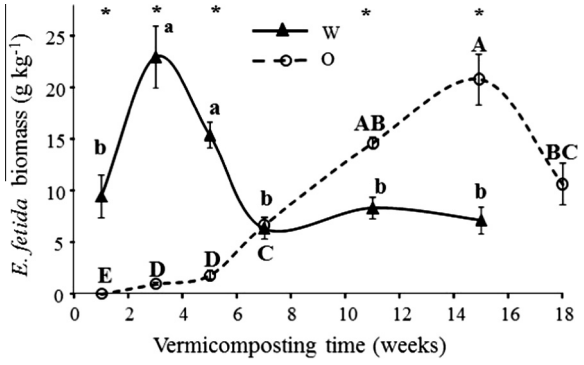

Fig. 1. Earthworm biomass during the vermicomposting period. Vertical bars represent the standard deviation. Same letter (lowercase W or Capital O) are not significantly different ($p < 0.05$). The asterisk (*) denotes a significant difference between substrates at a given time.

of 23 ± 4 g kg^{-1} substrate. Thereafter, the worm biomass declined significantly until the week 7 as available nutrients in the organic mixture became depleted, remaining constant until the end of the vermicomposting period. By contrast, the mixture of wet olive cake and goat manure (O) remained uncolonized until the 3rd week, when low earthworm biomass was recorded (0.96 ± 0.1 g kg^{-1} substrate). Afterwards, earthworm biomass increased significantly, reaching the maximum (21 ± 3 g kg^{-1} substrate) at week 15 of vermicomposting. From 15 to 18 weeks, earthworm biomass diminished significantly as a consequence of depletion of the nutrient content in this feed substrate. At week 15 in W and 18 in O, no clitellated earthworms were found, implying that more substrate would be necessary in order to sustain earthworm growth and reproduction. In previous vermicomposting processes using wet olive cake, alone or mixed with other organic wastes, but inoculated with a higher earthworm density, the substrate was also colonized in a slow manner, and increments in earthworm biomass were found after at least 2 months post-inoculation (Melgar et al., 2009). The high levels of fatty and oily compounds in the wet olive cake may initially favour a microaerophilic environment in the O substrate, and therefore the earthworms are deprived of oxygen and/or exposed to transitory toxic substances produced under those conditions. The fast increase of earthworm biomass in the W may have been due to the addition of small vine-shoot fragments (<5 cm) that promoted the rapid colonization of filamentous fungi on the wood substrate, creating a favourable environment for bacterial growth during the preprocessing period (Fig. 1). Wal et al. (2007) describe the size as a factor affecting the rate of initial wood decomposition, as small fragments have a relatively large surface area for interaction with the surrounding microbiota. Given that untreated wood wastes are unsuitable for the earthworm development (Kumar and Shweta, 2011), the rapid growth of these microorganisms must have helped to reduce the predecomposition time in W substrate (McMahon et al., 2008). In fact, the wide set of enzymes capable of decomposing recalcitrant lignocellulose wastes and the networks of hyphae of most fungal species may allow the translocation of nutrients from N-rich to N-poor substrates (Chigineva et al., 2011), stimulating microbial activity and promoting earthworm colonization, since fungal mycelium constitute a nutritional source for these Oligochaeta.

3.2. Chemical changes after the vermicomposting-maturation period

The chemical properties of the initial substrates (I) were significantly altered after the vermicomposting process and the maturation (M) period (Table 2). The pH values changed slightly, significantly rising only in the olive-oil-waste mixture (O). The electrical conductivity, 3-fold higher in O than in the winery mixture (W), decreased significantly, whereas in the W substrate the opposite occurred. In either case, total organic carbon (TOC), water-soluble carbon (WSC), cellulose, hemicellulose, lignin, and polyphenols significantly decreased. The reductions in the WSC, lignin, cellulose, and hemicellulose content in W were greater (64%, 33%, 73%, and 45%, respectively) than in O (41%, 20%, 65%, and 39%, respectively). However, the reduction in TOC content recorded in the olive substrate (O) was greater (45%) than in W (30%), due to the presence in O of specific organic compounds, such as carbohydrates or fats (7–19%) (Alburquerque et al., 2009) which were degraded during the vermicomposting-maturation processes. The total extractable carbon (TEC), as a measurement of the total carbon in the humic substances, was similar in the initial substrate and the mature vermicomposts. Nevertheless, the humic acid fraction (HAC) was significantly higher in the vermicomposted and matured product (M). The total N contents in the mature vermicomposts were higher than initial substrate, although significant differences were detected only in O. The significantly lower C/N values and the higher HAC:FAC ratio and percentage of humification (HR), in both mature vermicomposts (M) with respect to the initial values, indicate a high degree of stability and an extended synthesis of organic compounds resistant to microbial degradation (i.e. humification). Comparatively, the humification process was more widespread in the olive substrate (O), presumably related to its greater mineralization (% of TOC lost).

Table 2
Physico–chemical analysis of initial substrates (I) and mature vermicomposts (M) of vine shoots mixed with biosolid vinasse (W) and wet olive cake mixed with goat manure (O).

	W		O	
	I	M	I	M
pH	7.8 ± 0.2	7.6 ± 0.1	7.3 ± 0.1	7.8 ± 0.0*
EC (dS m^{-1})	1.4 ± 0.0	4.4 ± 0.2*	4.4 ± 0.2*	3.2 ± 0.2
TOC (g kg^{-1})	454 ± 1*	317 ± 6	496 ± 2*	274 ± 1
WSC (g kg^{-1})	15.6 ± 2.4*	5.6 ± 0.2	13.1 ± 0.4*	7.6 ± 0.0
Cellulose (g kg^{-1})	248 ± 1*	66 ± 1	193 ± 1*	68 ± 1**
Hemicell. (g kg^{-1})	186 ± 1*	102 ± 2	161 ± 1*	115 ± 1*
Lignin (g kg^{-1})	240 ± 2*	160 ± 1	189 ± 1*	151 ± 1.0
Polyphenols (g kg^{-1})	3.5 ± 0.1*	1.4 ± 0.0	4.8 ± 0.6*	1.9 ± 0.1
Total N (g kg^{-1})	26 ± 0.8	28 ± 0.5	17 ± 0.6	19 ± 0.3*
C/N	17 ± 0.5*	11 ± 0.0	30 ± 1*	14 ± 0.2
TEC (g kg^{-1})	55 ± 1	55 ± 3	142 ± 4	151 ± 1
HAC (g kg^{-1})	16 ± 2	30 ± 0.3*	87 ± 2	105 ± 0.7*
HR %	12 ± 0.2	17 ± 1*	29 ± 1	55 ± 1
HAC/FAC	0.43 ± 0.11	1.2 ± 0.2*	1.6 ± 0.0	2.2 ± 0.0*

EC: electrical conductivity, TOC: total organic carbon, WSC: water-soluble carbon, TEC: total extractable carbon, HAC: humic acid carbon, FAC: fulvic acid carbon, HR: humification ratio = 100 × TEC/TOC. The asterisk (*) denotes, in each substrate, significant differences ($p < 0.05$, paired-sample t-test) between the initial substrate and mature vermicompost for each parameter.

3.3. Enzyme activities during the vermicomposting and maturation time

In W, Dehydrogenase (Dhase) activities related to the metabolic state of microbial population showed significant differences ($F = 60.3$ $p < 0.001$). Values were highest during the first three weeks and afterwards fell significantly to remain almost constant until the end of the vermicomposting period (FV). However, the Dhase activity remained constant the first 5 weeks in O, and then intensified until FV (Fig. 2a). In both process, the Dhase activity in the maturation time (M) was significantly lower than in FV. The o-DPO enzyme, which is related to the capacity of the microorganisms to break down recalcitrant organic compounds, followed a similar pattern in both substrates, increasing during the

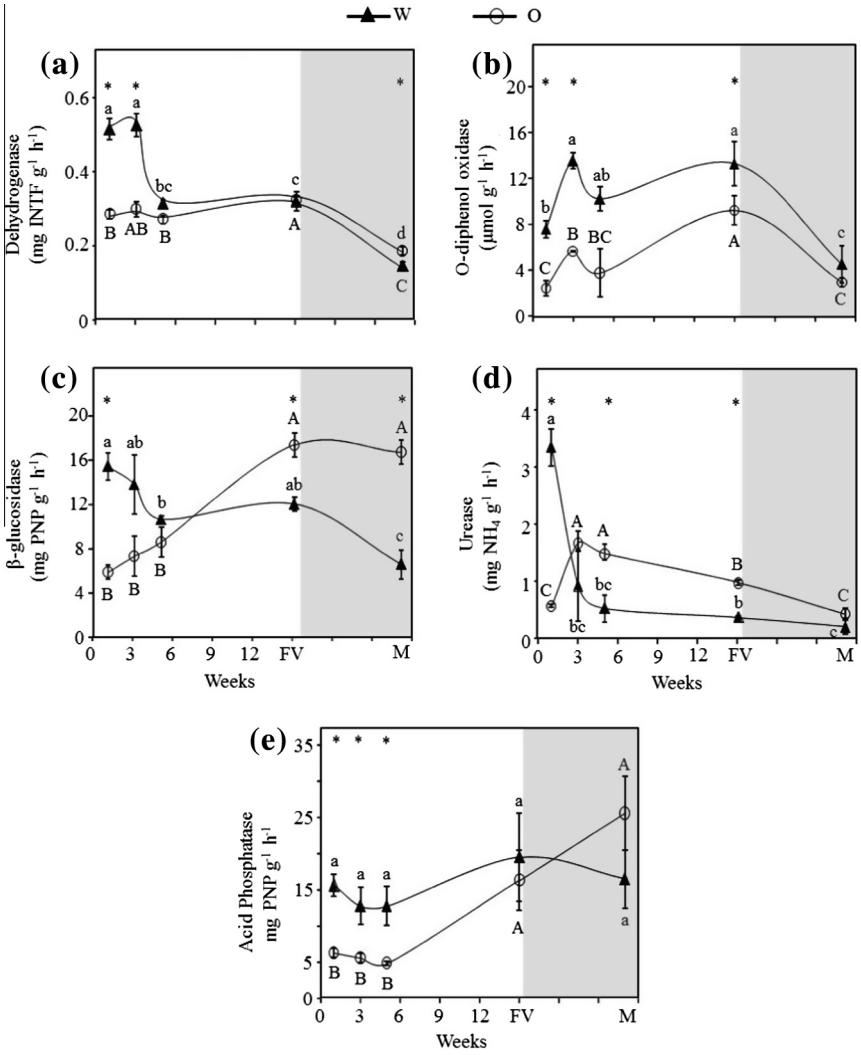

Fig. 2. Enzyme activities during the vermicomposting period (□) and after maturation period (■). Error bars represent the standard deviation. Data with the same lowercase (W substrate) or capital letter (O substrate) are not significantly different ($p < 0.05$). The asterisk (*) denotes a significant difference between substrates at a given time.

first three weeks when the amount of these compounds was larger, then declining, and finally increasing significantly in O until FV (Fig. 2b). In M, the activity diminished significantly for W and in O showed similar activity to the 1th week ($F = 26.3$ $p = 0.14$). In both organic substrates, the o-DPO activity decreased significantly in the maturation period. In the W, the highest values of β-glucosidase and urease activities were recorded in the first week of the vermicomposting period, diminishing until the 5th wk and then remaining constant. By contrast, β-glucosidase activity intensified significantly during the vermicomposting period in the O, while urease activity reached the highest values after 3 weeks, and then declined later until FV. In general, these both enzymes significantly declined after the maturation period (Fig. 2c and d), except to β-glucosidase, where FV and M no showed differences in O ($p = 0.54$). In relation to the acid phosphatase activity, vermicomposting and maturation time did not significantly differ over the time course ($F = 2.55$ $p = 0.12$) in W. However, O substrate registered higher acid phosphatase activities at FV and M times with respect to the first five weeks. In general, all these enzymes were significantly higher at the first week in the W than in O mixtures. This reveals that the W contained greater amounts of easily available organic compounds than did O. Thus, the higher activity of β-glucosidase can be explained by the higher hemicellulose and cellulose content in W (Table 1), in which hydrolysis produced cellobiose, this enzyme being responsible for converting cellobiose into glucose. In addition, the higher content in organic N and the addition of a substrate with an easily metabolizable organic-N, via vinasse biosolids, as has been reported in other biosolids (Parmar et al., 2001), would induce the urea production, thereby leading to the notable urease activity recorded at the beginning of the vermicomposting process. Consequently, a high ammonium content accumulated in this substrate (4 mg g^{-1} determined in the blank samples from the urease-activity measurements), which would strongly inhibit the urease activity (Castaldi et al., 2008), and hence a pronounced decrease occurred after one week. Likewise, it is well known that biosolids contain large amounts of organic phosphate compounds which induce acid-phosphatase enzyme synthesis. Due to high amounts of easily available organic compounds in W substrate, a greater dehydrogenase activity was recorded with respect to the O substrate.

Despite these clear differences, in both vermicomposting processes, although the evolution of *E. fetida* biomass was also different (Fig. 1), Mantel's test revealed a significant correlation between the worm biomass and the β-glucosidase and o-DPO activities (Table 3). This implies that both enzyme activities were directly related to *E. fetida* activity and depended on its population. The enzyme β-glucosidase can be strongly activated in the earthworm gut, as suggested above, resulting in greater cellulolytic activity

Table 3
Mantel's test of dissimilarity matrices obtained from multiple comparisons of worm biomass, enzyme activities, abundance, and relative abundance of microbial taxa, as well as microbial structure during the vermicomposting period.

O	Worm	Enzymes					Abundance		Relative abundance				Structure	
W	Biomass	Dhase	o-DPO	Gluco	Urease	Phosp	Bacteria	Fungi	Alpha	Beta	Gamma	Actino	Bacteria	Fungi
Worm bio.		0.34*	0.60**	0.90**	ns	0.92**	−0.43*	ns	ns	0.72*	ns	0.70**	0.61**	0.64**
Enzyme														
Dhase	ns		ns	0.32*	ns	0.30*	ns	ns	ns	ns	ns	ns	ns	ns
o-DPO	0.33*	ns		0.68*	ns	0.63*	−0.43**	−0.41*	ns	0.58**	ns	0.57**	0.57**	0.62**
Gluco	0.33*	0.54**	ns		0.02	0.87**	−0.52**	−0.40*	ns	0.80**	ns	0.80**	0.64**	0.68**
Urease	ns	ns	−0.47**	ns		ns	0.48*	ns	ns	ns	ns	ns	0.36**	0.53*
Phosp	ns	ns	ns	ns	ns		0.48*	ns	ns	0.70**	ns	0.66**	0.57**	0.56**
Abundance														
Bacteria	−0.44*	0.25*	−0.53**	0.35*	0.55**	ns		0.57**	ns	−0.53**	ns	−0.50**	0.40**	0.40**
Fungi	ns	0.69**	ns	ns	0.22*	ns	0.25*		0.34*	−0.40*	0.34*	−0.43**	0.27*	0.35*
R.Abundance														
Alpha	ns	0.40*	ns	ns	0.62*	ns	0.42*	0.51**		0.35	Ns	0.32*	ns	ns
Beta	ns	ns	ns	ns	−0.51**	ns	ns	ns	ns		Ns	0.69**	0.55**	0.58**
Gamma	ns	ns	ns	ns	0.60**	ns	0.57**	ns	0.60**	0.31		−0.32*	0.28*	0.33*
Actino	ns	ns	0.47**	ns	−0.46**	ns	−0.42**	ns	ns	0.68**	0.26**		0.71**	0.66**
Structure														
Bacteria	0.34*	0.56**	ns	0.37*	0.38**	ns	0.35*	n.s	0.39**	0.53**	0.30*	0.44**		0.92**
Fungi	0.68**	0.31**	0.50**	0.35**	0.71**	ns	0.66**	n.s	0.34*	0.32*	0.41**	0.54**	0.77**	

* Significant correlation at 0.05 level.
** Significant correlation at 0.01; ns: no significant.

in castings than in undigested material (Parthasarathi and Ranganathan, 2000). Therefore, this activity abates when the worm biomass decreases and less casting would be released into the organic substrate. Similar results were described in the vermicomposting of other wastes such as pig slurry (Aira et al., 2007) and tomato-fruit wastes (Fernández-Gómez et al., 2010). In addition, β-glucosidase was correlated with Dhase activity, since the vermicast released has a larger surface area, higher humidity, and greater nutrient concentrations, resulting in more vigorous microbial activity. Therefore, the enzyme β-glucosidase is a good biomarker to monitor the time course of an earthworm population in the vermicomposting processes using lignocellulose wastes as feed substrates. Finally, the enzyme o-DPO was related to the relative abundance of the Actinobacteria class and to worm biomass (Table 3). Thus, both would be expected to contribute to the degradation and/or stabilization of the phenolic compounds (Table 2). It has been reported that *E. fetida* has the capability to bioaccumulate high amounts of harmful chemicals (including phenol) in their tissues and either biodegraded or biotransformed by o-DPO enzymes, which are involved in the formation of melanin, a key compound in cellular pathogen defence (Procházková et al., 2006). On the other hand, the intimate mixing during passage through the earthworm gut encourages the stabilization of phenolic compounds by the stimulation of the microbial activity in castings (Sabrina et al., 2012).

The maturation period in vermicomposting processes is a final period that needs no treatment, which involves only microorganisms, and is related to the stabilization and humification of the organic matter contained in the substrates. However, the biochemical and microbiological changes occurring at this stage are still not well known. In W and O mixtures after maturation (M), significant decreases in all the enzymes activity were found with respect to the final time of the vermicomposting period (FV), except for acid phosphatase (Fig. 2). These reductions indicate depressed microbial activity due partially to the scarce available C and N sources after the vermicomposting period (FV) as well as to the partial drying over this phase (from 76% at FV to 30% after maturation). It is well known that drying depresses microbial activity, particularly the metabolic activity of bacteria, also inducing the inactivation of extracellular hydrolytic enzymes (Dick, 1994). The higher β-glucosidase, urease, and phosphatase activities in O with respect to W in the mature vermicomposts (M) suggest larger fractions of extracellular enzymes-humic acid bonds and more remains of inducers to these enzymes (Burns, 1982). This may be supported by the higher humic acid contents and humification ratio measured in the O substrate at this phase (Table 2).

