UNIVERSIDAD DE GRANADA

INSTITUTO DE BIOTECNOLOGIA

APLICACIÓN DE LA TECNOLOGIA DEL VERMICOMPOSTAJE PARA LA VALORIZACIÓN AGRONOMICA DE RESIDUOS Y DESTRIOS DE CULTIVOS DE INVERNADERO

TESIS DOCTORAL

Manuel Jesús Fernández Gómez

Granada, 2011

Editor: Editorial de la Universidad de Granada
Autor: Manuel Jesús Fernández Gómez
D.L.: GR 1571-2012
ISBN: 978-84-9028-032-4

UNIVERSIDAD DE GRANADA

INSTITUTO DE BIOTECNOLOGIA

APLICACIÓN DE LA TECNOLOGIA DEL VERMICOMPOSTAJE PARA LA VALORIZACIÓN AGRONOMICA DE RESIDUOS Y DESTRIOS DE CULTIVOS DE INVERNADERO

Memoria presentada por el Licenciado en Biología D. Manuel Jesús Fernández Gómez para optar al grado de Doctor Europeo por la Universidad de Granada.

Granada, 28 de Septiembre de 2011

Fdo: Manuel Jesús Fernández Gómez

Directores de la Tesis Doctoral

Fdo. Rogelio Nogales Vargas-Machuca
Doctor en Ciencias Biológicas
Investigador Científico del CSIC

Fdo. Esperanza Romero Taboada
Doctora en Farmacia
Investigadora Científica del CSIC

ESTACIÓN EXPERIMENTAL DEL ZAIDÍN, CSIC, GRANADA

Para la realización del presente trabajo de Tesis Doctoral el Lcdo Manuel Jesús Fernandez Gómez disfrutó de una Beca para la Fprmación de Doctores del Programa Nacional de Formación del Profesorado Universitario (FPU), referencia: AP2006-03452.

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 (CSIC). El presente estudio se encuadró dentro de los objetivos científicos-técnicos propuestos en el proyecto de Excelencia, subvencionado por la Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía y cofinanciado con fondos Feder, P05-AGR-00408 "Reutilización agroganadera de subproductos de invernaderos mediante tecnologías innovadoras y de bajo coste (vermicompostaje, bloques alimenticios). Implicaciones de la presencia de fitosanitarios, 2007-2010". Además algunas de las actividades científicas incluidas en este trabajo fueron realizadas en el Institute of Microbiology, University of Innsbruck, Austria y en la School of Environment and Natural Resources, The Ohio State University, USA, durante las Estancias Breves en el Extranjero realizadas por el Lcdo. M. Fernandez durante el disfrute de la Beca FPU concedida.

A mi familia

AGRADECIMIENTOS

Al término de la presente Tesis Doctoral, quiero expresar mi agradecimiento a todas aquellas personas y organismos que han colaborado en la elaboración de este trabajo, así como aquellas personas que con su apoyo moral me han ayudado durante los años que he dedicado al desarrollo de esta Tesis Doctoral.

A los organismos que han permitido la realización de esta Tesis: al Ministerio de Educación por la concesión de la beca de Formación de Personal Universitario (AP2006-03452) y a la Junta de Andalucía quién ha financiado esta Tesis a través del proyecto "P05-AGR-00408". Al Consejo Superior de Investigaciones científicas, organismo al que pertenece la Estación Experimental del Zaidín, lugar donde se ha realizado la mayor parte de esta Tesis. Al Instituto de Biotecnologia de la Universidad de Granada por toda su ayuda durante y posteriormente a la realización del Diploma de Estudios Avanzados.

A mis Directores de Tesis, el Dr. Rogelio Nogales Vargas-Machuca y la Dra. Esperanza Romero Taboada, por confiar en mí para el desarrollo de este trabajo experimental así como por la magnífica labor que han realizado a la hora de dirigir la investigación durante casi cinco años; periodo durante el cual me han prestado una ayuda personal y compresión de un valor incluso mayor al de su sobresaliente labor profesional.

Al Dr. Antonio Luís Extremera León por su dedicación y ayuda prestada durante el periodo de investigación tutelada del programa de Doctorado en Biotecnología.

A la Dra. Marta Goberna Estellés, ya que sin ella no habría sido posible desarrollar esta tesis; no sólo por su gran labor científica y por sus enseñanzas sobre biología molecular, sino también por los cuidados que me prestó durante mi accidentada estancia en Innsbruck.

Al Dr. Heribert Insam del Instituto de Microbiología de la Universidad Innsbruck por su cálida acogida y gentil hospitalidad durante el desarrollo de mi estancia y posterior visita, así como por su inestimable ayuda en la elaboración y supervisión de parte esencial de los estudios que se recogen en esta tesis.

Al Dr. Richard P. Dick, director del grupo del grupo de investigación de Ecología microbiana de suelos en la School of Environment and Natural Resources de la Universidad Estatal de Ohio, por aceptarme durante dos ocasiones en su grupo de investigación, durante

las cuales me trató de forma inmejorable, permitiendo que ambas estancias fueran inolvidables.

A la Dra. Montserrat Díaz Raviña del Departamento de Bioquímica del Suelo del Instituto de Investigaciones Agrobiológicas de Galicia por su colaboración en el análisis de las comunidades microbianas durante proceso de vermicompostaje, algo que ha enriquecido la información recogida en esta Tesis.

A Celia Cifuentes por todo el trabajo que ha realizado en la parte experimental de esta tesis, siempre de forma voluntariosa y con una sonrisa. Sin ella esta tesis no hubiese sido posible.

A Mar Quirantes por su ayuda desinteresada en el desarrollo de los ensayos recogidos en memoria de Tesis Doctoral, echándome una mano para contar lombrices y en el control de los procesos de vermicompostaje mientras he estado de estancia.

A Mari Ángeles Delgado por trabajo durante mis comienzos en el vermicompostaje, se lo agradezco realmente teniendo en cuenta su aversión ante las lombrices.

A Fernando Calvo, quién también ha "pringado" contando lombrices y analizando los vermicomposts, gracias.

A Amparo Salido por su ayuda en mis infructuosos inicios en la determinación de plaguicidas, además gracias por estar siempre dispuesta a resolver mis dudas sobre cualquier tema, dándome sabios consejos.

A Carlos Pérez Losada de la empresa Ros Roca y a Manuel Liria encargado de la planta de compostaje de Motril, así como a los operarios de la misma, Marcelo, Eduardo entre otros, porque siempre me han tratado fenomenal en mis visitas a la planta.

A Dr. Clive Edwards por su amabilidad y disposición que hizo posible la investigación desarrollada durante mi estancia en Columbus.

A la Dra. Nicola Lorenz y a la Dra. Linda Dick por su desinteresada ayuda profesional e increíble trato personal durante mi estancia en el grupo de investigación de Ecología microbiana de suelos de la School of Environment and Natural Resources. Aprovecho también para agradecer a los compañeros con los que coincidí en este grupo el trato recibido durante mis dos periodos de estancia.

Al Dr. Emilio Benítez por su ayuda resolviéndome cuestiones sobre actividades enzimáticas y demás dudas.

A la Dra. Astrid Vivas por recibirme de forma tan afectuosa y afable cuando todavía era un "sin papeles", ensañándome muchísimo en el campo de la biología molecular.