3.4. Abundance and structure of bacterial and fungal communities during the vermicomposting and maturation period

In the W substrate, the total bacterial abundance was the highest in the first week ($46.1 \times 10^9 \pm 7.3 \times 10^9$ copies g^{-1}), decreasing significantly from 1 to 3 weeks (Fig. 3a). In the O substrate the total bacterial abundance did not show significant differences ($F = 2.55$ $p = 0.12$), however in the first week the abundance was significantly lower ($5.6\ 10^9 \pm 1.6 \times 10^9$ copies g^{-1}) than in W. Nevertheless, a similar value (2.5×10^9 -1.3×10^9 copies g^{-1}) was reached at the end of the vermicomposting period (FV). Total abundance of fungi, followed a similar pattern to that of total bacteria (Fig. 3b). In fact, a significant correlation (Table 3) was found between the abundance of bacteria and fungi during the vermicomposting period, values being higher in O (0.58, $p < 0.01$) than in W (0.25, $p < 0.05$).

The bacteria: fungi abundance ratio tended to decrease during the vermicomposting period in the W substrate (255 in the 1st week, 204 at FV) but increased significantly in the O substrate (95 in the 1st week, 365 at FV). This was due to a similar reduction of bacteria and fungi with respect to the initial population in W (95% and 93%, respectively), while in the O substrates showed a more severe decline in fungal abundance with respect to bacterial abundance (94% and 77% respectively). Bacteria and fungi are considered important dietary sources for earthworms, and therefore, the digestion of the organic wastes by these Oligochaetes would have a negative effect on total bacteria and fungi abundance (Table 3). However, Sen et al. (2008), using the real-time PCR method, observed that total bacterial abundance remained constant during the vermicomposting of industrial sugar wastes, varying within the range of 1.7×10^8 -6.8×10^8 16S rRNA copies g^{-1}. By contrast, Pramanik and Chung (2011) found increases in the total number of bacteria and fungi during the vermicomposting process. These

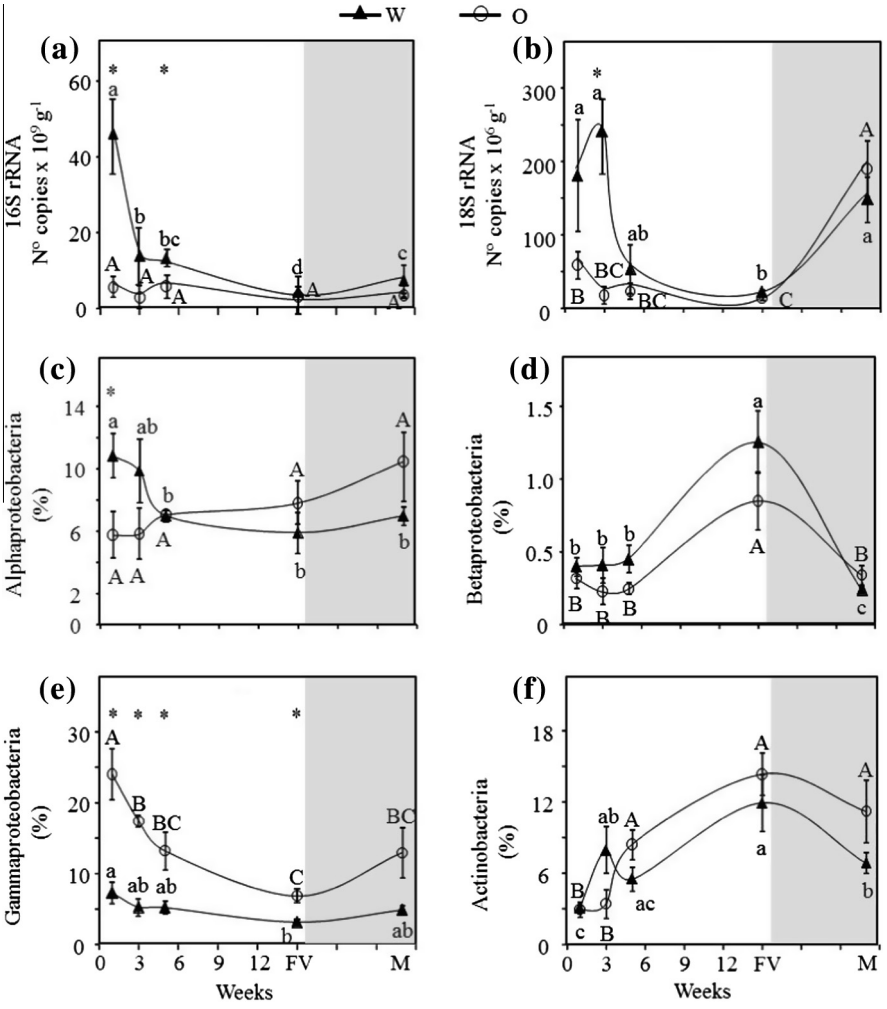

Fig. 3. Total abundance of bacteria and fungi, relative abundance of microbial taxa during the vermicomposting period (□) and after maturation period (■). Error bars represent the standard deviation. Data with the same lowercase (W substrate) or capital letter (O substrate) are not significantly different ($p < 0.05$). The asterisk (∗) denotes a significant difference between substrates at a given time.

contrasting results may be related to the carbon available. Tiunov and Scheu (2004) have reported a strongly decrease in microbial biomass under carbon-limited conditions, where earthworms and microorganisms compete for easily available carbon. On the other hand, the addition of labile carbon increases N conversion into microbial biomass, becoming a source of N available to earthworms.

The fungi abundance in both process increased significantly throughout the maturation period (Fig. 3a and b). These pronounced surge in fungi at this stage would confirm that these microorganisms have the ability to grow on recalcitrant compounds after the vermicomposting period under a low-moisture regimen (<30–40%).

The discrete DGGE band patterns found during the vermicomposting period were used as genetic fingerprints of bacterial and fungal community structure. The complex banding pattern with 35 and 25 bands per line in the two vermicomposting processes, respectively. Mostly, the bands were similar between treatment replicates, indicating good reproducibility of extraction, PCR amplification, and electrophoretic separation. (Fig. 4a–d). The principal component analysis (PCA) of the DGGE fingerprinters of bacteria in W substrate (Fig. 4a) revealed that the first principal component (PC1), with 30% of the variance, distinguished the bacterial structure at the early times of vermicomposting (1st and 3rd weeks) from that of the final vermicomposting time (FV). The second component (PC2) explained the 25% of the variance, discriminating the bacterial structure at the 5th week from the mature vermicompost (M). In the O substrate (Fig. 4b), the PCA of the bacterial structure revealed that 29% of the variance was explained by PC1 but with short spatial distances between the 1st, 3rd, and 5th weeks and between the FV and M. Multivariate analyses of variance (MANOVA) in these PC scores showed that the bacterial structure at the 1st and 3rd weeks significantly differed ($p < 0.01$) according to Tukey's test. PC2 explained 25% of the variability, discriminating the bacteria structure at the 5th week from that of the other times. The fungal-structure analysis by PCA in the W substrate showed that PC1 explained 33% of variance data (Fig. 4c), discerning clearly between the 1st week and M. In addition, the MANOVA analysis revealed a significant spatial separation ($p < 0.001$) between the fungal structure in the 3rd week and those corresponding to the 5th and FV. These fungal structures were also differentiated from the 1st week and M in the PC2 with 30% variance. The fungal-structure analysis in the O substrate with variance data in PC1 and PC2 of 34% and 27%, respectively, was similar to that described in the W substrate (Fig. 4d). The bacterial and fungal structure in both substrates was closely correlated with the time course of worm biomass over the vermicomposting period (Table 3). This supports the idea that the digestion of these

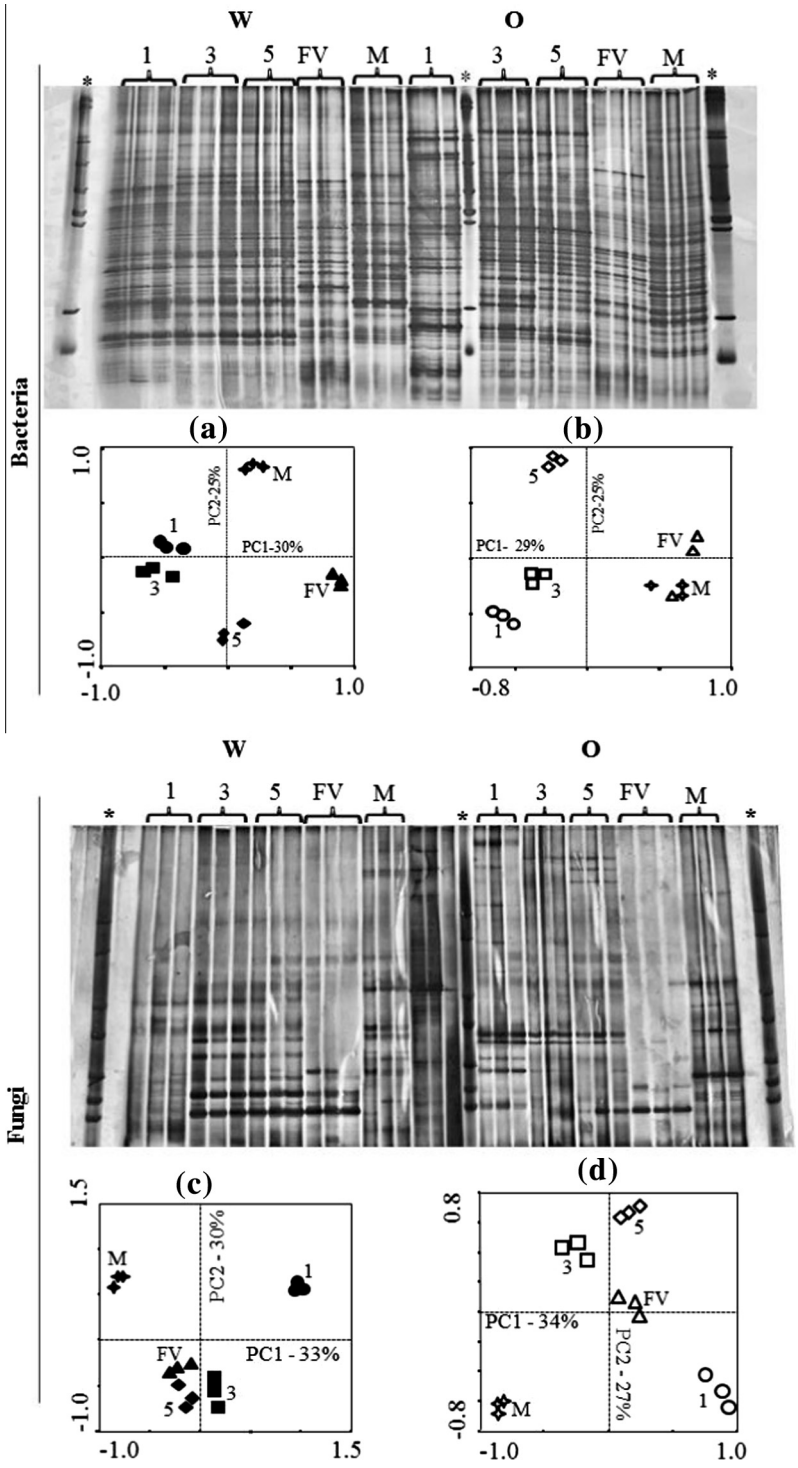

Fig. 4. Grey-scale image of DGGE gels and principal-component analysis of bacterial (a and b) and fungi (c and d) in W and O substrates during the vermicomposting period (1, 3, and 5 weeks and FV) after maturation (M). The asterisk (*) represents the ladder line.

organic wastes by *E. fetida* significantly modified the microbial structure, by discriminating feed and/or by stimulating or depressing specific microorganism taxa. Likewise, the bacterial and fungal structures were correlated with each other and with their respective abundances (Table 3). In addition, Mantel's test highlights the correlations between bacterial and fungal structure with β-glucosidase and urease activities in both substrates. This indicates that the structure was related to labile C and N sources.

3.5. Relative abundance of microbial taxa communities during the vermicomposting and maturation period

In both substrates, the abundance of Alpha-, Beta-, and Gamma-proteobacteria as well as Actinobacteria classes (Fig. 3) were correlated with bacterial structure (Table 3), indicating that these play a significant role in bacterial diversity changes occurring during the vermicomposting.

At the beginning of the vermicomposting period, the Alpha- and Gammaproteobacteria classes (Fig. 3c and e) were dominant, respectively, in winery wastes (10.9 ± 0.8%) and olive-oil wastes (23.9 ± 0.8%), diminishing significantly ($p < 0.001$) with vermicomposting time. In the W substrate, the relative abundance of the two bacterial groups were correlated with each other ($r = 0.60$, $p < 0.01$) and with the total bacterial abundance ($r = 0.42$, $p < 0.05$ Alphaproteobacteria; $r = 0.57$, $p < 0.01$ Gammaproteobacteria), but in the O substrates this did not occur. Alpha- and Gammaproteobacteria taxa are related to the degradation of lignin (DeAngelis et al., 2011) and both with the fungi would be responsible for the decline in these organic winery wastes during the early stages of the vermicomposting. This is supported by the correlation of the urease activity with the abundance of both bacterial groups in this substrate ($r = 0.62$, $p < 0.05$ Alphaproteobacteria; $r = 0.60$, $p < 0.01$ Gammaproteobacteria).

However, this did not occur in the O substrate, where the abundance of the Gammaproteobacteria class was considerable. This class is also an abundant group in microcosms treated with oil products (Viggor et al., 2013), due to the ability the some bacteria to produce biosurfactants and to survive in this substrate type. Betaproteobacteria and Actinobacteria classes, which initially were less abundant, proliferated significantly with vermicomposting time, correlating with each other in both substrates ($r = 0.68$, $p < 0.01$ in W, $r = 0.69$, $p < 0.01$ in O). At the end of the vermicomposting (FV), the Actinobacteria became predominant, their relative abundance reaching 11.9% in W and 14.3% in O, exceeding even the sum of the rest of the classes determined. Actinobacteria class have enzyme activity against a wide variety of substrates, as reflected by the significant correlations with o-DPO activity ($r = 0.47$, $p < 0.01$ in W; $r = 0.57$, $p < 0.01$ in O). This enzyme has been reported in Actinobacteria such as *Nocardia* sp. and several species of *Streptomyces* (Kirby, 2005).

Some genera of the Actinobacteria class are abundant in the *E. fetida* gut, and it is known that some produce chitinase enzymes to degrade chitin, which is a component of the cell wall of fungi (Yasir et al., 2009). This may explain the negative correlation between the abundance of fungi and the Actinobacteria class in the olive substrate (O) ($r = -0.43$, $p < 0.01$). By contrast, in the winery substrate (W), this negative correlation was not found, due presumably to the higher abundance of fungi after the preconditioning period, as mentioned above. Nevertheless, in the W and O vermicomposting, the relative abundance of Actinobacteria class was negatively correlated with the total bacterial abundance ($r = -0.42$, $p < 0.01$ in W; -0.50, $p < 0.01$in O) and particularly with the relative abundance of Gammaproteobacteria (-0.26, $p < 0.01$ in W; -0.32, $p < 0.05$ in O). These results must be related with the colonization ability of this taxa in the worm gut and castings (Jayasinghe and Parkinson, 2009) and by its antagonistic activity against other bacterial taxa. It is well known than many genera of Actinobacteria produce antibiotics, which depress or eliminate other bacteria and fungi, including pathogenic bacteria such as *Salmonella* sp., *Vibrio cholerae*, *Pseudomona* sp., and *Escherichia coli*, which belong to the Gammaproteobacteria class (Monroy et al., 2009). The absence of *Salmonella* and *E. coli* determined in the O product, as a consequence of the addition of manure (<1000 NMP g^{-1} product; Real Decreto 824/2005, Orden PRE/630/2011) leads to the hypothesis that the abundance of Actinobacteria class in the O vermicompost product (Fig. 3f) contributed to the reduction of human pathogens. Thus, the presence of a large abundance of Actinobacteria community when a potential toxic substrate (manure, sludges, etc.) is submitted to vermicomposting would ensure the quality and safety of the product.

At the end of maturation period, the relative abundance of Betaproteobacteria and Actinobacteria classes suffered a reduction, whereas Gammaproteobacteria had a significant increment (Fig. 3d, e and f).This bacterial taxa must be one of the most resistant bacteria under this maturation condition. On the other hand, the reduction of relative abundance of Actinobacteria class can minimize their antagonistic action against fungi and other bacteria (Jayasinghe and Parkinson, 2009).

4. Conclusions

Novel information is presented concerning the relationships between microbial communities, enzyme activities and worm biomass in vermicomposting and maturation periods of dissimilar lignocellulosic wastes. In the vermicomposting period of both substrates, the total bacterial abundance diminished but Actinobacteria class proliferated, this being the main microbial taxa at the end of this period. In addition, the bacterial community-structure changes were correlated to biomass worm and β-glucosidase activity. The maturation period resulted in a subsidence of microbial activity while the total abundance augmented. Actinobacteria constitute good indicators to ensure the safety of the vermicomposts, although further studies are needed in this sense.

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Biodegradation of 3,4 dichloroaniline by fungal isolated from the preconditioning phase of winery wastes subjected to vermicomposting

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HIGHLIGHTS

- Fungi from vermicomposting degraded 3,4-dicholoraniline (DCA).
- *Fusarium* sp. and *A. niger* grew on DCA as nitrogen source.
- Enzymes involved in the DCA biodegradation pathway.
- *A. niger* has a high detoxification potential for DCA.
- New metabolites indicate different strategies of DCA biodegradation by fungi.