A Dra. Beatriz Moreno por su infinita paciencia con mis despistes y "meteduras de pata" en el laboratorio, sé que he llegado a sacarte de quicio, pero siempre has estado ahí, como modelo de trabajo "pulcro" y eficiente, y dispuesta a ayudar.

A Rosita por toda su alegría y cariño, no hubiese sido lo mismo sin ese montón de risas que nos hemos pegado en el laboratorio.

A todos los demás compañeros Jean, Dani, Mario, Belén, Jesús, Sol, Rafita, que han sido unos verdaderos compañeros, siempre dispuestos a ayudar en todo lo que podían: dudas sobre protocolos, PCRs, estadística, gráficas, traducciones al inglés… y lo que es aun más importante, unas personas con las que te dan ganas de venir a trabajar cada día.

A las personas restantes de este grupo de investigación, Dra. Mercedes Campos, Dr. Juan Sánchez Raya, Dr. Paco Gallardo, Herminia, Luisa que me trataron muy bien desde el primer momento de mí llegada al grupo hasta ahora y con los que he pasado momentos inolvidables durante las celebraciones en la biblioteca .

A Pedrito, Mari Carmen, Toñi y Jesús del personal de seguridad de la Estación Experimental del Zaidín por dejarme siempre pasar con el coche aunque no quedasen plazas de aparcamientos, por abrirme la puerta de madrugada cuando estaban de ronda y por dejarme pasar los fines de semana aunque no estuviera apuntado.

A mis amigos y amigas fuera del mundo de la EEZ que me han ayudado a desconectar del absorbente mundo de la ciencia, aunque fueran culpables de que más de un día llegara al laboratorio sin dormir o con resaca. En particular, a mi colega Oscar, que aunque no sabe muy bien aun de que va mi tesis me alojó en su casa cuando terminó mi beca; aunque su influencia y la de sus vecinas también son responsables de que algún que otro día improductivo.

A la Selección Española de Fútbol por todas las alegrías que me ha dado durante los cuatro años dedicado a mi tesis doctoral, desde conseguir una Eurocopa hasta ser campeones del Mundo, haciendo que un experimento fallido no tenga importancia en comparación con el orgullo de que en Austria o en Ohio te identifiquen por español cuando llevas puesta la camiseta de "La Roja".

A todos los miembros de mi familia: mi padre, mi madre, hermana, mis abuelos, mi tío, mi tía… por estar siempre pendientes de mí desde Melilla, ofreciéndome vuestro cariño, apoyo, ayuda y comprensión, lo cual ha hecho posible que el desarrollo de esta tesis. ¡Gracias!.

ÍNDICE

SUMMARY

Nowadays, the recycling of huge quantities of plant debris and non-marketable fruits generated from greenhouse crops represent a worldwide environmental problem that should be solved by using low-cost technologies, such as vermicomposting. This is an eco-friendly bioprocess able to stabilize organic wastes from different nature by means of the joint action of earthworms and microorganisms under aerobic and mesophilic conditions. Thus, vermicomposting can bioconvert hazardous and worthless organic wastes into safe and valuable products which can be used as soil amendments, agricultural fertilizers or bioactive materials.

Hence, the present research was conducted in order to evaluate the feasibility of vermicomposting to recycle diverse types of greenhouse vegetal wastes: heterogeneous plant (HP), tomato-plant debris (P), or tomato-fruit wastes (T), into valuable vermicomposts. The vermicomposting of those wastes was performed at different scales (laboratory or pilot) by using different vermicomposting systems and bulking materials (chapters 1, 2, 3, 4). In a first study at laboratory scale, HP, P and T were mixed with cow dung or straw in 2:1 and 4:1 ratios in order to develop a vermicomposting process using the epigeic worm *Eisenia andrei*. Subsequently, fresh damaged tomatoes (T) were vermicomposted through a pilot vermireactor where an initial population of *Eisenia fetida* was introduced and fed continuously with this waste at a high organic loading rate (13.6 kg TOC m^{-3} wk^{-1}) for 150 days (chapter 4). Meanwhile, the feasibility of using paper-mill sludge as complementary waste for the vermicomposting of P was also investigated. In addition, the impact of imidacloprid residues, an insecticide frequently applied in greenhouse crops, on earthworms was also investigated during the vermicomposting of this waste (chapter 5).

During those vermicomposting processes, earthworm development and changes in physico-chemical features, enzyme activities, and microbial communities of the wastes were investigated to evaluate their proper biodegradation and biostabilization. The results indicated that high salt content in HP and P (>10 dS m^{-1}) prevented earthworm survival to vermicompost these greenhouse wastes. By contrast, T was successfully vermicomposted at laboratory scale or pilot scale, recording an adequate earthworm growth and reproduction. Likewise, although earthworms did not survive in P alone, mixing P with paper-mill sludge in a ratio of 2:1 or 1:1 allows earthworm survival and the vermicomposting of both wastes simultaneously. The results of vermicomposting of P containing imidacloprid reveal that greenhouse plant wastes containing imidaclorpid residues over 2 mg kg^{-1} are inadequate to be vermicomposted.

After the different vermicomposting processes, earthworm and associated microorganisms strongly changed the chemical features of the different types of greenhouse wastes, as indicated by the significant decrease in their TOC content, C:N ratios, and enzyme activities values, and the improvement of their degree of maturity which pointed out a successful degradation and stabilization of these wastes. Along with this chemical and biochemical changes, earthworm activity strongly transformed initial microbiota inhabiting the wastes, reducing their content in bacterial and fungal biomass and transforming their microbial structures, giving rise to vermicompost microbial communities which were different from those of initial wastes. Thus, the vermicomposting processes that were designed were able to biodegrade and biotransform greenhouse vegetable wastes (i.e. plant debris and damaged fruits) into valuable organic-materials with adequate features to be used as organic soil amendments for conventional, integrated and organic agriculture or as organic growing media for greenhouse crops

To evaluate the microbiological quality of the vermicomposts produced from greenhouse vegetal wastes, microbial communities inhabiting the vermicompost produced from damaged tomato-fruit by using a continuous vermicomposting process (vermicompost called DT) was investigated in-depth together with the resident microbial communities in other three vermicomposts produced from wastes of different origin (i.e. WW: winery wastes; OB: olive-mill waste and biosolids; and CM: cattle manure) which were also biotransformed by using *E. fetida*. Firstly (chapter 6), the resident bacterial community in these four vermicomposts was investigated by using PCR amplification of 16S RNA genes and subsequent denaturing gradient gel electrophoresis (DGGE) and COMPOCHIP (i.e. a microarray targeting typical bacteria of stabilized organic materials and pathogenic bacteria) analysis. Secondly (chapter 7), the chemical features, enzyme activities, community-level physiological profiles (CLPPs), fungal community structures in the four vermicompost was determined, investigating their interrelation with the microbial respiratory response of vermicompost microbiota to three different pesticides (i.e. metalaxyl, imidacloprid, and diuron). DGGE provided a distinctive fingerprint of each vermicompost type, which was statistically related with the particular vermicomposts' chemical features. The comparison of the vermicomposts' fingerprint showed that they housed bacterial communities with an average similarity coefficient near 80%. COMPOCHIP detected the common presence of *Sphingobacterium*, *Streptomyces*, Alpha-Proteobacteria, Delta-Proteobacteria, and Firmicutes in all the vermicomposts, and identified differences among the vermicomposts concerning the abundance of certain bacterial taxa which enables to estimate the

vermicompost microbiological quality. This approach can also provide a worthwhile guidance for future studies aimed at profiting from bacteria inhabiting vermicomposts for improving soil infertility and degradation, solving environmental pollution, controlling plant pest and diseases, and other biotechnological applications Vermicompost enzyme activities and CLPPs indicated that WW, OB, and DT had higher microbial functional diversity than CM. The microbiota of the former tolerated all three pesticides whereas microbial respiration in CM was negatively affected by metalaxyl or imidacloprid. The response of vermicompost microbiota to the fungicide metalaxyl was correlated to its fungal community structure. These results suggest that vermicomposts with higher microbial functional diversity can be useful for the management of pesticide pollution in agriculture.

To assess the influence of the vermicompost microbial community when this type of organic amendment is applied to soils, a study was performed with the aim of comparing the short-term effects on biological soil properties caused by the application of two different types of organic amendments as thermophilic-compost or vermicompost, which were produced independently by recycling of the same type of waste constituted of fruit and vegetables thrown out by grocery store (chapter 8). For this, a cropland soil was amended with thermophilic-compost, vermicompost, or a mixture of both in 4:1ratio by using rates equivalent of 0, 10 or 30 Tm ha^{-1} and incubated for 28 days. After incubation, both amendments increased the CO_2-C production, enzyme activities, and microbial biomass in soil amended at the rate of 30 Tm ha^{-1} as compared with unamended soil. A principal component analysis of fatty acid methyl esters (FAME) soil profile suggested that the microbial community structure developed in soil amended with the thermophilic-compost was different from that in soil amended with vermicompost.

Finally, to know the usefulness of the vermicompost produced from greenhouse tomato-fruit wastes (DT) in a complex soil-plant bioremediation system, a heavy metals (HM) contaminated soil was amended with DT or a commercial vermicompost and *Trifolium repens* plants were sown (chapter 9). The effect of inoculating arbuscular micorrhizal (AM) fungi to enhance plant growth and improve the bioremediation system was also investigated. The inoculation of the soils with AM fungi had weak effects on plant growth as well as on the availability and extractability of metals and enzyme activities compared to non-inoculation. The addition of both vermicomposts, but particularly DT, stimulate plant growth due to the supply of available nutrients, thus helping to establish on the HM-polluted soil a plant cover which can prevent contaminant migration via wind and water erosion, leaching, and soil dispersion. Moreover, amending soil with DT decreased HM extractability and

bioavailability in the contaminated soil and improved its microbial functional diversity and biochemical quality.

The results of this research provide a sound basis to improve the usefulness of vermicomposting as biotechnology for the recycling of greenhouse vegetable wastes into valuable organic-materials to be used in agriculture, soil restoration or bioremediation processes. The findings revealed by this research concerning vermicompost microbial communities represent novel information which can be useful for future studies aimed at unravelling the vermicompost microbiology.

INTRODUCCIÓN

I.1. CULTIVOS EN INVERNADERO O BAJO PLÁSTICO Y GENERACIÓN DE RESIDUOS

I.2. EL VERMICOMPOSTAJE COMO ESTRATEGIA PARA EL RECICLADO Y VALORIZACIÓN DE RESIDUOS ORGÁNICOS

I.3. ESTUDIO DE LAS COMUNIDADES MICROBIANAS EN PROCESOS DE VERMICOMPOSTAJE

I.1. CULTIVOS EN INVERNADERO O BAJO CUBIERTA Y GENERACIÓN DE RESIDUOS

I.1.1. DEFINICIÓN DE RESIDUO, CLASIFICACIÓN Y ESTADO ACTUAL DE GESTIÓN

En el año 2008, los 27 países de la Unión Europea, generaron más de 2600 millones de toneladas de residuos de diferente naturaleza, lo que correspondía a una producción anual de residuos por habitante europeo superior a 5 toneladas. En España, la producción de residuos supuso el 5.7% del total europeo, por lo que cada español generó 3.2 Tm de residuo al año (Figura I.1)(http://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=env_wasgen&lang=en). A pesar de la política desarrollada por la Unión Europea para favorecer la reducción de la producción de residuos y su reutilización, en la actualidad, su gestión y el apropiado reciclaje de ciertos residuos constituye uno de los mayores problemas con los que se enfrentan los países de la Unión.

Figura I.1. Porcentaje de residuos de diferente naturaleza generados por España respecto al total de la Unión Europea.

La Ley 22/2011 de residuos y suelos contaminados del Reino de España del 28 de julio del 2011, que incorpora al ordenamiento jurídico español la Directiva comunitaria 2008/98/CE, define residuo como cualquier sustancia u objeto que su poseedor deseche o tenga la intención o la obligación de desechar.

La Ley también incluye el término de subproducto, como la sustancia u objeto, resultante de un proceso de producción, cuya finalidad primaria no sea la producción de esa sustancia u objeto, y no se considera como residuo cuando se cumplan las siguientes condiciones: a) que se tenga la seguridad de que la sustancia u objeto va a ser utilizado

ulteriormente, b) que la sustancia u objeto se pueda utilizar directamente sin tener que someterse a una transformación ulterior distinta de la práctica industrial habitual, c) que la sustancia u objeto se produzca como parte integrante de un proceso de producción, y d) que el uso ulterior cumpla todos los requisitos pertinentes relativos a los productos así como a la protección de la salud humana y del medio ambiente, sin que produzca impactos generales adversos para la salud humana o el medio ambiente.

Aunque los residuos de diferente naturaleza y origen han sido tradicionalmente clasificados atendiendo a su estado físico, este criterio de clasificación resulta en ocasiones impreciso, ya que, por ejemplo, el calificativo de sólido puede ser en ocasiones ambiguo si consideramos que la mayoría de los efluentes líquidos y gaseosos, son realmente suspensiones de sólidos en otros medios. Por esta circunstancia, clasificar los residuos de acuerdo a los distintos sectores productivos de las actividades de nuestra sociedad que los originan puede ser, aunque amplio, un criterio más adecuado. Según éste, los residuos pueden agruparse en tres grupos:

- Residuos producidos por el sector primario: dentro de este grupo se engloban los diversos residuos agrícolas, ganaderos, pesqueros y forestales.
- Residuos producidos por el sector secundario: englobados en esta categoría se encuentran una gran variedad de residuos resultantes de diversos procesos industriales, así como los residuos derivados de la minería y la construcción.
- Residuos producidos por el sector terciario: principalmente representados por los residuos sólidos urbanos y las aguas residuales urbanas.

En la actualidad, la gestión (recogida, almacenamiento, transporte, tratamiento y eliminación) de las enormes cantidades de residuos que genera la actividad humana ha avanzado de forma simultánea a la mayor concienciación ciudadana y al interés medioambiental despertado por las administraciones públicas y privadas. Aunque, la mayoría de los profesionales implicados en la gestión de los residuos reconocen que no hay una estrategia global y única para gestionar todos los diferentes residuos generados por cada sector, si existe consenso en los objetivos claves que deben ser perseguidos:

1) Prevenir la generación de residuos, reduciendo el volumen y toxicidad de los residuos producidos.
2) Reciclar, reutilizar y valorizar los residuos generados, tanto como sea posible, buscando, en lo posible, un valor añadido para ellos.