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ABSTRACT

A hazardous contaminant, 3,4-dichloroaniline (DCA) is widespread in the environment due to its extensive use in the manufacture of chemicals and its application in different sectors. The ability of fungi grow on in winery wastes in the preconditioning period of vermicomposting to degrade DCA was investigated. Three filamentous fungi (F1, F2, and F3) were isolated and one identified as *Aspergillus niger* and two as *Fusarium* sp. strains. The culture media with the fungus alone or in consortium (Fmix) with DCA as the nitrogen source were analyzed by solid-phase microextraction and gas chromatography–mass spectrometry (SPME-GC/MS). The fastest degradation rate was measured in Fmix with a DT_{50} of 0.85 day^{-1}. *Fusarium* sp. and *A. niger* differed in the metabolism of DCA. Five metabolites were identified as a result of oxidation, co-denitrification, *N*-acetylation, and polymerization reactions. The major metabolites were 3,4-dichloroacetanilide and dichloroquinolines. The azo-metabolites tetrachloroazobenzene and tetracloroazoxybenzene and 3,4-dichloronitrobenzene were found in minor amounts but appeared to be the most persistent in the *Fusarium* cultures (half-lives ranging from 8.3 to 30.9 days). This study highlights the metabolic potential of microorganisms in the preconditioning period of the vermicomposting process and its possible application for *in situ* bioremediation strategies.

1. Introduction

The compound 3,4-dichloroaniline (DCA) is used in the synthesis of a wide range of substances. DCA residues from the chemical industries are released *via* wastewater into the hydrosphere and are again incorporated into soil or water compartments when pesticides such as vinclozolin or phenylureas are biodegraded by microorganisms [1–3]. This compound remains highly persistent in soils and waters [4–6], affecting the soil microbial population [7] and aquatic species [8]. Tetrachloroazobenzene (TCAZB) and tetrachloroazoxybenzene (TCAXB) are also formed as unwanted by-products from compounds containing the DCA moiety [3]. Martins et al. [9] have reported that aromatic amines (AA) have genotoxic and cytotoxic potential towards most living organisms. Therefore, exposure to this toxic contaminant has raised increasing concern over its fate and persistence in the environment.

Fungi, in contrast to bacteria, are capable of degrading a wide range of organic pollutants such as pesticide residues even at high concentrations because they have a complex enzymatic system which is usually induced by nutrient depletion, not by a particular pollutant. Basidiomycetes, white rot fungi (*Chrysosporium lignorum*, *Trametes versicolor*, *Phanerochaete chrysosporium*) have physiological capacity to degrade lignin and many xenobiotic compounds with a variety of structures due to its ligninolytic system

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[10]. However, the effectiveness of these fungi in xenobiotic degradation under environmental conditions is questionable, because the pH and C:N ratio are not always optimal for growth [11]. To overcome these disadvantages, the isolation of imperfect fungi of lignocellulose wastes may be an excellent alternative. Imperfecti fungi have ability to grow and occupy high volumes of wood and tolerate water stress [10]. Moreover, their lignin-degradation systems are not very substrate-specific and therefore can oxidize a great variety of compounds, including environmental pollutants such as dye or anthracene [12,13]. Imperfecti fungi play an important role in the pre-composting period from vermicomposting of lignocellulose organic substrates, contributing to the optimal conditions for earthworm growth [14,15]. However, no information is available on the biocatalyst potential of these imperfecti fungi during the first step of vermicomposting despite that they may offer an efficient alternative for bioremediation techniques meant to degrade and minimize the environmental impact of DCA, providing a low-cost environmentally friendly technology.

The objectives of this study were: (a) to isolate fungi that often grow in winery wastes submitted to preprocessing before vermicomposting, selecting and identifying those able to survive in DCA media; (b) to evaluate their growth capacity, tolerance, and biodegradation potential for DCA, determining the presence of metabolites of this compound; and (c) to elucidate the mechanisms responsible for DCA degradation.

2. Materials and methods

2.1. Chemicals

The chemical compound 3,4-dichloroaniline (DCA, 99.5% purity) was supplied by Chem Service. The metabolite standards, such as 3,4-dichloronitrobenzene (DCNB, 95% purity) was from ACROS Organics (Geel, Belgium), 3,4-dichloroacetanilide (DCAN) from MP Biomedicals, LLC (Illkirch, France), 4,7-dichloroquinoline (DCQ, 99% purity) was from Sigma–Aldrich, 3,3′4,4′-tetrachloroazobenzene (TCAZB, 98% purity) and 3,3′,4,4′-tetrachloroazoxybenzene (TCAXB, 99% purity) were supplied by Dr. Ehrenstorfer GmBH (Ausburg, Germany). 2,4,5-Trichloroaniline (99.0% purity) from Fluka (Steinheim, Germany) was used as internal standard. HPLC-grade acetonitrile from Scharlau Chemie, S.A (Barcelona, Spain) was used. Water was purified using the Milli-Q water-purification system (Millipore, MA, US).

2.2. Fungal Isolation and culture media

One gram of a representative organic sample from the preprocessing period of the vermicomposting of vine-shoot (Appendix A) was inoculated into 9 ml of peptone water and homogenized for 1 min by vortexing. Serial dilutions were made (10^{-1}, 10^{-3}, and 10^{-5}). Aliquots of 1 ml from each dilution were inoculated on potato dextrose agar (PDA) medium supplemented with streptomycin (30 mg l^{-1}) and incubated at 28 °C for 4 days. Colonies with the presence of mycelia were isolated from PDA medium. For degradation studies, a minimal salts medium (MM) was used, composed of (g l^{-1}) glucose 5.0, NH_4SO_4 7.5, K_2HPO_4 4.8, and $MgSO_4{\cdot}7H_2O$ 0.2, and supplemented with streptomycin (30 mg l^{-1}) and trace elements (mg l^{-1}): $MnSO_4$ 20, $FeSO_4{\cdot}7H_2O$ 1, $CuSO_4{\cdot}7H_2O$ 0.7, and NaCl 0.7. All media were adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 20 min.

2.3. Tolerance of the isolated fungi to DCA

For an assessment of the ability of the strains to grow on MM with DCA, the isolated fungi were inoculated into 50 ml of MM and incubated at 28 °C at 110 rpm for 4 days. For the elimination of any trace of C and N, cultures were centrifuged at 4500 rpm for 15 min and washed twice with phosphate buffer at pH 6.5 (KH_2PO_4 6.8 g l^{-1} + K_2HPO_4 8.8 g l^{-1}). Subsequently, 0.5 ml of the suspension was inoculated into solid MM (agar 15 g l^{-1}) that contains DCA at 20 mg l^{-1} as a carbon source (MMCS). DCA was also included in the solid MM as nitrogen source using in this case glucose (5 g l^{-1}) as a carbon source (MMNS). These were maintained at 28 °C under darkness for 5 days. The strains that grew were selected for degradation studies.

2.4. Identification of fungal strains

Chromosomal DNA was extracted from 20 mg of mycelium from each fungus according to Sambrook and Russell [16] with some modifications as an increment of 100 μl in SDS (20%) and 30 μl in proteinase K (20 mg ml^{-1}). Due to PCR inhibition factors, DNA was diluted to 10 ng μl^{-1}. The small subunit (SSU) 18S ribosomal RNA gene was amplified by PCR using 5 ng of DNA as template, 10 μM concentration of each primer un-SSU-0817F (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536R (ATTGGCAATGCYCTATCCCCA) described by Borneman and Hartin [17] and 1 U of Taq DNA polymerase (BioCat GmbH, Germany) in a 25 μl final reaction volume. Amplifications were performed in a Multigene gradient cycler (Labnet International, USA) under the following conditions: 5 min at 95 °C, 30 cycles of 45 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C, plus an additional 7 min cycle at 72 °C. The amplicon size was 762 bp. The PCR-amplified fragments were cloned in pGEMT-Easy vector (Promega) and sequenced using ABI PRISM 3130x l Genetic Analyzer. Sequences were compared with those available on the database using the BLAST programme at the National Center for Biotechnology Information (NCBI). Molecular-sequence data were analyzed using MEGA 5.05 package [18]. Those sequences were aligned and phylogenetically analyzed with the 18S SSU ribosomal DNA sequences from other DCA-degrading fungi using the MUSCLE programme (multiple sequence comparison by log-expectation) and choosing Neighbour Joining as clustering method to produce the distance matrices. The phylogenetic trees were plotted using likelihood as the statistical method. The alignment was bootstrapped using 100 replications. The model of nucleotide substitution was selected [19]. The consensus tree was constructed following the distance tree.

2.5. Pre-inoculums preparation and degradation experiments

Three fungi (F1, F2, and F3) selected by their tolerance to DCA were inoculated in Erlenmeyer flasks of 500 ml with 100 ml of MM for 4 days to grow abundant mycelia. Fungal biomass was washed twice with phosphate buffer as was described above.

Fungal biomass of F1, F2 and F3 (0.30 g l^{-1} dry weight) individually and three-fungi mixture (Fmix; 0.1 g l^{-1} d.w. each) were inoculated in Erlenmeyer flasks of 250 ml containing 50 ml of the MMNS and supplemented with 15 μg ml^{-1} of DCA, respectively. Controls (F1C, F2C, F3C, and FmixC) without DCA and heat-killed inoculum (121 °C by 15 min) were set up to control the abiotic degradation and sorption effect. The treatments were incubated in triplicate at 28 °C on a rotary shaker at 130 rpm in darkness. The fungal growth, pH, and concentrations of DCA were analyzed at 0, 3, 5, 7, 15, 29, and 42 days.

2.5.1. Evaluation of growth of fungal strains on DCA

For the determination of the growth of these fungal strains on DCA, the fungal culture from each flask was harvested by using a pre-weighted filter paper (Whatman GP filters) and dried for 24 h at 105 °C. Fungal growth was quantified as a gram of dried mycelium per litre of culture medium. Different parameters were measured in the exponential phase [20]: biomass productivity (BP), specific

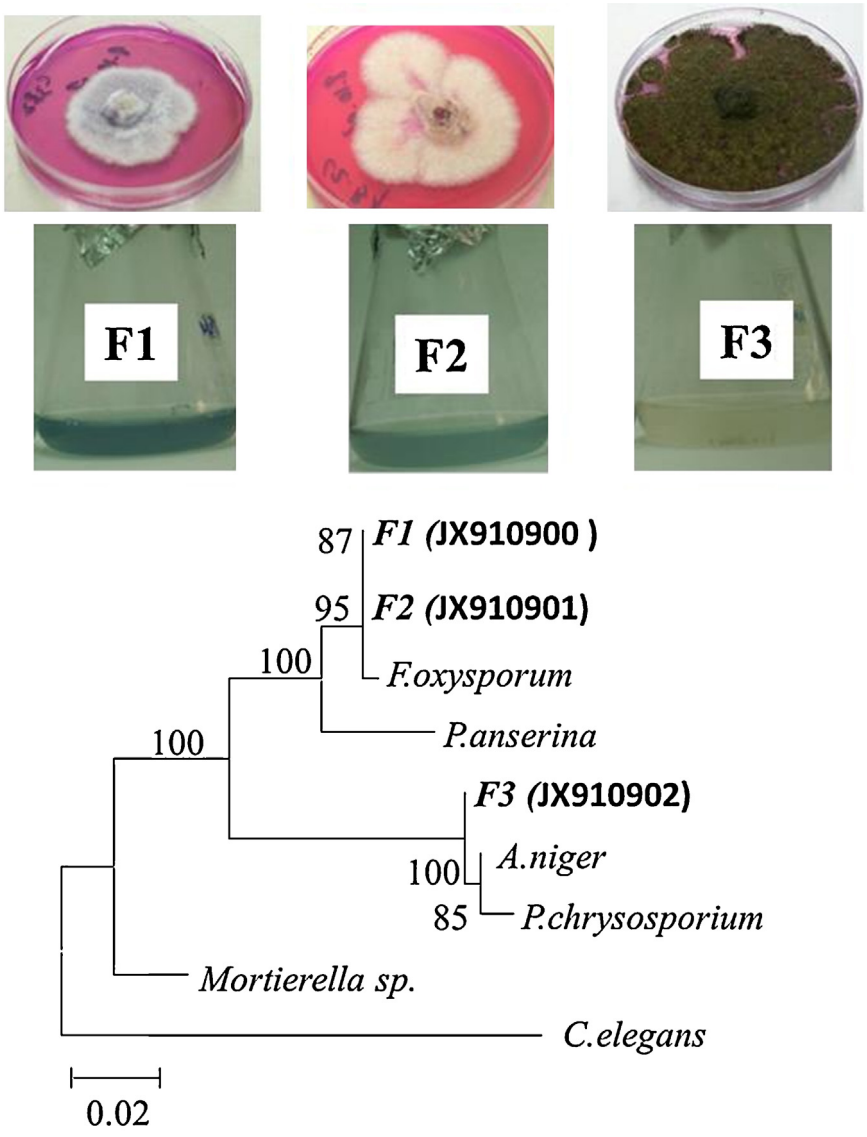

Fig. 1. Morphological characteristics of the fungal F1, F2 and F3 isolated at the preconditioning period from the winery waste vermicomposting and phylogenetic tree. The branch support was estimated using 100 bootstrap replicates.

growth rate (μ), and biomass duplication time. All culture media were buffered at pH 7.0, this being similar to the pH measured in the winery wastes in the pre-processing period of the vermicomposting process.

2.6. Chemical analysis

Liquid media contained in the flasks at different times were centrifuged (8000 rpm) and supernatants were filtered through 0.22 μm PTFE syringe filter (Fisher scientific) to be analyzed by solid-phase micro-extraction coupled to a gas chromatograph with a mass-spectrometry detector (SPME-GC/MS), followed the methodology of Risticevic et al. [21]. Two fibres, 85 μm polyacrylate (PA)-coated fibre and a 100 μm polydimethylsiloxane (PDMS)-coated fibre from Supelco (Bellefonte PA, USA) were employed for DCA and metabolite analysis (Figs. A.1 and A.2). The fibres were conditioned at 300 °C for 1 h (PA) or 250 °C for 0.5 h (PDMS) prior to use. The fibres were immersed directly into 9 ml of each sample for 10 min (PA) or 1 h (PDMS), immediately drawn back into the needle and transferred without delay (less than 5 s) into the injection port of the GC. The incubation and extraction temperature was 35 °C. The speed of the magnetic stirring was 250 rpm. A desorption time of 7 min at 280 °C (PA) or 10 min at 250 °C (PDMS) was used. Blanks were periodically desorbed. 2,4,5-Trichloroaniline at 30 μg l^{-1} was used as an internal standard. Splitless injection was employed. The analyses were performed on a Varian Model 450GC coupled to a 240 MS detector. Two columns (Varian FactorFour™ 5 ms) of different sizes (30 m × 0.25 mm × 0.25 μm film and 20 m × 0.15 mm × 0.15 μm film) from Agilent (Waldbronn, Germany) were used for the metabolite analysis with the PA and PDMS fibres, respectively. Helium was the carrier gas. The GC oven programme for the PA fibre was: initial temperature 50 °C held for 7 min, ramped at 20 °C min^{-1} to 250 °C and held for 2 min, for a total run time of 19 min. For the PDMS fibre the oven programme was: 50 °C for 10 min, ramped at 20 °C min^{-1} to 250 °C, ramped at 30 °C min^{-1} to 300 °C and held for 2 min, for a total run time of 23 min. The interface was kept at 280 °C, the ion trap at 240 °C. The mass spectra were determined at electron energy of 70 eV. The analyses were operated in full scan/SIM mode method. The parent compound and metabolites were quantified by using calibration curves ranging from 5 to 100 μg l^{-1} from the corresponding analytical standards

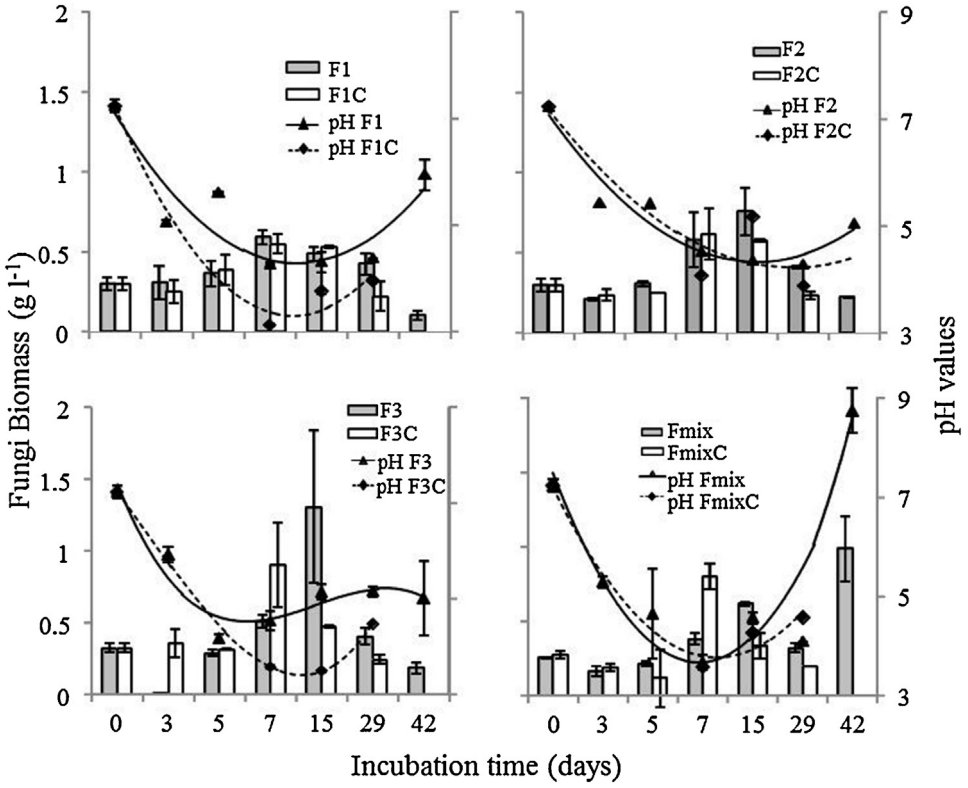

Fig. 2. Growth and pH values of the isolated and the consortia fungal incubated in culture media with (F1, F2, F3 and Fmix) and without (F1C, F2C, F3C and FmixC) 3,4-dichloroaniline.

($R^2 > 0.994$). The most probable metabolite structures were based on their retention times, fragment ions of the standard (Table A.1) and by using NIST library spectra included in the MS Workstation software 6.9.1.