3) Recuperar energéticamente los residuos sobrantes mediante tecnologías de bajo impacto ambiental.
4) En último caso, si no es posible alcanzar los objetivos anteriores, eliminar los residuos no reciclables de forma segura bajo control medioambiental es la medida a adoptar.

La Ley de residuos y suelos contaminados deja claro esos conceptos claves:

- Prevención: medidas adoptadas antes de que una sustancia, material o producto se haya convertido en residuo, para reducir: a) La cantidad de residuo, incluso mediante la reutilización de los productos o el alargamiento de la vida útil de los productos; b) Los impactos adversos sobre el medio ambiente y la salud humana de los residuos generados, incluyendo el ahorro en el uso de materiales o energía; c) El contenido de sustancias nocivas en materiales y productos.
- Reutilización: cualquier operación mediante la cual productos o componentes de productos que no sean residuos se utilizan de nuevo con la misma finalidad para la que fueron concebidos.
- Reciclado: toda operación de valorización mediante la cual los residuos son transformados en nuevos productos, materiales o sustancias que son útiles para la finalidad original o para cualquier otro uso. Incluye la transformación del material orgánico, pero no la valorización energética ni la transformación en materiales que se vayan a usar como combustibles o para operaciones de relleno.
- Valorización: cualquier operación cuyo resultado principal sea que el residuo sirva para sustituir la finalidad útil de otros materiales, que de otro modo se habrían utilizado para cumplir una función particular.
- Eliminación: cualquier operación que no sea la valorización, incluso cuando la operación tenga como consecuencia secundaria el aprovechamiento de sustancias o energía.

En el caso concreto de los residuos orgánicos biodegradables, definidos como residuos que en condiciones de vertido pueden descomponerse de forma aerobia o anaerobia, su gestión es variable, dependiendo de la naturaleza del residuo y del lugar donde se origina. Particularmente en Europa, el reciclaje de los residuos orgánicos hasta materiales orgánicos estabilizados aptos para ser utilizados como enmiendas orgánicas de suelos agrícolas, ha sido establecido por la Directiva europea 2006/12/CE como una de las estrategias de gestión más aconsejables, sobre todo si se considera que el 75% de los suelos del Sur de Europa poseen

un contenido en material orgánica por debajo del 2% (Zdruli et al., 2004). La producción de materia orgánica estabilizada que pueda ser óptimamente aplicada a este tipo de suelos mejoraría sus propiedades estructurales, fisicoquímicas y biológicas, evitando su posible degradación a la vez que se incrementa su fertilidad (Aguilar & González, 2002; Albiach et al., 2000, 2001). Desde este punto de vista, el reciclado de residuos orgánicos en materiales orgánicos estables, maduros, ricos en nutrientes, y con propiedades biológicas adecuadas constituye no sólo una estrategia de reutilización de residuos, sino además, una forma de valorizar estos residuos en materiales orgánicos de valor añadido como enmiendas de suelos o como productos con valor agrícola.

I.1.2. CULTIVOS EN INVERNADERO O BAJO PLÁSTICO Y SUS RESIDUOS GENERADOS

El desarrollo de cultivos bajo plástico y/o en invernaderos se está expandiendo rápidamente a nivel mundial debido a que estos sistemas de cultivo permiten la producción de vegetales de alta calidad a lo largo de todas las épocas del año. Actualmente, se estima que más de dos millones de hectáreas se encuentra ocupadas por este tipo de cultivos en todo el mundo (Pardossi et al., 2004), destacando esta práctica agrícola en China, donde los cultivos de invernadero ocupan alrededor de un millón y medio de hectáreas (Jiang et al., 2003). La otra gran zona de localización de esta práctica agrícola es la cuenca mediterránea, con aproximadamente 200000 hectáreas, principalmente concentradas en España e Italia (Pardossi et al., 2004). Según los datos facilitados por la última encuesta sobre superficies y rendimientos de cultivos de España (Ministerio de medio ambiente y medio rural y marino, 2010), actualmente 62505 hectáreas se encuentran ocupadas por cultivos de invernadero; concentrándose el 70% de esas hectáreas en Andalucía. Estos cultivos se encuentran dedicados principalmente a la producción de una gran variedad de hortalizas y algunas frutas, destacando la superficie dedicada a la producción de tomates (8468 ha), fresas-fresones (6060 ha), pimientos (4698 ha) y pepinos (2999 ha); también es importante la producción de flores y plantas ornamentales en invernadero que ocupa 1446 ha.

El gran rendimiento de los cultivos en invernadero o bajo plásticos implica que se genere conjuntamente una gran cantidad de residuos derivados de este tipo de práctica agrícola (Parra et al., 2001; Stanghellini et al., 2003). La imposibilidad de que los invernaderos asuman y reintegren la gran cantidad de residuos que producen (a diferencia de lo que ocurre

en otros sistemas agrícolas) supone que estos flujos residuales hayan sido considerados como uno de los principales problemas a resolver en las áreas donde esta intensa práctica agrícola se encuentra extendida (Parra et al., 2008). Los principales residuos generados por los cultivos en invernadero pueden clasificarse, en las siguientes categorías principales según su naturaleza: residuos plásticos, residuos de envases, residuos de substratos, residuos líquidos y residuos vegetales (Tabla I.1). Además de estos residuos mayoritarios, los invernaderos producen otros residuos minoritarios de carácter heterogéneos como maderas, alambres, etc., de los que se dispone de menor información.

Tabla I.1. Producción anual media de residuos de cultivos en invernadero (Cara & Riviera, 1998; Cuadrado, 2001).

Tipo residuo	Producción por hectárea
Residuo vegetal	29.1 toneladas
Sustratos	6-10 toneladas
Plásticos	0.7 toneladas
Líquidos y lixiviados	0.3 toneladas
Envases fertilizantes y fitosanitarios	68 unidades

I.1.2.1 Residuos plásticos

La mayoría de los residuos plásticos generados por los invernaderos provienen de las láminas de cubiertas plásticas utilizadas en la infraestructura de estos sistemas, las cuales van siendo periódicamente desechadas una vez finalizada su vida útil, pudiendo llegar a acumularse entre 2 y 2.6 t ha^{-1} tras dos o tres años de cultivo (Escobar, 1998) (Figura I.2a). Junto a estos plásticos, las cuerdas plásticas de polipropileno, denominadas rafias, que se usan en los cultivos de invernadero para mantener extendidas las diferentes plantas cultivadas son otro residuo plástico generado, aunque éste sólo representa el 3-4% del total de los residuos plásticos generados anualmente por los invernaderos (Tolón & Lastra, 2010) (Figura I.2b). Otros residuos plásticos como los provenientes de los acolchados usados en algunos cultivos, representan un porcentaje incluso inferior al de las rafias.

Figura I.2. Residuos de plásticos de invernaderos (a) y residuos de rafias mezclados con residuos vegetales de invernaderos (b).

I.1.2.2. Envases de fertilizantes y fitosanitarios

En los cultivos de invernadero españoles se ha estimado una utilización anual de alrededor de casi 70 unidades de fertilizantes y fitosanitarios por hectárea de invernadero con una capacidad media de 1.6 litros/envase (Cara & Riviera, 1998); aunque en el caso de los cultivos que desarrollan en invernadero siguiendo un sistema de producción integrado la generación de envases de plaguicidas es menor. Estos envases se caracterizan por presentar una naturaleza plástica y metálica predominantemente, mientras que el vidrio y los cartones son menos frecuentes; poseyendo un gran poder contaminante si son desechados de manera inadecuada debido a su contenido en restos de estos insumos (Figura I.3).

Figura I.3. Envases de fertilizantes abandonados en zonas adyacentes a los invernaderos.

I.1.2.3. Residuos de substratos

Los substratos utilizados en cultivos de invernadero del tipo hidropónico llegan a convertirse en un residuo cuando su vida útil llega a su fin. Los substratos más comúnmente empleados en el sudeste español son la lana de roca y la perlina, seguidos por la fibra de coco (Urrestarazu et al., 2005). Aunque en España los invernaderos hidropónicos sólo representan alrededor del 20% de los cultivos de invernadero (Tolón & Lastra, 2010), estos pueden llegar a generar tras dos o tres años de cosecha cantidades promedio alrededor a 75 m^3 ha^{-1} de lana de roca o 128 m^3 ha^{-1} de perlita (Escobar, 1998).

I.2.2.4. Residuos líquidos

La mayor parte de estos residuos provienen de los sobrantes del regadío, de la utilización de dosis excesivas y del lavado de los residuos sólidos gestionados de manera inadecuada (Cuadrado, 2001). Aunque no representan una gran proporción en el global de residuos generados por los invernaderos (Tabla I.1), los residuos líquidos poseen un potencial riesgo ambiental ya que suelen contener cantidades variables de restos de fertilizantes y plaguicidas debido a prácticas de lavado de sus envases (Cuadrado, 2001; Tolón & Lastra, 2010). En este sentido, problemas de contaminación de acuíferos han sido asociados a cultivos en invernadero debido a un inapropiado diseño para el control de este tipo de residuos (Song et al., 2009).

I.1.2.5. Residuos vegetales

Aunque inicialmente este tipo de residuos no fue considerado como tal debido a su presunta fácil biodegradabilidad, las enormes cantidades producidas de estos restos vegetales en las áreas con importante concentración de cultivos de invernadero, junto a su residual contenido en restos de plaguicidas y otros xenobióticos, otorgaron a estos materiales vegetales la denominación legal de residuos. Dentro de este tipo de residuos se debe distinguir entre el residuo vegetal constituido por los residuos de las plantas cultivadas en invernadero – matas de plantas que son desechadas tras la cosecha – y el residuo vegetal constituidos por los frutos – destríos – que son descartados bien porque no son aptos para su comercialización debido al efecto de magulladuras, tamaño inadecuado, podredumbre, etc., o bien porque a

pesar de ser aptos no son comercializados debido a cuestiones mercantiles (principalmente para elevar el precio de estos frutos en el mercado).

Los residuos vegetales – matas de plantas – generados por los cultivos de invernadero (Figura I.4) constituyen un importante problema ambiental debido a que son el residuo que se genera en mayor cantidad y volumen entre todos los originados por estos cultivos (Tabla I.1). La cantidades de restos de plantas generadas anualmente varían entre los distintos tipos de cultivo, siendo el cultivo de tomate el que genera mayor volumen de residuos vegetales por hectárea, entre 40-50 Tm de restos de plantas por hectárea y año (Manzano-Agugliaro, 2007; Tolón & Lastra, 2010). Concretamente, en la región del litoral de Granada, se ha estimado que los diferentes cultivos de invernadero generan una media de 28.5 t ha^{-1} $año^{-1}$ de restos de plantas (Manzano-Agugliaro, 2007). Los restos de plantas procedentes de los diversos cultivos de invernadero constituyen en conjunto un residuo con unas características especiales, que los diferencian de los otros residuos vegetales generados por los sistemas de cultivo tradicionales. En comparación, los restos de plantas generados por los cultivos en invernadero presentan un mayor grado de humedad (alrededor al 60% en el momento de su retirada), un elevado contenido en sales, y una baja relación C/N; además de una concentración mayor de restos de productos fitosanitarios.

Figura I.4. Residuos de plantas –matas de plantas- de cultivos de invernadero.

En cuanto al residuo vegetal constituido por los frutos descartados, denominado frutos de destríos, aunque son generados en menor cantidad, en comparación a la que representa los restos de plantas, ésta no debe ser menospreciada ya que puede representar hasta el 25 % de la cantidad total de residuos vegetales generados por los cultivos en invernadero. Además, a este porcentaje de destríos de invernadero, debería sumarse la importante cantidad de frutos y vegetales, que a pesar de ser comercializados, son finalmente descartados, como destríos,

por fruterías y supermercados una vez el producto deja de ser apto para el consumidor. En España, la cantidad de frutas y hortalizas desechadas por los supermercados y minoristas no ha sido investigada aun, a diferencia de otros países como Brasil, donde se realizó una investigación a gran escala que determinó que el 16% de las frutas y verduras de los comercios del país no llegan a ser vendidas antes de que se deterioren, siendo finalmente descartadas (Fehr et al., 2002). Algunas encuestas y estimaciones no oficiales han afirmado que entre el 10 y el 15% de las frutas y verduras procedentes de los cultivos en invernaderos en España terminan siendo desechadas en la última fase del proceso de comercialización.

I.1.3. GESTIÓN DE RESIDUOS SÓLIDOS GENERADOS POR LOS CULTIVOS DE INVERNADERO

I.1.3.1. Residuos plásticos, envases de fitosanitarios y fertilizantes, y residuos de sustratos

El reciclaje de residuos inorgánicos (plásticos, fitosanitarios, fertilizantes y otros) generados por los cultivos de invernadero se encuentra relativamente bien gestionado en la actualidad. Por ejemplo el reciclaje de las láminas de las cubiertas plásticas mediante su transformación en otros materiales plásticos, como vallas, postes, bolsas, etc. es prácticamente total hoy en día (Castilla, 2004). Aunque persiste el problema del ineficiente reciclaje de plásticos que están muy degradados (Tolón & Lastra, 2010).

En lo que se refiere a los envases de fitosanitarios y otros insumos, éstos están sometidos a una reglamentación especial ya que poseen un enorme potencial contaminante. La Ley obliga a los fabricantes, envasadores y los comerciantes de productos fitosanitarios a establecer un Sistema de Depósito, Devolución y Retorno (SDDR) o bien participando en un Sistema Integrado de Gestión como SIGFITO (Sigfito Agroenvases, 2009), que funciona a través de una red de puntos de recogida, denominados Centros de Agrupamiento. Sin embargo, se estima que aproximadamente sólo el 70% de los productores los devuelven al almacén o los depositan en contenedores apropiados, mientras que un 15% son depositados en vertederos y el otro 15% son abandonados de manera inadecuada (Tolón & Lastra, 2010). Menos problemática es la gestión de los substratos agotados generados por los cultivos hidropónicos en invernadero, los cuales usualmente suelen ser reutilizados en otros sectores como por ejemplo en la construcción, o bien se desinfectan para ser usados como enmiendas

en suelos con problemas de estructura y textura (Urrestarazu et al., 2005). Igualmente, los lixiviados y otros residuos líquidos son eficientemente gestionados en invernaderos donde se desarrollan los cultivos hidropónicos, y actualmente el incremento de la mecanización y las nuevas tecnologías en los invernaderos donde el cultivo se realiza sobre el suelo ha minimizado el riesgo de contaminación debido a estos residuos (Castilla, 2004; Song et al., 2009).