2.7. Kinetic models

The experimental data of DCA degradation were fitted to two kinetics models [22]: the single first-order model (SFO) and the first-order multi-compartment model (FOMC). The SFO model assumes that the number of pesticide molecules is small relative to the number of degrader microorganisms and their enzymes, or water molecules in the case of hydrolysis. The first-order multi-compartment (FOMC) corresponds to bi-phasic parameters; a quick initial decrease in pesticide concentrations is often followed by a slower decline. DCA and metabolite persistence were calculated by using the DT_{50} and DT_{90} values. The chi-squared test (χ^2) was used to evaluate the goodness of fit for each model and the quality of the measured data. A significance level of $\alpha = 0.05$ was used to validate the models. The model with the smallest χ^2 values described the experimental data best. The statistical *t*-test was used to check the value found for the degradation rate constant (k).

It is important to point out that when metabolites were determined in quantities of less than 10% of the parent compounds, i.e. <1.5 $\mu g\,ml^{-1}$, they were considered minor metabolites. Furthermore, the model fit gives more weight to parent compound and major metabolites; hence those minor metabolites cannot be optimized together within the model. In these cases, only the experimental declination curve was considered for modelling.

2.8. Statistical analysis

Statistical differences among the biomass productivity for each treatment were performed using Kruskal–Wallis non-parametric test. A *post hoc* pairwise comparison was performed by using Tukey's multiple-comparison test at a risk level of $\alpha = 0.05$. Analysis of variance (ANOVA) was used to determine significant differences in the specific growth rate (μ) and duplication time (Td), where Tukey's parametric test was used to compare each treatment.

Table 1
Biomass productivity (BP) and kinetic parameters (μ and Td) for the fungal growth with (F1, F2, F3 and the mixture consortia Fmix) and without (F1C, F2C, F3C and FmixC) DCA.

Treatment	BP ($g\,l^{-1}\,day^{-1}$)	Mean rank	F1	F1C	μ, $days^{-1}$	Td, days
F1	0.02 ± 0.01	4.3			0.17 ± 0.11 c,d	3.12 ± 0.64 b,c
F1C	0.01 ± 0.02	4.0	0.30		0.19 ± 0.04 c,d	3.79 ± 0.77 b,c
F2	0.05 ± 0.01	18.3	14.00[a]	14.33[a]	0.42 ± 0.24 b,c	2.38 ± 1.92 b,c
F2C	0.03 ± 0.01	9.7	5.33	5.67	0.44 ± 0.13 b,c	1.68 ± 0.50 b,c
F3	0.07 ± 0.05	18.0	13.67[a]	14.00[a]	0.10 ± 0.05 d	8.28 ± 4.26 a
F3C	0.04 ± 0.02	15.7	11.33[a]	11.67[a]	0.50 ± 0.17 b	1.48 ± 0.49 c
Fmix	0.03 ± 0.00	12.0	7.67	8.00	0.13 ± 0.11 d	6.13 ± 2.33 a,b
FmixC	0.05 ± 0.01	18.0	13.67[a]	14.00[a]	0.96 ± 0.06 a	0.73 ± 0.08 c

[a] Significant differences in BP from Kruskal–Wallis statistic analyze lower case letters are significant differences from Tukey test.

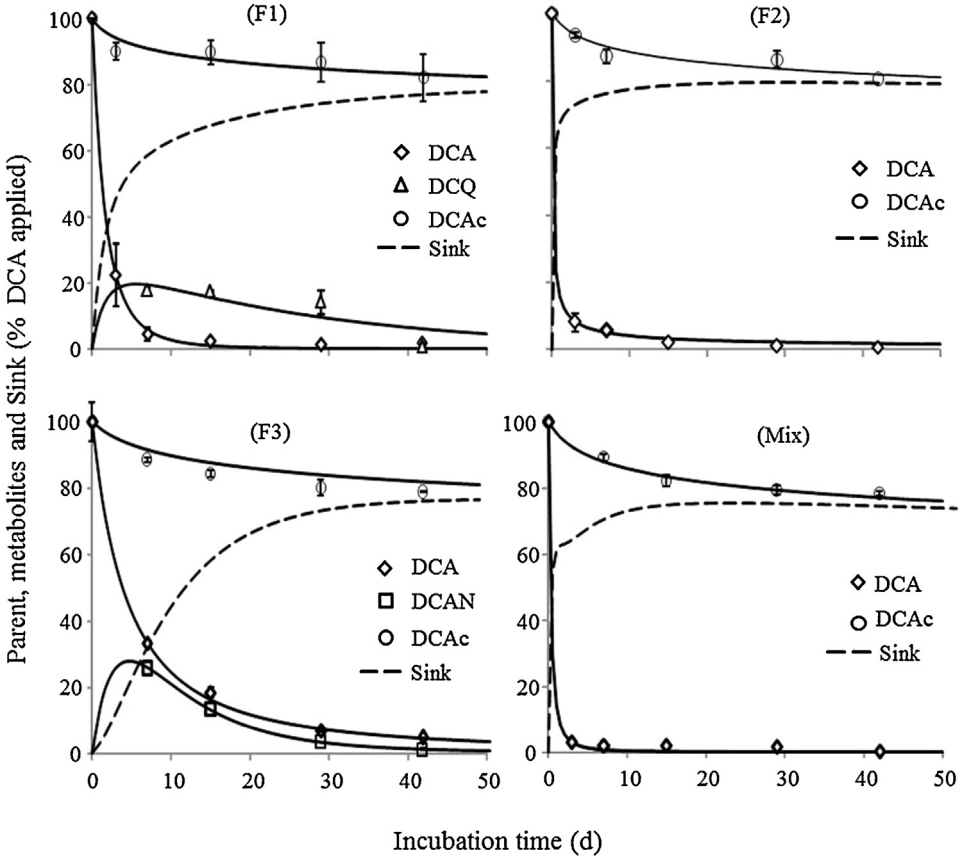

Fig. 3. Biodegradation of DCA and of the metabolites DCQs and DCAN by *F. oxysporum* strains (F1 and F2), *A. niger* (F3) and the fungal consortia (Fmix). Controls of heat-killed mycelium were made for each treatment. Error bars are standard deviations in the triplicate samples.

3. Results and discussion

3.1. Isolation and identification of DCA degrading fungi

Three fungi (F1, F2, and F3) were selected for their growth on DCA as a nitrogen source. After 7 days from inoculation, F1 and F2 strains showed cottony mycelia white or grey on top and pale pink underneath whereas colonies from F3 had black mycelia (Fig. 1). These fungi are part of the aerobic microbial population capable of growth in competition with other microorganisms inhabiting the winery wastes. Under the assayed experimental conditions, the liquid media from F1 and F2 proved blue in colour but the F3 media did not. However, the three fungal strains used glucose as carbon and energy source because the 3,4-DCA was not an easily available carbon source. Emtiazi et al. [23] reported that *Fusarium* sp. isolated from activated sludge was able to utilize aniline as the sole nitrogen, carbon, and energy source with the production of acetanilide and catechol but its chloroaniline derivatives were utilized as a nitrogen source only in the presence of glucose.

The sequences of the small-subunit (SSU) 18Sr ribosomal RNA gene of these fungi were compared with those available in the GENEBANK database by using the Blast programme (Figure 1). The sequences of F1 and F2 with 764 bp were 99% identical to that of *Fusarium oxysporum* and were deposited in NCBI database (F1 and F2 Accession Number JX910900 and JX910901, respectively). The sequence from F3 with 767 bp was 99% identical to that of *Aspergillus niger* (Accession No. JX910902). The phylogenetic analysis of these sequences by distance methods showed that F1 and F2 were closely related to *F. oxysporum*, which is also a DCA-degrading fungus [24]. The branches of these strains were related with the *Podospora anserina* strain (Fig. 1). On the other hand, F3 was closely associated with *A. niger* which in turn had a short branch distance from *P. chrysosporium*. The relationship among branches of these fungi and the capacity of growth in the presence of DCA indicated that these have a common ancestor, a contention supported by high bootstrap values (1 0 0).

3.2. Growth of fungal strains on DCA

In all treatments, there was a lag phase of 3 or 5 days before the exponential phase, which may be associated with adaptation to the culture medium (Fig. 2). A significant decrease in mycelia on the 3rd day was measured for F3. The exponential fungal-growth phase reached its maximum value on the 7day of incubation for F1 and for all the controls without DCA (F1C, F2C, F3C, and FmixC) while it was reached a week later (15 days) for F2, F3, and Fmix. An increment was detected after 42 days in the Fmix culture, probably due to the slower F3 growth.

Significant differences in biomass production (Table 1) were found with the Kruskal–Wallis statistical test ($p < 0.05$). Pairwise comparisons between treatments by Tukey's test showed that BP from F1 and F1C was different from F2, F3, F3C, and FmixC.

The specific growth rate (μ) values revealed that when DCA was added to the media, fungal growth decreased significantly only for F3 and Fmix cultures, these having the longest duplication times. These results suggest that DCA significantly affects fungal growth, especially for F3 and Fmix.

A decrease in pH values during the exponential growth stage gave values between 3.0 and 4.0 (Fig. 2). This may be due to the release of organic acids as the result of the glycolysis during fungal growth. In fact, *A. niger* is used in industry to produce citric acid.

Table 2
Kinetic parameters for the biodegradtion of DCA by the fungal F1, F2, F3 and the fungal consortia (Fmix). Mean of duplicate samples ± standard deviation.

	F1	F2	F3	Fmix
Pseudo first order kinetic model				
Ci (%)	99.94 ± 2.95	99.98 ± 1.97	98.32 ± 1.41	100 ± 1.15
k (day^{-1})	0.48 ± 0.08	0.74 ± 0.17	0.13 ± 0.01	0.80 ± 1.24
DT_{50} (days)	1.41	0.93	5.07	0.85
DT_{90} (days)	4.70	3.09	16.85	2.85
χ^2	5.2	7.1	14.3	3.3
t-Test	<0.001	<0.05	<0.001	<0.05
First order multicompartment kinetic model				
Ci (%)	99.98 ± 2.64	100 ± 0.66	99.78 ± 2.84	–
α	3.25 ± 0.31	0.53 ± 0.11	1.61 ± 0.63	–
β	6.13 ± 0.72	0.05 ± 0.04	7.22 ± 4.41	–
DT_{50} (days)	1.45	0.13	3.81	–
DT_{90} (days)	6.30	3.57	22.82	–
χ^2	5.4	3.1	8.3	–

Except for F3, after the exponential phase the pH rose, reaching the highest value in Fmix culture.

3.3. Biodegradation of DCA and analysis of metabolites

Despite the lag phase detected in fungal growth (Fig. 2), the biodegradation of DCA occurred mainly in the first 7–15 days of incubation, either separately or in the consortium (Fig. 3). The amount of DCA degraded after 7 days was higher than 90%, except for F3, with 70% degradation. However, the mass balance (quantification of DCA adsorption, metabolites, and remaining DCA concentration) indicated a progressive increment of unidentified degradation products known as Sink (Figs. 3 and 4). This rapid degradation of DCA by *Fusarium* species, and to a lesser extent by *A. Niger*, must be related to different strategies that *F. oxysporum* has for survival under limited conditions such as denitrification, heterolactic, acid fermentation, or ammonia fermentation [25], implying a high metabolic potential against a variety of xenobiotics [12,26]. Despite the larger amount of DCA remaining in F3, the biomass productivity in this medium was higher than in the media inoculated with F1 and F2 (Table 1). This may be due either to the transformation of DCA into less toxic compounds by F3 or else to its higher tolerance to DCA and biodegradation products unlike F1 and F2 (Figs. 2 and 3).

Both the SFO and FOMC models were well adjusted to the experimental degradation curves from F1, F2, and F3 media, but only the SFO model was fitted to the experimental curve from Fmix. The goodness of fit for each model showed that FOMC was the best model to explain the DCA degradation by F1, F2, and F3 treatments (Table 2 and Fig. 3). The β values indicate a significantly lower degradation rate for DCA in the F3 culture with respect to the others. The DT_{90} values, which are more appropriate to estimate the compound persistence in biphasic models [22], indicate that DCA in the F3 culture was 3- and 8-fold more persistent with respect to F1 and F2. These differences in the degradation kinetics agree with the fungal-growth parameters measured (Table 1).

The degradation of DCA by the fungi generated various metabolites (Fig. 4) which were not detected in the controls, pointing to biodegradation as the main degradation pathway. In the cultures with *F. oxysporum* (F1 and F2) at 7 days of incubation the metabolites TCAXB, TCAZ, DCQs, and DCAN were detected (Fig. 4) and quantified. The DCQ isomers registered the highest amounts (17.7% and 4.6% of applied DCA, respectively) followed by TCAXB and TCAZB. The metabolite DCNB was quantified at day 15 in F1 and at day 7 in F2 (0.015 and 0.24 μg ml^{-1}, respectively). After 29 days of incubation, a new peak was determined at 0.004 and 0.011 μg ml^{-1} in F1 and F2, respectively (<10% of applied DCA). This peak matched the DCAN structure. All the metabolites TCAXB, TCAZB, DCNB, and DCAN remained below the 10% of total applied

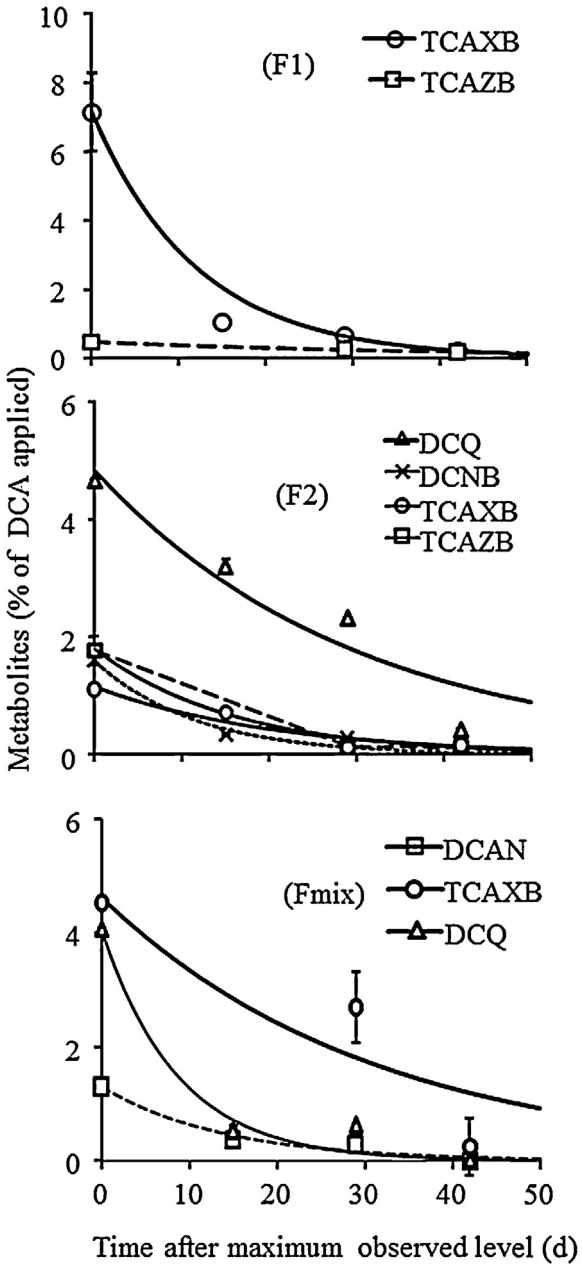

Fig. 4. Decline of the metabolites DCAN, DCNB, DCAXB, DCAZB and DCQs from the maximum observed during the biodegradation of DCA by the fungi strains. Error bars are standard deviations in the triplicate samples.

Table 3
Kinetic parameters for the biodegradation of DCA metabolites by fungal F1, F2, F3 and the fungal consortium (Fmix). Mean of duplicate samples ± standard deviation.

Metabolites	F1	F2	F3	Fmix
***kd* (day^{-1})**				
3,4-DCNB	–	0.08 ± 0.03	–	–
3,3′,4,4′-TCAXB	0.08 ± 0.013	0.04 ± 0.02	–	0.03 ± 0.012
3,3′4,4′-TCAZB	0.02 ± 0.030	0.06 ± 0.04	–	–
3,4-DCAN	–	–	0.17 ± 0.045	0.07 ± 0.028
DCQ	0.04 ± 0.013	0.05 ± 0.01		0.11 ± 0.068
ffM (%)				
3,4-DCNB	–	0.06 ± 0.01	–	–
3,3′,4,4′-TCAXB	0.14 ± 0.01	0.02 ± 0.00	–	0.12 ± 0.08
3,3′4,4′-TCAZB	0.005 ± 0.02	0.04 ± 0.03	–	–
3,4-DCAN	–	–	0.79 ± 0.15	0.03 ± 0.06
DCQ	0.28 ± 0.06	0.07 ± 0.01	–	0.19 ± 0.03
DT_{50}				
3,4-DCNB	–	8.11	–	–
3,3,4,4′-TCAXB	8.32	14.54	–	21.47
3,3′4,4′-TCAZB	30.90	11.04	–	–
3,4-DCAN	–	–	4.0	9.62
DCQ	19.19	14.71	–	6.02
DT_{90}				
3,4-DCNB	–	26.96	–	–
3,3′,4,4′-TCAXB	27.64	48.30	–	71.32
3,3′4,4′-TCAZB	102.67	36.67	–	–
3,4-DCAN	–	–	13.4	31.96
DCQ	63.74	26.96	–	20.01
χ^2				
3,4-DCNB	–	8.7	–	–
3,3′,4,4′-TCAXB	0.4	7.9	–	0.46
3,3′4,4′-TCAZB	2.0	–	–	–
3,4-DCAN	–	–	4.9	10.10
DCQ	13.0	–	–	12.41
***t*-Test**				
3,4-DCNB	–	<0.05	–	–
3,3′,4,4′-TCAXB	<0.05	<0.05	–	<0.05
3,3′4,4′-TCAZB	<0.05	<0.1	–	–
3,4-DCAN	–	–	<0.001	<0.001
DCQ	<0.05	<0.05	–	<0.05

ffM, formation fraction of metabolite.

DCA. Other peaks detected matched the 3,4-dichlorobenzene (DCB) structure (Fig. 4). In the *A. niger* culture at the day 3 of incubation, only DCAN was detected at concentration higher than 3.93 μg ml^{-1} (25.8% of applied DCA).

In the Fmix culture, the first metabolite determined was DCAN, but in lower amounts with respect to F3 (0.2 μg ml^{-1} and 0.045 μg ml^{-1} for day 3 and 29, respectively). On day 7, 0.62 μg ml^{-1} of DCQ isomers was determined. TCAXB appeared in a small amount (0.16 μg ml^{-1}) and the TCAZB peak appeared but was below the detection limit.

Experimental declination curves of the metabolites showed a proper fit to the SFO model with a significant χ^2 (Table 3). The declination constants (*kd*) in F1, followed the sequence: TCAXB > DCQ > TCAZB. By contrast, the sequence in F2 was: DCNB > TCAZB ≈ DCQ > TCAXB. The highest amount of the metabolite DCAN was determined in the F3 cultures but its persistence was also lowest (*kd* = 0.17 day^{-1}, DT_{50} = 4 days). In the Fmix the metabolite persistence was: DCQ > DCAN > TCAXB.

In general, the DT_{50} values indicate a high potential to degrade theses metabolites in the early stages, but the DT_{90} values reveal a slower degradation with time, perhaps due to the limited nutrients under these experimental conditions. Nevertheless, the fact that the persistence measured was relative should not be disregarded, since it depended on the amount of metabolite formed and the time when it occurred for each treatment. Detailed information of chromatograms (Table A.1) and mass spectra of the metabolites determined in the biodegradation of 3,4-DCA (Figs. A1 and A.2) are shown in Appendix A.

3.4. Degradation pathway of DCA by fungi

The fungi clearly varied in the rate of degrading DCA. It was also clear that DCA affected the fungal growth. Furthermore, differences were found in the formation and decay of these metabolites. For all these reasons, various strategies in the metabolism of DCA were considered to propose the pathway illustrated in Fig. 5. Thus, the presence of DCNB, DCB, TCAZB, TCAXB, and DCQs in *F. oxysporum* cultures and only of DCAN in the *A. niger* culture indicated different catabolic reactions. The metabolite DCNB determined came from the sequential oxidation of the aniline's amine group to nitro group. Peroxidases such as chloroperoxidases are fungal enzymes having a structure similar to cytochrome P450 and are able to convert the arylamine functional group to the nitroso oxidation state [27] by the introduction of an oxygen atom from hydrogen peroxide to the substrate, acting as monooxygenases catalyzing the heteroatom oxidation (nitrogen in this case). Within this oxidation path, other intermediates may be formed, such as *N*-(3,4-dichlorophenyl)-hydroxylamine (DCPHA) and dichloronitrosobenzene (DCNOB) [28] (Fig. 5). These intermediates were not detected, however, the detection of DCB (Table A1 and Fig. A1) indicates a reduction by denitrification. Shoun et al. [29] reported that *F. oxyspurum* and *F. solani* have a peculiar denitrifying system where NO is reduced to N_2O by nitric oxide reductase cytochrome P450nor. This process is called co-denitrification, in which a hybrid N_2 or N_2O species is formed upon combination of nitrogen atoms from NO and other nitrogen compounds (nitrogen donor). According to Oshima et al. [30], the denitrifying system in *F. oxysporum*

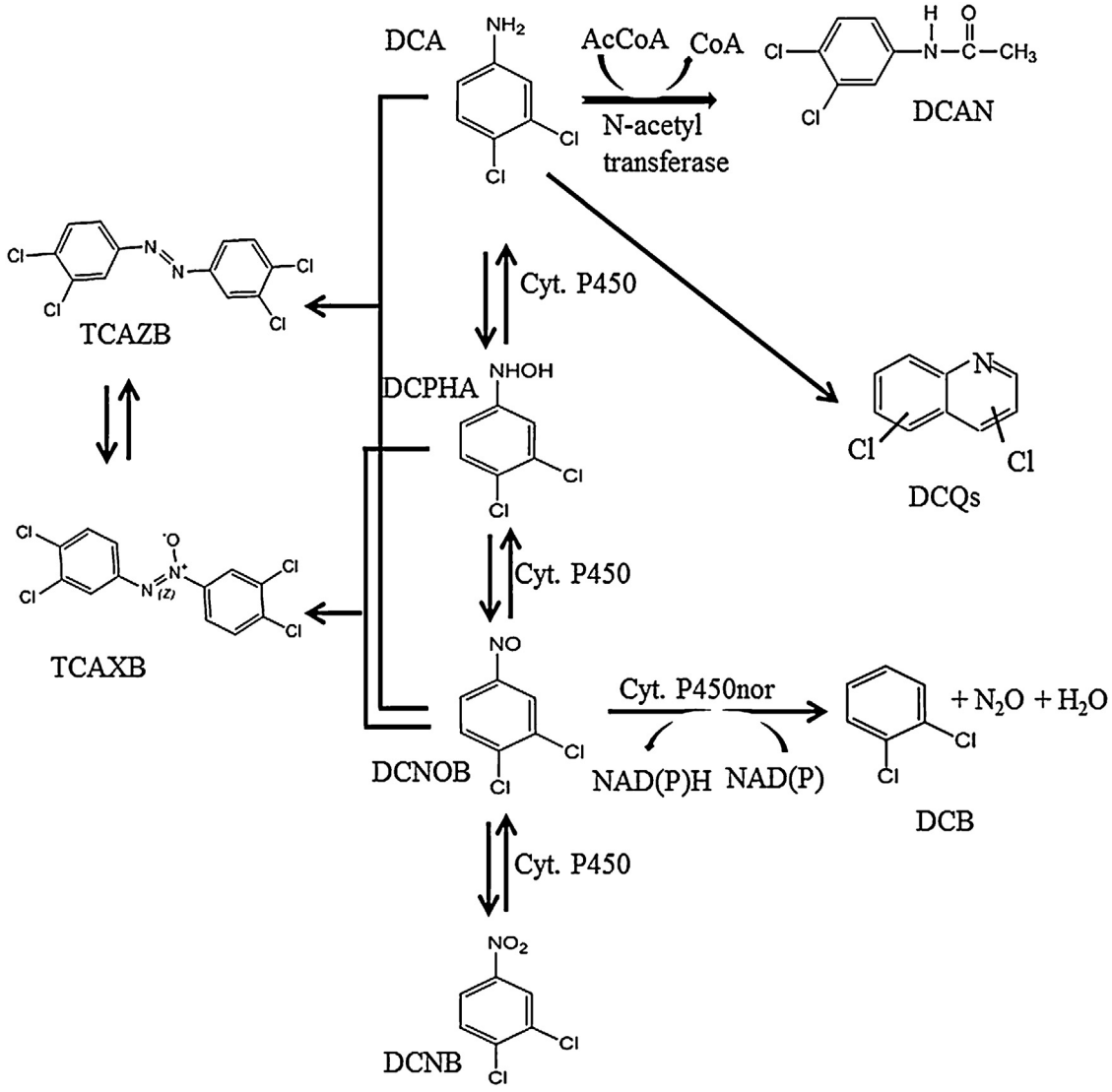

Fig. 5. Biodegradation pathways for DCA by *F. oxysporum* strains (F1 and F2) and *A. niger* (F3).

MT811 can produce N_2 as the co-denitrification product, but it cannot form N_2 by denitrification. Cytochrome P450nor involves a multi-functional detoxifying enzyme. Thus, the nitrite group from DCNB would be reduced to NO generating the metabolite DCNOB. Two DCNOB molecules by the co-denitrification reaction by P450nor released N_2O and the DCB metabolite (Fig. 5).

Azo and *azoxy* dimers (TCAZB and TCAXB) were present probably because DCA and hydroxylamine (DCPHA) can react chemically with the C-nitroso compound (DCNOB) [28]. These *azo* compounds have been found in *Filaboletus* sp., *P. chrysosporium* and *Geotrichum candidum* cultures growing in the presence of DCA, where lignin peroxidase and aniline oxidase have been involved [31]. These *azo* dimers can undergo a metabolic activation where an electrophilic nitrenium ion can be produced and subsequently react with DNA and RNA, becoming a mutagenic compound [32]. The strategy of *F. oxysporum* strains may likely be the formation of harmless polymeric compounds. Polyphenoloxidase enzymes such as laccase catalyze the coupling of phenolic compounds by fungi under limited nitrogen conditions. Laccase can catalyze the four-electron reduction of molecular oxygen to water, coupled with the concomitant oxidation of organic substrate, producing four radicals, which can react to give dimers, oligomers, and polymers [28]. Within these oligomers, the formation of DCQs can be a detoxification mechanism. Aromatic amines (AA) with ortho-position free as DCA can be a substrate in different chemical reactions in the synthesis of quinolines. Furthermore, it is known that fungi can produce quinolines [33,34].

In the case of the *A. niger* media the presence of DCAN as a major metabolite supports the idea that the *N*-acetylation reaction must be the main biodegradation path. This reaction leads to the detoxification of AA and is also involved in mechanisms of tolerance to xenobiotics [35] through the production of chemically stable arylacetamides. The *N*-acetyltransferase (NAT) catalyses the transfer of acetyl groups from the acetyl-Coenzyme A (AcCoA) to arylamines, where AcCoA acts as an acetyl donor, while the primary amine of DCA is the acceptor. Sandermann et al. [3] reported the biodegradation of DCA by *P. chrysosporium*, mediated by a coenzyme A transferase. Metobromuron was also acetylated to *p*-bromoacetanilide by *F. oxysporum* and *Talaromyces wortmanii* [36]. Martins et al. [35] identified and characterized a NAT2 enzyme that is required for the growth and survival of the filamentous ascomycete *P. anserina* in the presence of toxic AA. The catalytic efficiency of *A. niger* to transform DCA may be related to the presence of 5 putative genes encoding the NAT enzymes [35]. This enzyme provides an alternative strategy to confer herbicide tolerance to microorganisms and a detoxification pathway, since the

acetylated metabolite is less toxic for *Vibrio fischeri* than DCA [37]. This fact may be related to the peak biomass observed for *A. niger* after 15 days (Fig. 2), where the amount of DCA was even more than 2-fold higher with respect to *F. oxysporum* strains (Fig. 3) and support the contention that *A. niger* despite its slower development (Fig. 1) was more tolerant to DCA.

4. Conclusions

The literature reveals that DCA is a persistent toxic metabolite from the phenylureas biodegradation by bacteria. An alternative to degradation of DCA is the use of fungi. *F oxysporum* and *A. niger*, isolated during preconditioning period in the vine-shoot vermicomposting, that had the ability to degrade DCA, alone or in consortium, across multiple degradation pathways. The presence of different metabolites such as dichlorobenzene, dichloroquinoline, dichloroacetanilide among others, indicated a high biodegradation potential of these fungi and a stronger tolerance to the DCA, contributing to the possible mineralization under environmental conditions. These results demonstrate the biocatalytic potential of the microbiota involved in the vermicomposting of agroindustrial wastes with respect to the biodegradation of persistent metabolites such as the DCA. Thus, vermicomposting process can also be considered to be a microorganism source with a high catabolic potential that offers a new low-cost alternative in applying environmental biotechnologies in the DCA degradation. Similarly, this microbial potential can be applied under controlled conditions as a bioremediation system, although this requires further investigation.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2013.12.052.

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Abstract: Soil organic amendment affects biotic and abiotic processes that control the fate of pesticides, but the treatment history of the soil is also relevant. These processes were assessed in a multidisciplinary study with the aim of optimizing pesticide mitigation in soils. Soil microcosms pre-treated (E2) or not with diuron (E1) were amended with either winery (W) or olive waste (O) vermicomposts. Herbicide dissipation followed a double first-order model in E1 microcosms, but a single first-order model in E2. Also, diuron persistence was longer in E1 than in E2 (E1-DT50>200d-1, E2-DT50<16d-1). The genetic structure of the bacterial community was modified by both diuron exposure and amendment. O-vermicompost increased enzymatic activities in both experiments, but diuron-degrading genetic potential (puhB) was quantified only in E2 microcosms in accordance with reduced diuron persistence. Therefore, O-vermicompost addition favoured the proliferation of diuron degraders, increasing the soil diuron-depuration capability.

***Novelty Statement**

Given the environmental issue caused by the extensive use of pesticides, there is a need to develop techniques to mitigate their use and ensure a safe use of them. In this context, this study applies a multidisciplinary approach (physico-chemical and biochemical/molecular analysis) to evaluate the use of vermicomposted agroindustrial waste amendments in arable soil as strategy to mitigate the persistence of the phenylurea herbicide diuron in soils by different processes. We show that vermicomposts affected the bioavailability and degradation kinetics of diuron, and provide novel information about the impact of this mitigation technique on the evolution of PUH-degrading genetic potential.

Highlights

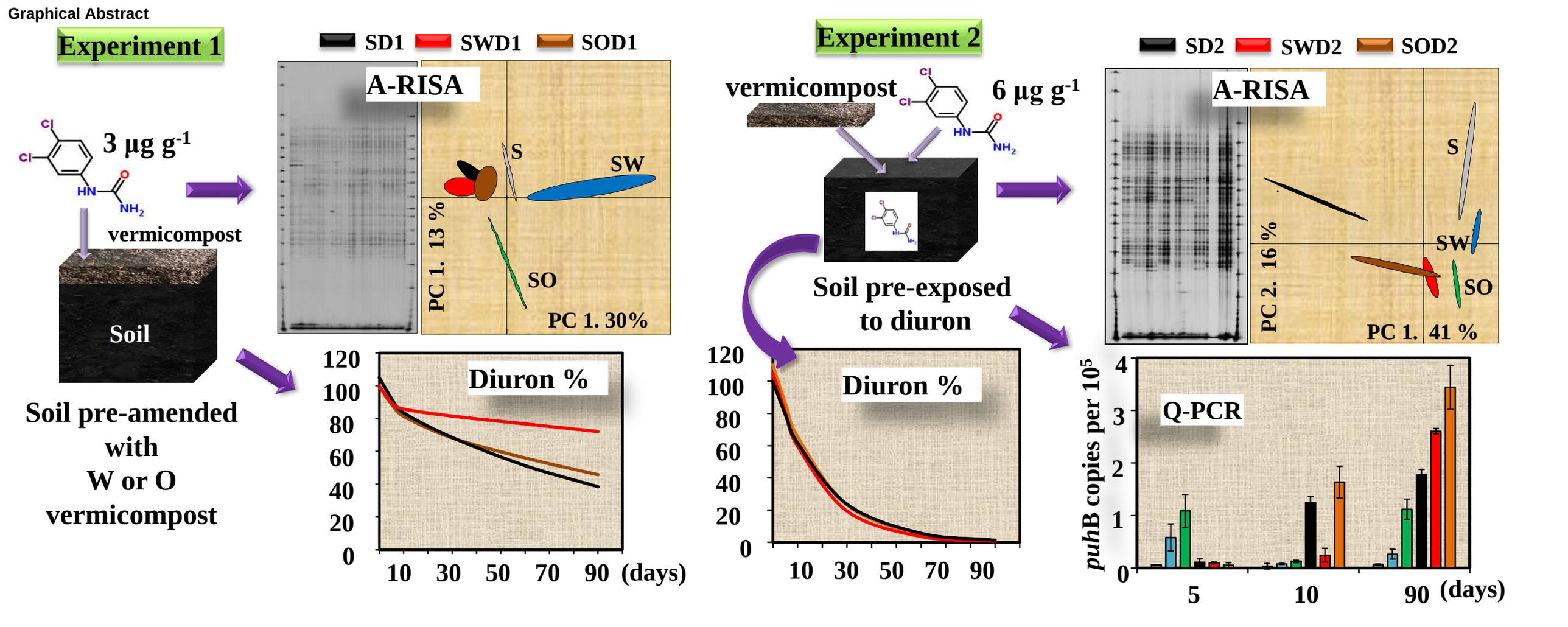
- The genetic structure of soil bacterial community was transiently affected by diuron.
- Soil amended with vermicompost regulated diuron persistence in soil.
- *puhB* abundance increased after bacterial-community pre-exposure to diuron.
- O-vermicompost mitigated diuron fate by improving microbial activity.

Abstract.

Soil organic amendment affects biotic and abiotic processes that control the fate of pesticides, but the treatment history of the soil is also relevant. These processes were assessed in a multidisciplinary study with the aim of optimizing pesticide mitigation in soils. Soil microcosms pre-treated (E2) or not with diuron (E1) were amended with either winery (W) or olive waste (O) vermicomposts. Herbicide dissipation followed a double first-order model in E1 microcosms, but a single first-order model in E2. Also, diuron persistence was longer in E1 than in E2 (E1-DT_{50}>200d^{-1}, E2-DT_{50}<16d^{-1}). The genetic structure of the bacterial community was modified by both diuron exposure and amendment. O-vermicompost increased enzymatic activities in both experiments, but diuron-degrading genetic potential (*puh*B) was quantified only in E2 microcosms in accordance with reduced diuron persistence. Therefore, O-vermicompost addition favoured the proliferation of diuron degraders, increasing the soil diuron-depuration capability.

Keywords: Diuron; Vermicompost; Biodegradation; ARISA fingerprinting; Phenylurea hydrolase genes.

Graphical Abstract



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Multidisciplinary assessment of pesticide mitigation in soil amended with vermicomposted agroindustrial wastes

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1. Introduction

To ensure the yield of crops, modern agriculture worldwide relies on the extensive use of pesticides such as the phenylurea herbicides [1]. Among these, diuron is a systemic herbicide widely applied in soils to control weeds in different agricultural crops [2]. In addition to be considered relatively persistent in soil [3], diuron has been detected in surface waters [4] and groundwater [5-7]. Consequently, diuron had been included in the list of priority pollutants by the EU Water Framework Directive (Directive 2000/60/EC).