I.1.3.1. Residuos vegetales de invernadero

Hoy en día, la gestión de los residuos vegetales producidos por los invernaderos, restos de matas y destríos es ineficaz, dada la enorme cantidad que alcanzan estos residuos en áreas de alta densidad de cultivos en invernadero (Parra et al., 2008). Tradicionalmente, los restos de diferentes plantas cultivadas en invernaderos, acompañados normalmente por las rafias plásticas, solían ser vertidos en las inmediaciones de estos cultivos, o en zonas abiertas como vertederos semicontrolados, donde incluso pastaba ganado (ovino o caprino), con el consiguiente riesgo que supone la incorporación a su metabolismo de restos de fitosanitario empleados en estos cultivos (Garrido-Frenich et al., 2003). Además, la acumulación incontrolada de estos residuos pueden crear focos de infección de plagas para los propios cultivos (Suárez-Estrella et al., 2004) así como la contaminación de acuíferos y aguas superficiales (Song et al., 2009). En la actualidad, la apropiada gestión de este tipo de residuos representa un importante problema ambiental, debido a la particular naturaleza de estos residuos que acarrea la generación de gases con efecto invernadero cuando éstos son acumulados en vertederos, donde además incrementan el riesgo de aparición de fuegos incontrolados (Parra et al., 2001). Particularmente, la gestión de los frutos de destríos, aunque originados en menor cantidad que las matas de plantas, es un problema que no ha sido solventado eficazmente. Además, su acumulación en vertederos no es una forma eficaz de gestión ya que el elevado contenido hídrico de los destríos origina importantes cantidades de líquidos con un alto contenido nutritivo y microbiano, los cuales pueden lixiviar y contaminar ambientes adyacentes.

En los últimos años en España se ha realizado un notable esfuerzo para conseguir una adecuada gestión de los residuos generados por los cultivos de invernadero. Así, en la provincia de Almería, en el término de Nijar se ha instalado una planta gasificadora, que transforma 20000 Tm de residuos vegetales en energía eléctrica (Figura I.5).

Figura I.5. Planta de Gasificación de Residuos Vegetales de Invernadero de Nijar.

Sin embargo, el poco poder calorífico de las matas de plantas no leñosas hace que este proceso sea de baja eficiencia comparándolo con la gasificación de otros residuos más leñosos. Además, la separación previa de las rafias de los residuos vegetales es necesaria si estos van a ser sometidos a gasificación ya que la presencia de estos materiales plásticos tiene como consecuencia liberar sustancias tóxicas peligrosas durante estos procesos, como dioxinas y otros contaminantes (Hedman et al., 2006). Para ello se requiere un inicial proceso de secado del entramado de matas envueltas seguido de un proceso de trituración y criba que libera los restos de plantas de las rafias. Por otro lado, la valorización energética de los residuos vegetales de invernadero requiere de una elevada inversión económica en la infraestructura necesaria para estos procesos. Además, varios estudios de evaluación de impacto ambiental han mostrado que desde el punto de vista medioambiental es mejor reciclarlos, y transformarlos en materiales orgánicos estabilizados para su posterior uso como enmiendas del suelo. Por ejemplo, Antón et al. (2005) concluyendo que el tratamiento de los residuos vegetales de tomate procedentes de cultivos en invernaderos mediante compostaje es una práctica de menor impacto ambiental en comparación con la que muestra la incineración y el vertido en vertederos de estos residuos, especialmente si se considera el impacto de estas prácticas en el cambio climático.

La transformación de estos residuos orgánicos en materiales orgánicos estabilizados y maduros permite recuperar los nutrientes empleados para el crecimiento vegetal en los cultivos de invernadero, pudiéndose usar los restos vegetales estabilizados como enmiendas orgánicas de suelos o fertilizantes orgánicos. En este sentido, es bien conocido que en Andalucía la mayoría de sistemas de invernadero se desarrollan en suelo enarenado al que se le incorpora una capa de materia orgánica, que es normalmente reemplazada cada año

(Castilla, 2004; Cuadrado, 2001; Parra et al., 2008). Por este motivo, se instaló en el año 2001 una planta de compostaje en El Ejido (Almería) (Figura I.6), y otra en 2006 en Motril (Granada) (Figura I.7).

Figura I.6. Planta de compostaje de residuos vegetales de invernadero (El Ejido, Almería).

Figura I.7. Planta de compostaje de residuos vegetales de invernadero (Motril, Granada).

Aunque el compostaje de esto residuos permite actualmente bioestabilizarlos reduciendo su volumen, las características particulares de estos residuos (alto contenido en humedad y baja relación carbono:nitrógeno) impiden el desarrollo de un adecuado proceso de compostaje (Alkoaik & Ghaly, 2005, 2006), por lo que el compost resultante es de baja calidad y de difícil comercialización. En las plantas de compostaje, los destríos son un problema adicional, ya que su elevado contenido hídrico provoca, por una parte, una ralentización del proceso de compostaje de los residuos vegetales en los túneles de fermentación y por otra, la generación de grandes cantidades de lixiviados llegan a colmatar las balsas de recepción y almacenamiento de agua de lluvia y líquidos derivados de los propios residuos.

Debido a que la cantidad de residuos vegetales generados por los cultivos en invernaderos es actualmente demasiado elevada para ser eficientemente reciclada por los sistemas de gestión que existen actualmente, la búsqueda de otras alternativas innovadoras que permitan su eficaz valorización debería ser uno de los objetivos perseguidos para conseguir que este tipo de cultivo intensivo sea sostenible a largo plazo. Las nuevas alternativas, a diferencia de las ya existentes, deben cumplir una serie de requisitos para su viabilidad:

1) Utilizar tecnologías de bajo coste que sean sostenibles medioambientalmente y susceptibles de uso por los propios agricultores en los lugares en que se generan los residuos, evitando así su transporte desde los invernaderos a los centros de acopio y tratamiento de estos residuos.
2) Favorecer la reutilización y diversificación de usos de los residuos vegetales de invernadero en diferentes sectores (agrícola, ganadero, etc.) aumentando la rentabilidad de los sectores implicados.

Así, mediante una gestión adecuada, los residuos vegetales producidos por los cultivos de invernaderos, en vez de ser un problema ambiental, podrían ser considerados como un recurso para producir materiales orgánicos útiles como enmiendas de suelos o productos orgánicos fertilizantes, que podría ser empleados de nuevo en los cultivos de invernadero, cerrando así el ciclo de la materia orgánica.

I.2. EL VERMICOMPOSTAJE COMO ESTRATEGIA PARA EL RECICLADO Y VALORIZACIÓN DE RESIDUOS ORGÁNICOS

I.2.1. DEFINICIÓN Y CARACTERÍSTICAS DEL VERMICOMPOSTAJE

El vermicompostaje es un proceso biotecnológico que permite degradar y estabilizar residuos orgánicos bajo condiciones aerobias y mesófilas mediante la acción de ciertas especies de lombrices de tierra capaces de alimentarse del residuo a la vez que aceleran su degradación microbiana. Así, en este proceso se aprovecha la capacidad detritívora de las lombrices, que ingieren, trituran y digieren el residuo orgánico, descomponiéndolo mediante la acción de sus enzimas digestivas y de la microflora aeróbica y anaeróbica presente en el interior de su intestino (Edwards, 1988).