As there is no alternative immediately available to remove pesticides entirely from agriculture, there is an urgent need to develop new techniques to ensure their environmentally safe use. Among innovative techniques, mitigation solutions to reduce the persistence and the risk of transfer of pesticides are of prime interest. The most widely known include the use of plant buffer strips [8, 9] and organic amendments [10, 11] such as the application of agro-industrial wastes, which could be previously stabilized by vermicomposting [12, 13] and composting [14], increasing the soil organic matter (SOM), microbial activity, and mineralization of recalcitrant material as well as the removal of xenobiotic residues by promoting biotic (biodegradation) and abiotic (adsorption) processes [15, 16]. This application promotes soil-purification capabilities and constitutes a low-cost, eco-friendly solution, offering an alternative to mitigate xenobiotic compounds such as pesticides. Nevertheless, developing and assessing the efficiency of the mitigation solutions remains a challenge. For this objective, biochemical approaches could be applied to estimate the activity of some oxidoreductases and hydrolytic enzymes (e.g. dehydrogenase and urease enzymes, respectively) in soil and used as proxies of the ability of its native microbiota to degrade several organic substrates and xenobiotics and as enzymatic indicators of soil

microbiological activity as well [12, 17]. Similarly, molecular approaches can also be applied to estimate the abundance of specific pesticide-degrading gene sequences, providing insight into the pesticide-degrading potential of the soil microbial community [18]. Recently, Pesce et al. [19] have developed a qPCR assay targeting *puhA* and *puhB* genes coding for the enzyme phenylurea hydrolase (PUH) that has been found in *Arthrobacter globiformis* D47 [20] and *Mycobacterium brisbanense* [21]. This allows the quantification of these genes in river sediments, demonstrating the value of this technique for monitoring genetic potential of diuron-degrading capacity. However, as underlined by Bers [22], these types of studies relating pesticide-degrading gene potential to pesticide-degrading activity remain scarce in the soil environment, and no studies available have evaluated the impact of mitigation techniques on the evolution of PUH-degrading genetic potential.

To fill this gap of knowledge, the present work seeks to evaluate the effect of mitigation approaches that use vermicomposts on PUH-degrading gene potential and degrading activity. Thus, microcosm experiments were established to evaluate the effect of two vermicomposts prepared from two-phase olive-oil waste (wet olive cake or *alperujo*) (O) and wine waste (vine shoots) (W) on diuron degradation. The fate of diuron (sorption, biodegradation, and metabolites formation) was monitored and interpreted in light of biochemical (dehydrogenase and urease activities) and molecular (genetic structure of the bacterial community and abundance of *puh*A and *puh*B genes) parameters, describing the evolution of the soil microbial community, including diuron-degrading populations.

2. Materials and methods

2.1 Soil amendments and chemicals

The soil was collected in an agricultural area under crop rotation in south-eastern Spain (37º 22´19.41´´N, 3º 36´5.54´´W). Soil samples from the uppermost 20 cm were air dried and sieved through a <2 mm mesh. The soil was a calcareous (43% $CaCO_3$) silty-clay loam (11% sand, 49% silt, and 40% clay). Soils were amended either with O or W-vermicompost obtained as described by Elvira et al. [23]. Both vermicomposts were ground and sieved through a < 2 mm mesh. Physico-chemical properties of both matrices were determined following established methodologies [12, 24] (Table 1).

Diuron (D) and its N-demethylated products, 3-(3,4-dichlorophenyl)-1-methylurea (DPMU) 99% purity, 3,4-dichlorophenylurea (DPU) 97.5 % purity and 3,4-dichloroanyline (DCA) with 99.5% purity, were supplied by Dr. Ehrenstorfer (Augsburg, Germany).

2.2. Sorption measurements

Sorption-desorption isotherms of diuron and its metabolites were determined following the classical batch-sorption method [24]. For sorption measurement, 5 g of dry soil sample, in triplicate, were placed in 50-ml glass tubes, and 20 ml of an aqueous standard solution containing diuron at five concentrations ranging from 1 to 5 mg L^{-1} were added. Desorption was measured using an herbicide concentration of 5 mg L^{-1} in 0.01 M $CaCl_2$ solution. The adsorption and desorption rate constants (K_{ads} and K_{des}) and apparent hysteresis index (H) were established fitting the data to a linear Freundlich model [24].

2.3. Experimental setup

Two different experiments were conducted to test the vermicompost effect on diuron degradation in soil. The first experiment, E1, involved the study of diuron dissipation in soil samples submitted to organic amendments with two types of vermicompost. Soil samples were amended twice with 5% either with O-vermicompost (SO) or W-vermicompost (SW) and aged after each vermicompost addition by a 3-month incubation at 20ºC in darkness and at 80% field capacity (i.e. in total 10% amendment). Thereafter, 40 g (dw) of soil (S), SO and SW soils were placed in triplicate in a 100 ml glass flask and spiked with diuron at 3 µg g^{-1} (equivalent to 7.8 kg ha^{-1} at a depth of 20 cm). Diuron treatments (SD1, SWD1, and SOD1), were left to evaporate overnight, homogenized, and finally incubated for 3 months under the conditions described above.

The second experiment, E2, was designed to evaluate the effect of the vermicompost amendment in a soil previously treated with diuron. Thus, the soil samples were firstly fortified with 3µg g^{-1} of diuron and aged by incubation for 3 months under the same condition as E1. Afterwards, soil fractions of 40 g (d.w.) in triplicate were amended with 10% of either W or O vermicompost and spiked with diuron to reach a final concentration of 6 µg g^{-1}± 0.42. The treatments of E2 (SD2, SWD2, and SOD2) were incubated as described for E1. Soil samples from E1 and E2 were analysed at different incubation times (0, 7, 10, 30, 60, and 90 days) to monitor the dissipation of diuron.

2.4. Chemical Analysis

An Agilent series 1100 high-performance liquid chromatography (HPLC) (Agilent, Germany) equipped with a diode array detector and a Zorbax Rx-C8 2.1x150 mm analytical column packed with diisopropyl n-octyl (5 µm) and an Eclipse XDB-C8

guard cartridge (2.1 × 12.5 mm i.d.) filled with the same material were used. Operating conditions are described by Romero et al. [17]. Recovery values for diuron and its metabolites were close to 100%, except for DCA, which gave recoveries of 81% ±1.2 for S, 56% ±7.1 for SW and 54% ±0.9 for SO. The low DCA recoveries agree with the results of Polati et al. [25], suggesting that it is strongly retained in the soils fractions.

2.5. Enzyme activity analyses

Soil samples of 1 g of wet soil in triplicate were used to determine dehydrogenase (DHS) and urease activity. DHS was determined as reported by García et al. [26]. The iodonitrotetrazolium formazan (INTF) produced was measured in a spectrophotometer at 490 nm (Shimadzu UV Mod. 684). Urease activity was determined using urea as a substrate as described by Kandeler and Gerber [27] and samples were read at 690 nm. Samples without soil were used as control to DHS activity and samples without urea were run simultaneously as control to urease activity.

2.6. DNA Extraction and quantification of 16S rRNA, *puhA* and *puhB* gene sequences in soil DNA samples

Nucleic acids were extracted in triplicate from 250 mg of soil sampled at different incubation times. The procedure deriving from the initial protocol [28] is described in the ISO standard 11063 [29]. DNA extracts were purified by using polyvinyl-polypyrrolydone columns (PVPP, Sigma-Aldrich, USA). Bacterial abundance (BA) was estimated by qPCR according to ISO/DIS 17601, and qPCR assays were performed with a StepOnePlus qPCR machine (Applied Biosystems, USA) using the universal

bacterial primers 341f and 534r, as described by López-Gutiérrez et al. [30]. The abundance of *puh*A and *puh*B genes were estimated by qPCR with puHAf/puHBr and puHBf/puhBr primers, respectively, as described by Pesce et al. [19]. Functions that described the relationship between Ct (threshold cycle) and the number of 16s rRNA, *puh*A and *puh*B sequences (log copy number) for each DNA sample (x) were Ct16S rRNA = -3.28 x + 39.26 (R^2=0.996), Ct *puh*A= -3.36 x + 35.91 (R^2= 0.991), and Ct *puh*B = -3.30 x + 35.25 (R^2= 0.999). The efficiency values of the qPCR assays were 101%, 98%, and 100%, respectively. Three replicates were run for each qPCR assay. Three non-template controls (NTC) were also run for each gene target.

2.7. A-RISA fingerprinting

The genetic structure of the bacterial community was analysed by automated ribosomal intergenic spacer analysis (A-RISA) [31]. The 16S–23S intergenic spacer of the bacterial rRNA was amplified with primers 38r/72f and PCR conditions as described by Martin-Laurent et al. [28]. The primer 72f was labelled at position 5' with IRD 800day fluorochromo (MWG SA Biotech, Ebersberg, Germany). The PCR products were loaded onto a 3.7% polyacrylamide gel (66 cm in length) and run on a LiCor 4300 DNA Analysis System (Biosciences, USA) for 15 h at 1500 V/80 W. The gels were further analysed using 1-D Scan (ScienceTec, France), which converts fluorescence data into electropherograms. The size of the bands (in base pairs) were calculated using a standard DNA ladder with 15 bands ranging from 200 to 1200 bp.

2.8. Data analysis

Modelmaker 4.0 was used to analyse the dissipation data. Experimental data were fitted to Double First Order in Parallel (DFOP) and Single First Order (SFO) models. Values of dissipation time DT_{50} and DT_{90} in DFOP were calculated using an iterative procedure [32]. Spearman correlations and analysis of variance (ANOVA) of experimental data were made with Statistic software SPSS® 15 (Chicago, Illinois, USA). The A-RISA results were converted into a distance matrix based on band presence (i.e. peak at a given position) and intensity (i.e. height of peak) using PrepRISA (http://pbil.univ-lyon1.fr/ADE-4/microb/). The A-RISA matrix was then used to perform the principal component analysis (PCA) using ADE-4 software (http://pbil.univ-lyon1.fr/ADE-4/home.php). The correlations between microbiological and biochemical variables as diuron degradation were assessed using Mantel's multivariate test. Distance matrices for A-RISA profiles of 16S rRNA were determined using a Jaccard distance transformation and for the rest of variables, the Euclidian distances were inferred.

3. Results and discussion

After 6 months of incubation, the effect of double amendments done either with W or O vermicompost led to the increment of the soil properties in the amended soils SW and SO (Table 1). The increase in soil organic matter (SOM) was two-fold higher in the amended soils than for the control. This gain in SOM was positively correlated with the increase in the lignin and fulvic acid (FA) concentration in the soils amended with vermicompost ($r = 0.82$ and 0.81, $p<0.01$ respectively). On the other hand, SOM was negatively correlated with the fall in pH by the amendments ($r = -0.91$, $p<0.01$), these results being consistent with those of Fern nde -Bayo et al. [24].

3.1 Sorption-desorption of diuron

The sorption-desorption Freundlich coefficients determined for diuron and its main metabolites were affected by the vermicompost amendment (Table 2, Figure. A.1). The K_{fads} increased in SW and SO by 2.3 and 1.7 fold, respectively, as compared to the control (S). The Freundlich exponent ($1/n_{ads}$) was close to 1, indicating good linearity in the amended soils, especially in SO, which is consistent with a partition-sorption process. In addition, as a result of vermicompost amendment, the sorption of the metabolites DPMU and DPU increased 1.6 fold in SO and 1.9-fold in SW as compared to S (Table 2). Similarly, sorption of DCA increased 2.2 fold in SW and SO with respect to S. The k_{fdes} were also higher in the amended soils than in control soil (Table 2). However, the H index revealed that metabolites in S, SW, and SO were retained at more irreversible sorption sites. The lowest reversibility was observed for DCA in the amended soils (Figure. A.1d).

The k_{fads} increment for diuron and its metabolites in the amended soils (SW and SO) correlated with the increase in SOM, FA, and lignin ($r > 0.75$, $p < 0.05$).The SOM is heterogeneous and composed mainly of an expanded component such as lignin, humic and fulvic acids (HA, FA); and a condensed component such as humin. The proportion and structure of these components affect sorption and linearity of the sorption isotherms [33]. Although the increment of lignin and humic substances (HA and FA) in the amended soil were similar, the k_{fads} differences with diuron and metabolites could be associated to structure and nature of SOM. It is known that different humic fractions from the same soil can have different sorption coefficients [34]. Therefore, in SO the content of these fractions might be involved in the near linearity of sorption isotherm (1/n close to 1), but the structure and composition of these fractions in SW counteracted this effect, showing a nonlinear sorption isotherm (1/n= 0.85). It is known that humic

fractions in W vermicompost have a low E_4:E_6 ratio, indicating a higher level of polymerization and condensation of aromatic constituents, with a dominance of humic acids of higher molecular weight [35]. Therefore, a change in SOM can affect the adsorption process [36].

In relation to K_{fdes} values, these were also higher in SW and SO than in S. The H index with values of less than 1 reflects that the molecules diffuse throughout the micropores of the organic matter, these being less accessible [10]. Consequently, by increasing the sorption capability of soil, the vermicompost amendments may contribute to the accumulation of diuron metabolites, especially DCA, if the degrading ability of the soil microbial community is low.

3.2. Dissipation of diuron

Significant differences in the dissipation kinetics of diuron were found between the different treatments (Table 4) when diuron was applied in the pre-amended soil (E1). The experimental data from SD1, SWD1, and SOD1 were fitted to DFOP kinetic with a fast initial decrease of diuron the first 10 days of incubation, followed by a slower decline (Figure 1). Significant differences in the degradation constants k_1 (F= 9.43 p= 0.014) and k_2 (F= 21.42 p= 0.002) were found (Table 3), with a higher degradation rate (k_1) in the amended soils (SWD1 and SOD1), but lower values in the second phase (k_2). The high DT_{90} value reflected the diuron persistence in the soils amended with vermicompost (Table 3).This was supported by Pearson's correlation between DT_{90} and SOM (r = 0.92, p<0.01) and negative correlation between k_2 and lignin concentration after amendment (r = -0.97 p<0.01). The DFOP kinetic for diuron in E1 might have been due to the relative increase in the diuron sorption and decline in microbial activity [37]. In fact, in k_2 the slow diuron degradation constant was significantly correlated with

the K_{fads} in the amended soils (r = 0.82 p<0.01) and was consistent with the longer dissipation time registered in SWD1. According to Wauchope et al. [38] a bi-exponential decline in pesticide can be accepted as sufficient evidence for long-term sorption kinetics.

In relation to E2, the diuron dissipation results were fitted to SFO model, showing rapid dissipation in all treatments (Figure. 1, Table 3) and lower DT_{50} values than observed in E1 treatments. In this case, 50% of the diuron added disappeared within 2 weeks. However, no significant differences (F= 0.336 p= 0.728) were found between the k values in the different treatments (Table 3). Unlike diuron degradation in E1, the accelerated disappearance of diuron in E2 could be attributed to the repeated application of diuron, which led to the adaptation of microorganisms to diuron degradation [39, 40]. This deduction was supported by the detection of diuron metabolites. Indeed, the metabolite DPMU in the soil managed in E1 was detected in the SD1 and SOD1treatments and quantified at concentration levels of 0.64 and 0.74 $\mu g\ g^{-1}$, respectively, which corresponded to 20.41% and 22.82% of the diuron applied (Figure. 1a and c). The metabolite DPU was detected in SWD1 within the first 5 days (10.85%) and in SOD1 after 90 days at the highest concentration (26.07%). However, in E2, the concentrations of DPMU were lower than 5% of the diuron applied, but the DPU metabolite was not detected.

The detection of DPU and DPMU indicated that the metabolism of diuron started with N-demethylation [1, 2]. In E1, the slow formation of DPMU in SWD1 may be the consequence of the slow diuron degradation, a hypothesis supported by k_2 values (Table 3). In E2, the slight increase in DPMU production in all treatments may be associated with the faster diuron degradation. These results are consistent with previous studies by ern nde -Bayo et al. [24] in which diuron degradation followed an SFO kinetic with a

relatively short DT50 (DT_{50} = 22 days) and a low accumulation of the metabolite DPMU. The DCA metabolite formed by hydrolysis reaction was not detected, probably because of its (i) strong and less reversible sorption to soil components (Table 2) or (ii) low recovery from soil (< 81%).

3.3. Soil-enzyme activities

As expected, the addition of vermicompost stimulated the soil microbial activity, which was stable in S, but reached its highest values at 30 and 60 days for SW and SO, respectively (Figure 2a). For SWD1 and SOD1 in E1, the application of diuron depressed DHS activity, especially in SWD1 (Figure 2b). However, in SOD1 the disturbance was weaker than that in SD1 and SWD1. In relation to E2, SD2 showed lower DHS activity than SWD2 and SOD2 at the end of the incubation time, this latter showing a significant enhancement (F= 249.77 p<0.05). This great surge of DHS in SOD2 despite the lower sorption of diuron in SO with respect to SW (Table 2) point to a possible adaptation ability of microbial community in SOD2. Furthermore, in both experiments, E1 and E2, greater soil microbial activity resulted from the addition of the O-vermicompost, which could be attributed to the vermicompost quality.

The urease activity in S declined over the first 30 days, but then increased again but without reaching initial values (Figure 2d). The SW treatment stimulated the urease activity during the first 30 days, which then remained constant until 90 days. At the end of the incubation time, only SO showed significantly higher urease activity than S. With diuron application in the SD1 and SWD1 treatments (Figure 2e), the urease activity rose until 60 days and then fell at 90 days. Conversely, SOD1 showed a fluctuating rise, reaching an activity level similar to that of SD1 and SWD1 at 90 days. On the other

hand, in the E2 treatments the urease activity slightly increased, but the values remained below of 300µg NH_4^+ g^{-1} h^{-1} over the incubation time.

The performance of DHS and urease activities might be used as proxies to estimate the resistance and the resilience of the soil microbial community, reflecting its ability to cope with environmental stress. Within this context, vermicompost amendment caused only minor disturbance and prompted the recovery (resilience) of enzymatic activity. Notably, the E1 treatments had a marked impact on the recovery of DHS activity. Perhaps due to physico-chemical factors such as the pesticide sorption in organic and inorganic matrices, this has major implications for the competition between immobilized enzymes and pesticides, with the subsequent release of free enzymatic molecules from matrices. Conversely, in E2 the recovery of DHS was immediate, especially in SOD2, probably because of the addition of O vermicompost, which can counterbalance the toxic effect of diuron. The urease activity in E1 was high but unstable over time. This might be explained by biological factors such as the cell-wall permeability or cell lysis, increasing the accessibility of the substrate to intracellular urease [41]. In E2, the high DHS activity and low urease activity can be explained by the inhibition of urease activity by the important high diuron concentration in soils.