En líneas generales, las diferentes especies de lombrices involucradas en el proceso son capaces de ingerir al día cantidades de residuos equivalentes al 50-100% de su propio peso, dependiendo del tipo de residuo y la especie de lombriz empleada (Edwards & Bohlen, 1996; Garg et al., 2008; Riggle & Holmes, 1994). La digestión del residuo orgánico por la lombriz conlleva una alteración física ya que éste es fragmentado, reduciéndose su volumen hasta aproximadamente el 50% (Sinha et al., 2010b), a la vez que aumenta su relación superficie-volumen; lo cual facilita la colonización microbiana del residuo excretado (Domínguez, 2004). El paso del residuo orgánico a través del intestino de la lombriz también altera su composición química de forma que se incrementa la concentración de nutrientes fácilmente asimilables para los microorganismos, los cuales proliferan rápidamente en el residuo recién excretado terminando su degradación (Parthasarathi & Ranganathan, 1999). Además, indirectamente, el mucus segregado por la lombriz y sus excreciones estimulan la proliferación de microorganismos degradadores de materia orgánica en el residuo durante el proceso de vermicompostaje, pues estas sustancias son una fuente de compuestos de fácil asimilación para los microorganismos (Domínguez et al., 2010). Igualmente, el movimiento de las lombrices a través del residuo del que se alimentan promueve su aireación estimulando su bioxidación microbiana (Domínguez, 2004). De este modo, gracias a la acción conjunta de lombrices y microorganismos el residuo orgánico es degradado hasta mineralizarse parcialmente, humificarse y estabilizarse.

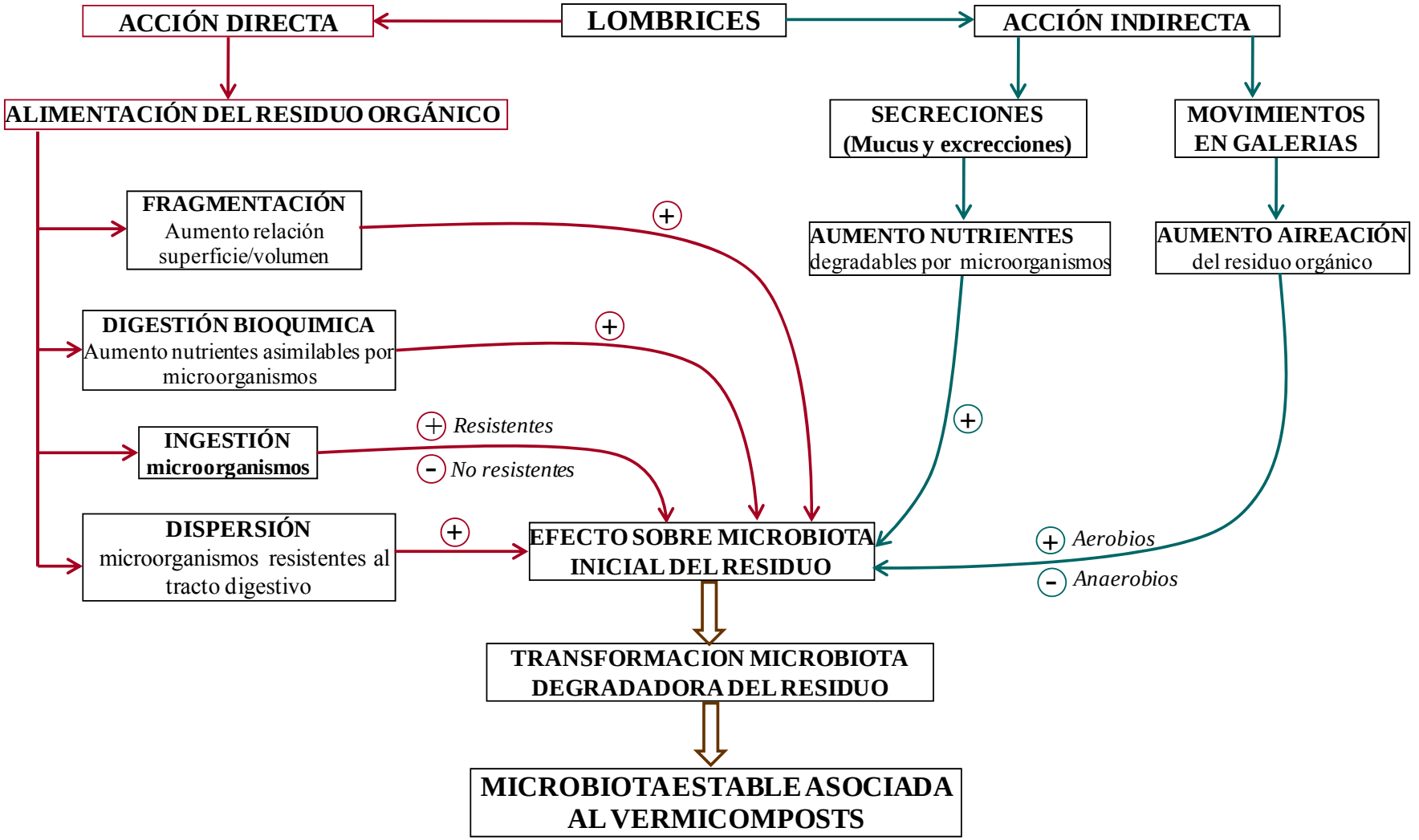
La degradación, mineralización y estabilización de los residuos orgánicos causada por el vermicompostaje se refleja en una disminución de los valores de carbono orgánico total del residuo orgánico, la cual ocurre en un grado variable (entre un 10 y un 55%, respecto al contenido inicial en carbono orgánico del residuo) dependiendo de la naturaleza del residuo orgánico, su biodegradabilidad, la especie de lombriz utilizada y su densidad, así como de las condiciones en las que se desarrolla el vermicompostaje y su duración (Nogales et al., 2008). Todos estos factores también condicionan el grado de polimerización y policondensación de la materia orgánica del residuo durante el vermicompostaje, originándose tras este proceso la aparición de compuestos similares a los ácidos húmicos y fúlvicos de los suelos (Li et al., 2011; Romero et al., 2007).