3.4. Bacterial abundance and structure

Given the differences found in the diuron-degradation kinetics between the E1 and E2, a subset of soil samples showing marked differences were chosen (i.e. 5, 30, and 90 days for E1 and 5, 10, and 90 days for E2) to extract soil DNA. In S and SW, 16S rRNA gene abundance reached its maximum after 30 days of incubation and then declined (Figure 2g, h, and i), while values in SO progressively rose to 12.8 x 10^9 copies g^{-1} soil

after 90 days (Figure. 2g). In E1, at 5 days, the BA in SD1 was significantly lower than in the amended soils SWD1 and SOD1 ($F = 7.34$ $p<0.05$). However, after 90 days of incubation, the BA was similar in all treatments, suggesting that the bacterial community had recovered from the stress induced by diuron (Figure 2h). In E2, at the initial time, the lowest abundance was determined in SWD2, but after 90 days the BA in SWD2 and SOD2 were significantly higher ($F = 22.47$; $p<0.05$) than that in SD2, which showed no significant variations (Figure 2i).

As well as the enzyme activity, changes in BA can be interpreted in the ecological concept of resistance and resilience [42]. In E1 and E2, BA was reduced by the effect of diuron and vermicompost, as observed in SD1, SD2, and SWD1. In these treatments the BA had weak resistance and poor resilience at the end of the experiment. SOD1 in E1 showed no significant differences in the BA over time, while SOD2 in E2 registered a recovery of BA. The resilience observed in SWD2 and largely in SOD2 may be a consequence of repeated exposure of the soil to a lower herbicide level (3µg g^{-1}), which promotes the development of a bacterial community adapted to pesticide, as well as to the changes in the physico-chemical properties of the soil and nutrients available in the amendment.

BA changes in both E1 and E2 can be attributed to alterations in the genetic structure of the bacterial community. In an effort to test this hypothesis, the evolution of genetic structure of the bacterial community was monitored by A-RISA (Figure A.2), and its fingerprints were compared by PCA (Figure 3). In both experiments, the addition of vermicompost and the treatment with diuron altered the soil bacterial structure. In E1, 5 days after treatment, the factorial map showed that the ordination on PC1 explained 53% of the variance in the data, allowing the discrimination of the microbial communities in S and soil treated with 3µg g^{-1} of diuron (SD1). Meanwhile the PC2

explained 22% with a differentiation of S from SO and SOD1 (Figure 3a), revealing the impact caused by vermicompost and diuron application, respectively. Thirty days after treatment, only the microbial communities of SO could be discriminated from all other treatments throughout PC1 (Figure 3b). Ninety days after treatment, the PC1 (29%), discriminated the microbial communities of SW from all treatments. In addition, ordination on PC2 (13%) discriminated the SO bacterial community from all the other treatments (Figure 3c).

In E2, 5 days after treatment, the factorial map showed that ordination on PC1 (33%) did not enable the discrimination of any microbial communities, but PC2 (20%) discriminated the microbial communities of S from SD2, SOD2 and SWD2, which could not be distinguished from each other (Figure 3d). After 10 days of incubation, similar trends were found, except SD2, which was discriminated from SOD2 and SWD2 throughout PC2 (16%) (Figure 3e). This revealed that the diuron treatment had an impact on the bacterial genetic structure of soils amended or not with vermicompost. After 90 days of incubation, the bacterial structure of the soils treated with diuron could be discriminated from its respective controls throughout PC1 (40%). Nonetheless, the bacterial community of SD2 could be discriminated from that of SWD2 and SOD2 throughout PC2 (16%) (Figure 3f).

In summary, the A-RISA analyses revealed that, in both experiments, only O-vermicompost modified the bacterial community structure as compared to the control soil. This suggests that the nature of the vermicompost determines the impact provoked on the bacterial structure of the soil. This agrees with a previous report holding that the nature of the organic amendment determines the shifts caused in the microbial genetic structure in agricultural soils [43] with different organic-matter management. The impact induced by the addition of organic matter was counterbalanced by diuron

treatment in both experiments. This effect was more evident in E1 than in E2. It should be borne in mind that fingerprinting techniques allow the dominant group to be studied and therefore does not enable the detection of new specific phylotypes involved in diuron degradation that is a small fraction of the total microbiota [18]. It could be hypothesised that some of the dominant groups were altered by the diuron treatment, in particular those favoured by organic amendment (Figure 3c and f). However, under diuron selective pressure (i.e. pre-treatment applied in E2), organic amendment was shown to contribute to the bacterial survival and adaptation of new microbial communities capable of degrading this pesticide, as was found in E2. Therefore, our results suggest that repeated diuron exposure promotes the development of microbial communities that utilize the pesticide as a carbon or nitrogen source [4]. This hypothesis was tested by monitoring the abundance of the bacterial community able to mineralize diuron, followed by qPCR assays targeting *puh*A and *puh*B genes coding for phenylurease enzymes PuhA and PuhB, respectively.

3.5. Quantification of *puh*A and *puh*B genes

The *puh*A and *puh*B gene sequences in E1 were detected in low copy number close to the detection limit (10 copies, C_T =33.52), which may be related to the low diuron degradation (Figure 1). In soil samples of E2, only the *puh*B gene sequence was detected in high abundance in the microcosms pre-exposed to diuron (SD2, SWD2 and SOD2; Figure 4a), confirming that this treatment exerted selection pressure, favouring the growth of diuron degraders.

In the soils not treated with diuron (S, SW and SO), the copy number of the *puh*B gene determined at day 5 in SW and SO was 9- and 18-fold greater than in S, respectively.

This suggests that this enzyme has promiscuous hydrolysis activity with the ability to catalyse the amide bond (C-N) or ester bond (P-O). This was probably because the PuhB enzyme has a Michaelis constant (Km) for diuron lower (7.6 μM) than for similar molecules and has a substrate-binding cavity more efficient in the hydrolysis of *N*-dimethylphenylurea compounds [21]. However, it was evident that the largest copy number of *puhB* sequences was quantified in the soils pre-exposed to diuron (E2). The *puh*B sequences in SOD2 and SWD2 after 90 days was similar but significantly higher than in SD2 (1.8×10^5 copy number g^{-1}). The relative abundance of diuron degraders within the total bacterial community was estimated as the *puh*B/16S rRNA gene ratio (Figure 4b). The *puh*B ratio throughout the incubation period was higher in the diuron treatments than in its respective controls, although SO showed a relatively greater *puh*B ratio, which also altered the genetic structure of the bacterial community (Figure 4b).

The Mantel correlation test confirmed that increases in the *puh*B gene-copy number positively correlated with a faster rate of diuron degradation ($r = 0.64$ $p<0.01$) and bacterial structure and BA ($r >0.40$; $p<0.05$). These results support the importance of quantifying the abundance of functional groups responsible for key steps of pesticide degradation [44, 45] and confirm the importance of agricultural practices such as organic amendment to regulate the microbial degradation of pesticide which is the key driver of the biofiltration, an important ecosystemic service supported by arable soils.

4. Conclusions

This multidisciplinary study reports the positive impact on pesticide dissipation exerted by soil amendment with agro-industrial vermicomposts. These results demonstrate that the nature and composition of vermicompost affect the fate of diuron as well as of the

microbial community, promoting the development of diuron-degrading populations. The history of diuron application counterbalanced the vermicompost effect. O-vermicompost increases bacterial and *puh*B abundance, corroborating its effectiveness as a tool to enhance the diuron biodegradation in soil. Further work is needed to understand the mechanisms by which vermicomposts promote pesticide biodegradation in order to define the best management options to mitigate environmental pesticide risk.

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Table1

Table 1. Physico-chemical properties of W and O-vermicomposts, unamended (S) and amended soil twice with 5% of each vermicompost (SW and SO), and aged after each vermicompost addition by a 3-month incubation.

Properties	S	SW	SO	O	W
TOC (%)	1.40±0.01	3.66±0.09	3.31±0.12	29.2±1.4	29.5±0.8
OM (%)	2.42±0.01	6.30±0.12	5.71±0.21	51±2.4	59±1.6
Lignin (g kg^{-1})	4.70±0.20	20.7±0.20	19.2±0.20	200±4	255±9
HA (g kg^{-1})	4.96±0.02	6.70±0.26	6.90±0.17	152±6	146±7
FA (g kg^{-1})	1.19±0.42	3.78±0.12	3.71±0.10	100±4	101±3
WSC (g kg^{-1})	0.04±0.00	0.13±0.01	0.49±0.30	8.8±0.3	3.4±0.4
pH	8.6	8.4	8.2	8.6	7.3

Table 2

Table 2. Adsorption and desorption Freundlich coefficients (Kf and 1/n) for diuron and its metabolites DPMU, DPU, and 3,4-DCA in the unamended (S) and 10%-amended soils by two consecutive additions of 5% with O or W vermicompost (SW and SO).

	k_{fads} ±ES	$1/n_{ads}$±SE	R^2_{ads}	k_{fdes} ±SE	$1/n_{des}$±SE	R^2_{des}	k_{oc}±SE	H
Diuron								
S	7.45±0.72c	0.77±0.01b	0.909	10.29±0.10c	0.40±0.01b	0.997	530±52a	0.52
SW	16.77±1.05a	0.85±0.01a	0.973	17.13±0.01a	0.47±0.01a	0.993	459±39ab	0.56
SO	13.14±0.08b	0.92±0.07a	0.989	14.39±0.52b	0.46±0.01a	0.991	397±16b	0.51
DPMU								
S	11.68±0.11c	0.66±0.01a	0.996	13.95±0.10c	0.19±0.01a	0.970	153±0.6b	0.29
SW	22.95±0.97a	0.68±0.09a	0.963	19.20±0.20a	0.12±0.01c	0.910	272±12a	0.18
SO	18.78±0.33b	0.70±0.01a	0.996	17.98±0.07b	0.15±0.01b	0.935	261±4.6a	0.22
DPU								
S	12.21±0.07c	0.65±0.01a	0.996	13.81±0.04c	0.24±0.01a	0.961	160±0.9b	0.37
SW	23.15±1.55a	0.72±0.11a	0.956	17.94±0.16a	0.09±0.15c	0.792	274±19a	0.13
SO	19.12±0.41b	0.71±0.01a	0.997	17.58±0.06b	0.16±0.02b	0.919	266±5.7a	0.23
DCA								
S	9.48±0.09c	0.69±0.01a	0.997	13.09±0.04c	0.11±0.00a	0.984	124±0.8c	0.16
SW	23.07±0.96a	0.69±0.04a	0.979	19.11±0.11b	0.04±0.04b	0.911	278±15b	0.06
SO	21.26±0.33b	0.69±0.01a	0.998	18.72±0.06a	0.02±0.00c	0.872	296±4.1a	0.03

kf unit: $\mu g^{1-1/n}\ g^{-1} mL^{1/n}$; SE standard error.

Table 3

Table 3. Kinetic parameters estimated from diuron degradation in soil samples from E1 and E2 based on DFOP and SFO models, respectively. Different letters indicate significant differences (α=0.05).

Soil	Treatment	Ci (%)	g	k_1 (d^{-1})	$k_{2\ X\ 10\text{-}2}$ (d^{-1})	DT_{50} (d)	DT_{90} (d)	R^2	$X^{2\gamma}$	*t-test*
				***DFOP* model**						
	SD1	104.98	0.12	0.224^{c}	0.95^{a}	62	210	0.99	1.1	< 0.05
E1	**SWD1**	108.99	0.18	0.514^{a}	0.22^{c}	200	830	0.99	0.9	< 0.05
	SOD1	108.90	0.19	0.439^{b}	0.75^{b}	58	260	0.99	0.8	< 0.05
				***SFO* model**						
	SD2	104.92	n.d	0.051^{a}	n.d	13.41	44.60	0.98	2.1	< 0.05
E2	**SWD2**	99.62	n.d	0.042^{a}	n.d	16.34	54.30	0.98	1.7	< 0.001
	SOD2	110.17	n.d	0.046^{a}	n.d	15.15	50.34	0.97	1.0	< 0.001

$^{\gamma}$ Error level of X^2, n.d: No data

Figure Captions

Figure 1. Degradation kinetics of diuron and DPMU in unamended soils SD1 and SD2 (A), amended soils with W-vermicompost SWD1 and SWD2 (B) and amended soils with O-vermicompost SOD1 and SOD2 (C) from the experiments E1 (no pre-treated with diuron) and E2 (pre-treated with diuron). The bars represent the standard deviations calculated from the triplicates. The curves correspond to data fitted to DFOP and SFO models.

Figure 2. Enzyme activities, dehydrogenase (a, b, and c), urease (d, e, and f), and copy number of 16S rRNA gene (g, h and i), determined in unamended (S) and amended (SW and SO) soils as well as in corresponding soils treated with diuron (unamended: SD1, SD2; amended with O-vermicompost, SOD1, SOD2) or with W-vermicompost (SWD1, SWD2) from E1and E2 experiments.

Figure 3. PCA ordination at the initial (a, d), intermediate (b, e), and final time (c, f) from E1 and E2 experiments, based on the analysis of the total bacterial communities, analysed by ARISA fingerprints of PCR products from DNA extracted directly from unamended (S=1) or amended soils (SW=3 and SO=2) without pre-treatment or pre-treated with diuron (SD1 and SD2=4, SWD1 and SWD2=5, SOD1 and SOD2=6).

Figure 4. Quantification of diuron-degrading community (*puhB* gene sequence) (a). *puhB* ratio of community in the total bacterial abundance, it was estimated by calculating the puhB/16S rRNA ratio (given as a percentage) (b). The qPCR assays were carried out from DNA extracted directly from soil (S) or amended soils (SW and SO) and soil pre-treated with diuron (SD2, SWD2, and SOD2) from experiment 2. The bars represent the standard deviations calculated from the triplicates. Data with the same letter are not significantly different ($p < 0.05$).

Figure 1

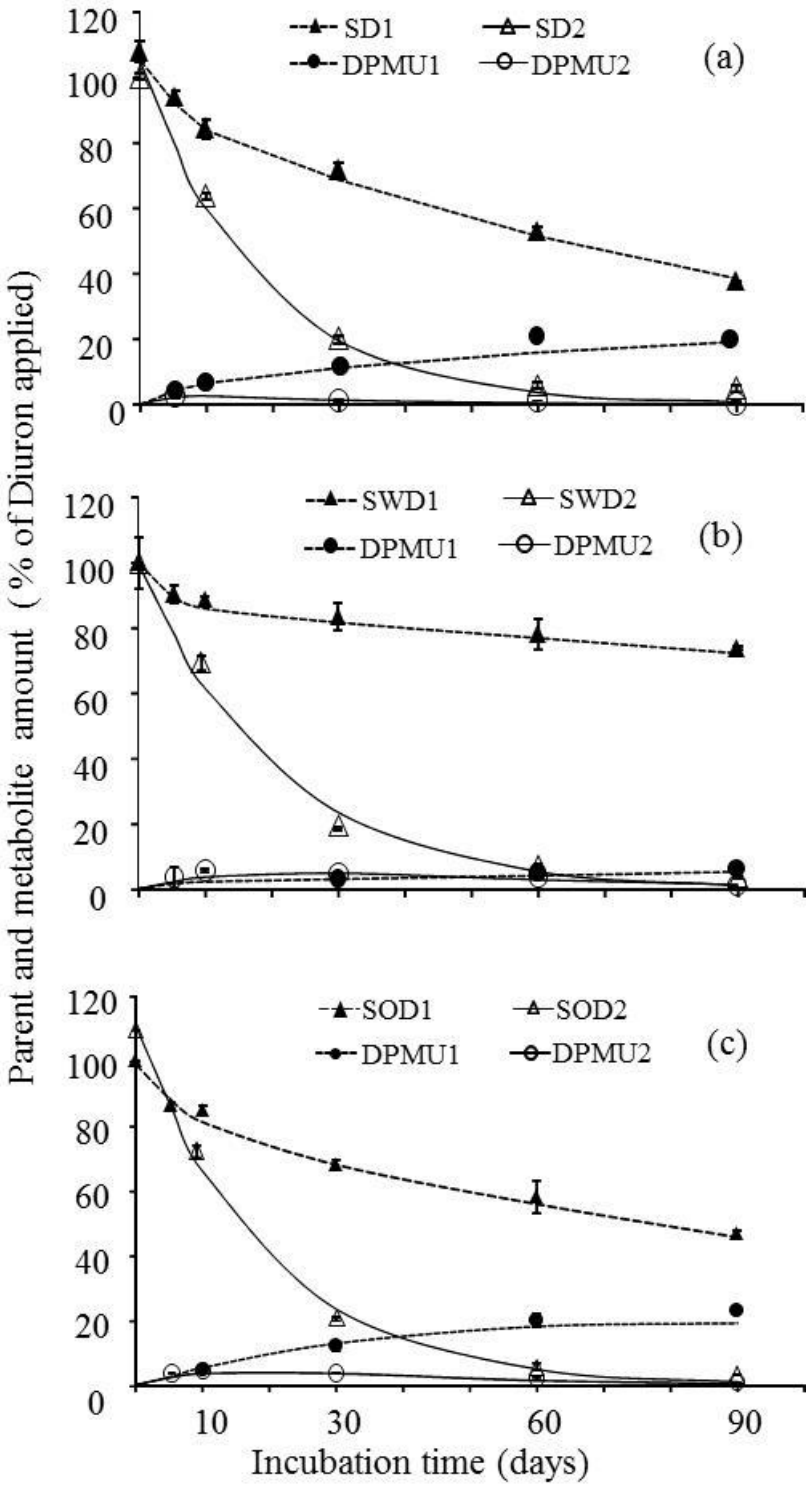

Figure 1

Figure 2

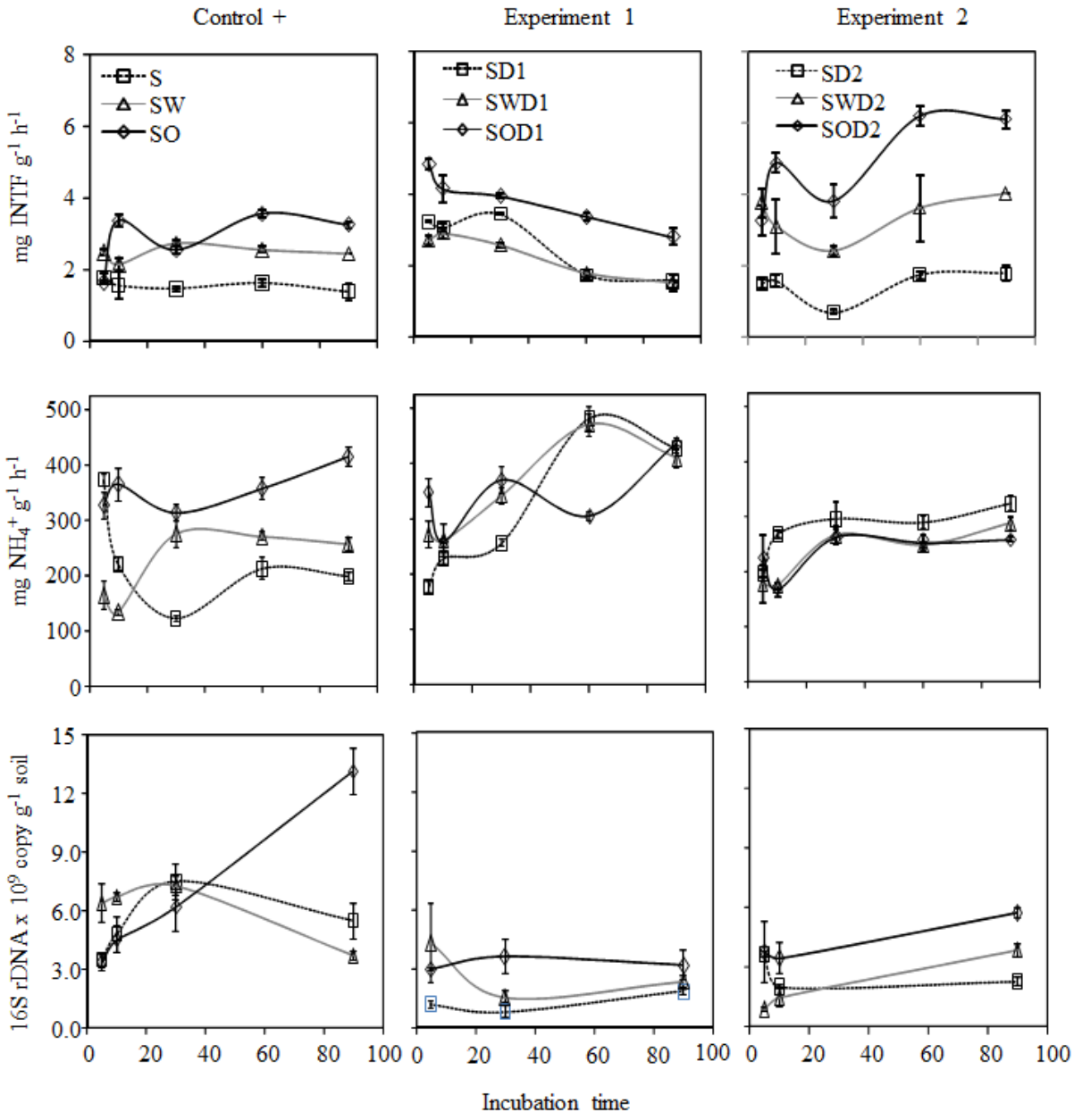

Figure 2

Figure 3

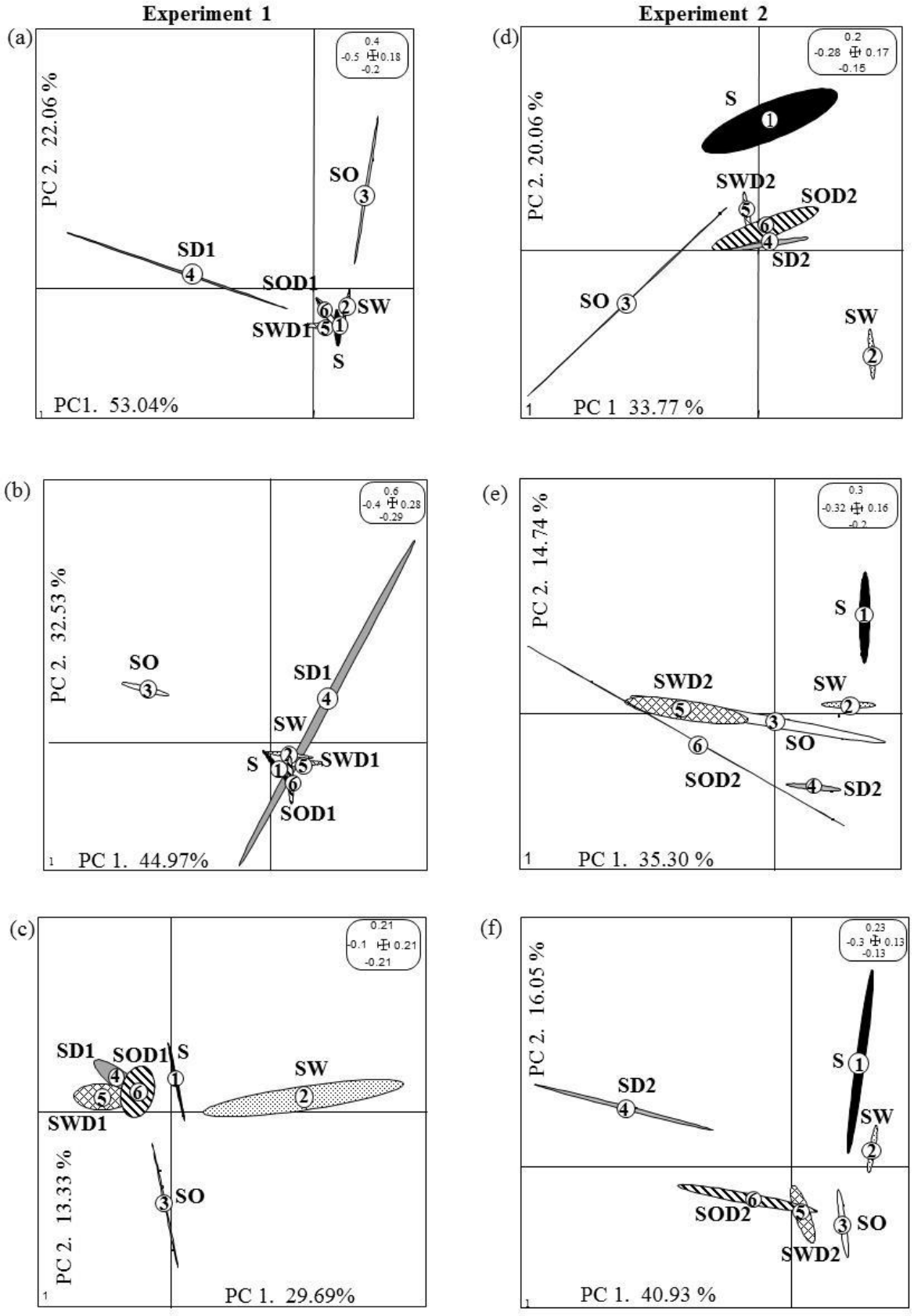

Figure 3

Figure 4

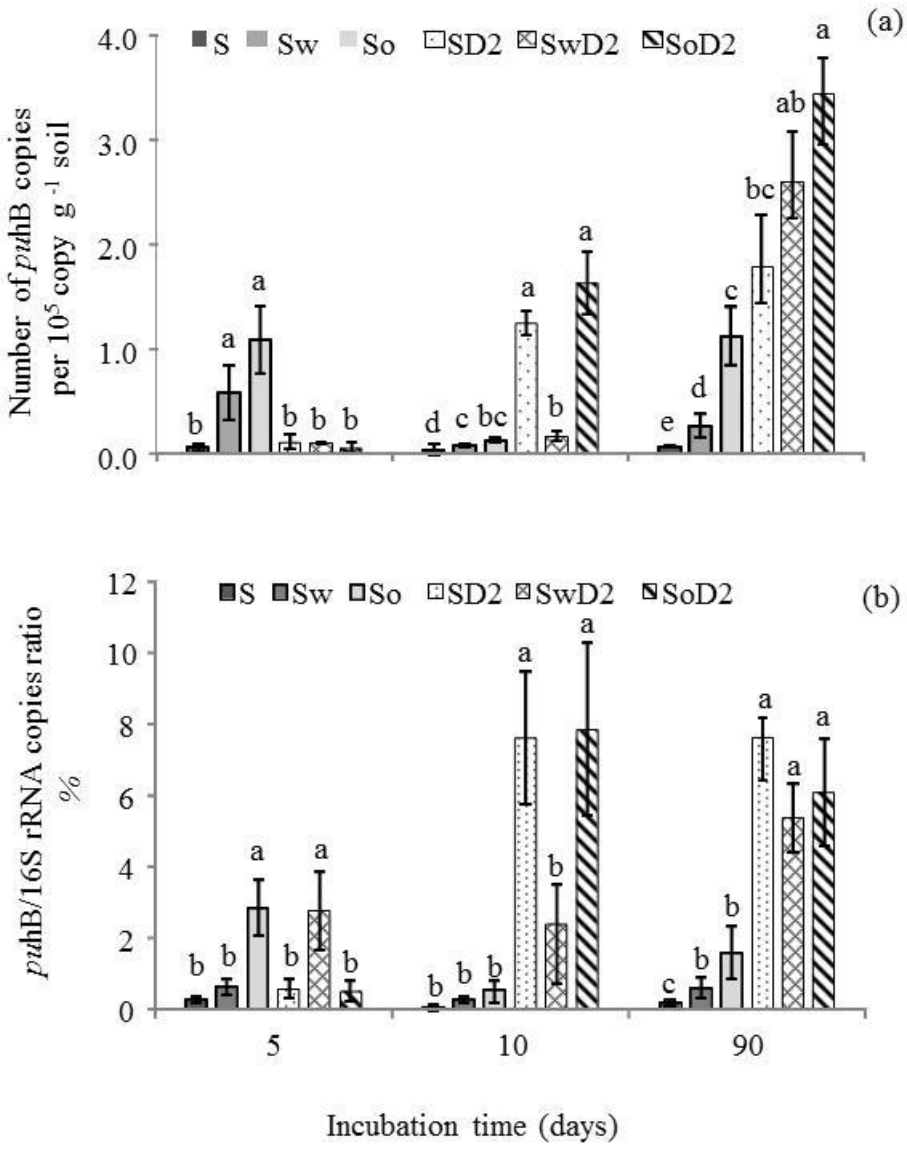

Figure 4

Supplementary data for

Multidisciplinary assessment of pesticide mitigation in soil amended with vermicomposted agroindustrial wastes

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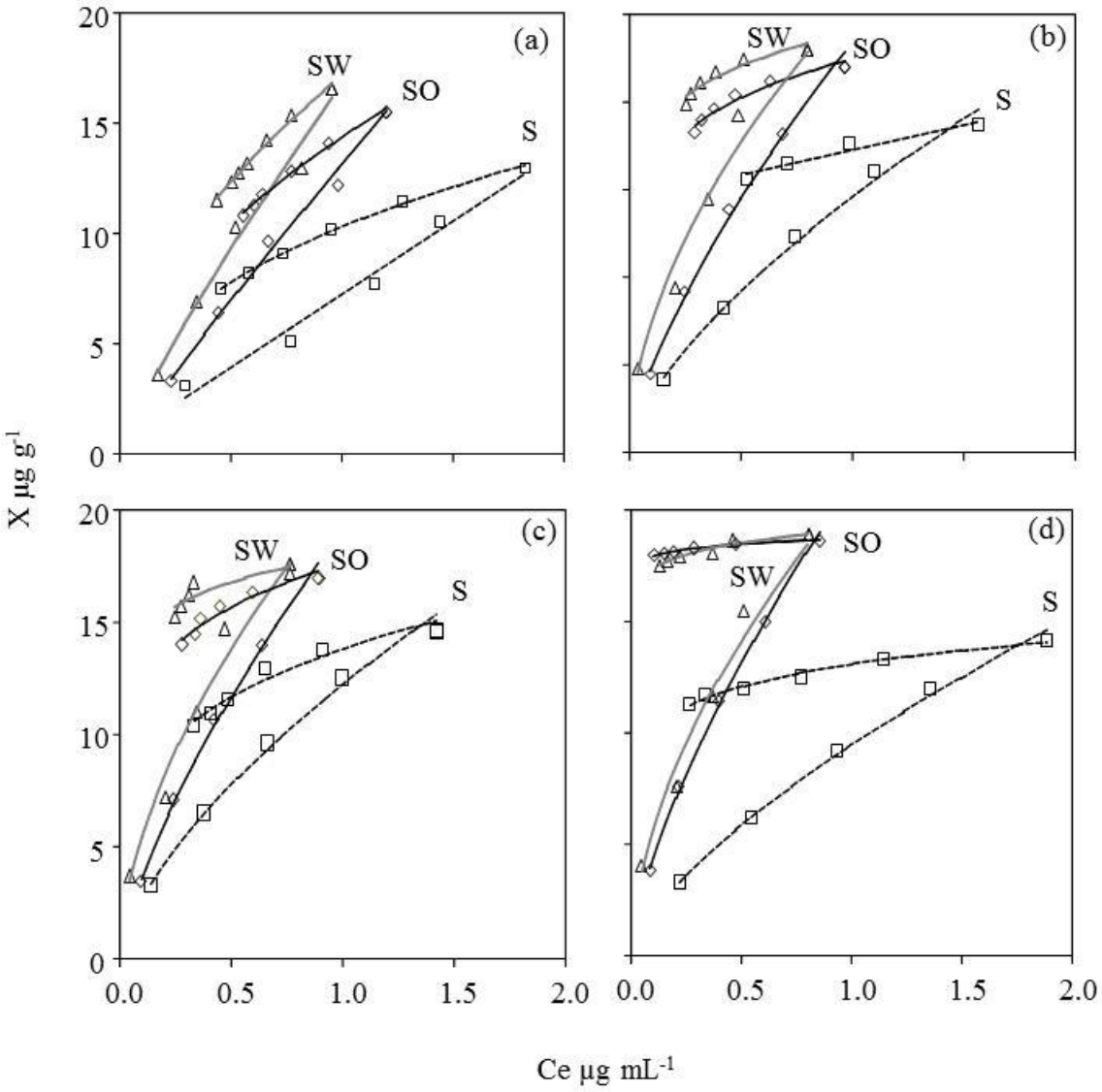

Figure A. 1. Adsorption-desorption Freundlich isotherms for diuron (a) and the metabolites DPMU (b), DPU (c), and DCA (d) in the ageing unamended soil (S) and 10%-amended soil (SW and SO). Experimental data are the symbols while lines represent the theoretical calculation.

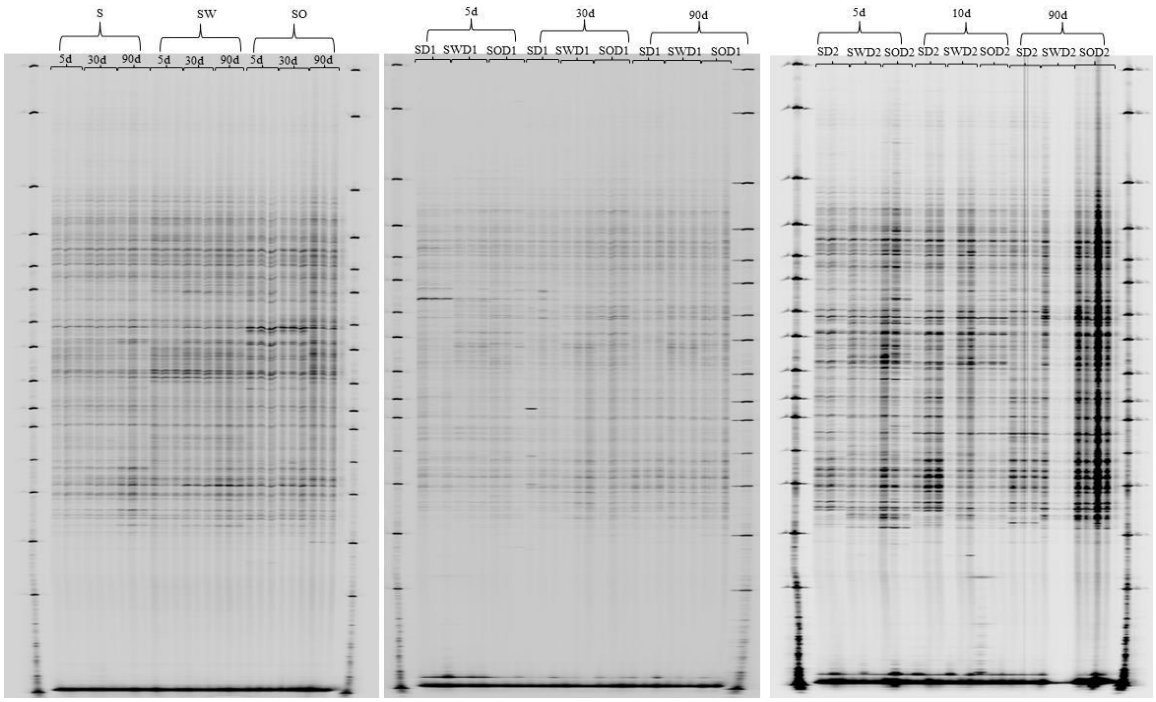

Figure A. 2. ARISA fingerprint gel of PCR products from DNA extracted at the initial, intermediate, and final time of the incubation period, in the control soils S, SW, and SO and in the soils treated with diuron from E1 and E2 experiments.