El vermicompostaje también suele producir un aumento general de la concentración de nutrientes vegetales en el residuo vermicompostado (Buchanam et al., 1988). Este fenómeno es consecuencia de la mineralización la materia orgánica del residuo y de la reducción de su volumen ocurrida tras el vermicompostaje, la cual resulta en un aumento de la concentración

de nutrientes en el residuo orgánico vermicompostado, especialmente apreciable en el caso del fósforo, calcio, magnesio y algunos micronutrientes (Nogales et al., 2008). Además, el vermicompostaje favorece que los elementos de la materia orgánica se mineralicen hasta formas químicas solubles que son fácilmente asimiladas por las plantas (Edwards & Burrows, 1988; Orozco et al., 1996). En el caso particular del nitrógeno, el vermicompostaje tiene un efecto variable en su concentración observándose tanto aumentos como disminuciones, dependiendo del tipo de residuo orgánico y de las condiciones particulares de vermicompostaje. En este sentido, se han registrado disminuciones de las concentraciones de nitrógeno tras el vermicompostaje, principalmente en residuos que poseen una cantidad elevada de este nutriente, debido al propio consumo por parte de las lombrices inoculadas (Bansal & Kapoor, 2000), a procesos de desnitrificación (Hobson et al., 2005), o a pérdidas por volatilización de este elemento en forma de amonio (Nogales et al., 1999). Por el contrario, el vermicompostaje de residuos orgánicos con bajo contenido en nitrógeno causa generalmente un incremento de la concentración de elemento en el producto vermicompostado, debido al efecto de la mineralización del residuo (Atiyeh et al., 2000), a procesos microbianos de fijación de nitrógeno atmosférico (Bhattacharya & Chattopadhyay, 2004), a la adición de compuestos nitrogenados contenidos en el mucus de la lombriz y en sus excreciones (Tripathi & Bhardwaj, 2004), e incluso en ocasiones a la descomposición de lombrices que mueren en algunos procesos de vermicompostaje (Suthar & Singh, 2008b). Por el contrario, algunos nutrientes, como el potasio, pueden perderse tras el vermicompostaje, especialmente cuando éste se lleva a cabo usando sistemas que permiten el drenaje de agua contenida en el lecho orgánico. En estos sistemas de vermicompostaje, un exceso de agua aplicada causa que las formas químicas altamente solubles liberadas tras la mineralización de los residuos orgánicos pueden sean arrastradas con el agua de drenaje (Garg & Kaushik, 2005).

Junto a las transformaciones físico-químicas ocurridas en el residuo durante el vermicompostaje, su microbiota autóctona también se transforma Debido a que lombrices sobre el residuo orgánico condicionan, directa o indirectamente, las poblaciones de microorganismos que se desarrollan durante el proceso, favoreciendo o dificultando la supervivencia de los diversos microorganismos (Domínguez et al., 2010; Monroy et al., 2009). La figura I.8 resume las interacciones entre las lombrices y los microorganismos autóctonos del residuo orgánico que determinan transformación de la microbiota del residuo durante el proceso de vermicompostaje.

Figura I.8. Acción de las lombrices sobre residuos orgánicos e interacciones con los microorganismos durante el proceso de vermicompostaje.



I.2.2. ESPECIES DE LOMBRICES HABITUALMENTE USADAS EN VERMICOMPOSTAJE

De las más de 4400 especies de lombrices terrestres identificadas, solamente unas pocas, pueden ser utilizadas en la degradación de residuos orgánicos. Estas lombrices, pertenecientes taxonómicamente al Orden *Haplotaxida y* Familia *Lumbricidae*, se agrupan en la categoría ecológica de epigeas, que poseen una estrategia reproductiva "r" (rápida y prolífica), lo cual permite que sucesivas generaciones de lombrices se vayan sustituyendo de manera continua, manteniendo por ello unas altas tasas de consumo del sustrato orgánico, lo cual acelera su degradación. Acorde con estos requerimientos, destacan las siguientes especies de lombrices usadas en vermicompostaje (Figura I.9, Tabla I.2).

Figura I.9. Principales especies de lombrices epigéicas utilizadas en procesos de vermicompostaje.

Eisenia fetida (Savigny, 1826) y *Eisenia andrei* (Bouché, 1972)

Estas especies son las más empleadas en procesos de vermicompostaje debido a su gran capacidad para adaptarse a las diferentes características de los distintos residuos orgánicos a vermicompostar, ya que poseen un amplio rango de tolerancia al pH, temperatura y humedad del substrato orgánico en el que se desarrollan (Domínguez, 2004). Además, son lombrices fuertes, resistentes y fáciles de manejar, ya que viven en cautiverio sin fugarse de su lecho. Igualmente, son muy voraces, y bajo condiciones óptimas pueden llegar a consumir diariamente una cantidad de residuos equivalente a su peso (Riggle & Holmes, 1994).

Ambas especies son muy parecidas a nivel morfológico, pudiendo convivir y desarrollarse en el mismo substrato orgánico (Bouché, 1972); hechos por los que antiguamente fueron consideradas como una única especie de lombriz (*E. foetida*). Sin embargo, actualmente ha sido demostrado que son especies de lombrices diferentes (Domínguez et al., 2003), distinguiéndose *E. fetida* por su color marrón con bandas intersegmentarias pálidas y/o amarillentas, recibiendo así el sobrenombre de lombriz tigre; mientras que *E. andrei* es conocida como la lombriz roja californiana debido a su pigmentación homocrómica rojo oscuro sin bandas. Pese a estas diferencias ambas especies presentan similar ciclo reproductor en un proceso de vermicompostaje el cual es uno de los más rápidos comparado con el de otras lombrices usadas en procesos de vermicompostaje (Tabla I.2).

Dendrobaena rubida (Savigny, 1826) y *Dendrobaena veneta* (Rosa, 1886)

Ambas especies de lombrices son características de climas templados donde han sido recomendadas para el vermicompostaje gracias a una relativamente rápida velocidad de crecimiento y una capacidad de proliferación, mayor en el caso de *D. rubida* (Domínguez, 2004). Particularmente, *D. veneta* se considera una buena lombriz para producción de proteína animal debido a su mayor biomasa (Domínguez, 2004).

Lumbricus rubellus (Hoffmeister, 1843)

La utilidad de esta especie en el desarrollo de procesos de vermicompostaje en climas templados ha sido descrita (Adi & Noor, 2009; Elvira et al., 1997), pero su utilización no es tan extendida, debido a su menor tasa de crecimiento y a un ciclo de vida más largo respecto a otras lombrices (Tabla I.2).

Eudrilus eugeniae (Kinberg, 1867)

Conocida como lombriz africana por ser nativa de ese continente, esta especie de lombriz es la de mayor tamaño de entre todas las usadas en vermicompostaje (Tabla I.2), proporcionando al proceso un interés extra ya que permite la producción de proteína animal a la vez que se estabiliza el residuo orgánico. Bajo unas condiciones adecuadas, esta especie es muy prolífica, presentando un crecimiento y ciclo reproductor relativamente rápido. Sin embargo, su estrecho margen de tolerancia respecto a la temperatura ambiental limita su utilización a procesos de vermicompostaje desarrollados en climas tropicales o subtropicales,

Tabla I.2. Características de la biología de las diversas especies de lombrices utilizadas en procesos de vermicompostaje (Domínguez, 2004; Parthasarathi, 2007; Tripathi & Bhardwaj, 2004).

Especies	Biomasa promedio lombriz adulta	Ciclo vida	Cápsulas día^{-1}	Lombrices capsulas^{-1}	Rango T^{a} (óptima)	Rango humedad (óptima)
E. fetida *E. andrei*	0.55 g	45 – 51 días	0.4	3.2	10 – 35 (25) ºC	50 – 90 (80) %
D. rubida	0.25 g	75 días	0.2	1.7	15 – 25 (25) ºC	65 – 85 (75) %
D. veneta	0. 92 g	100 – 150 días	0.3	1.1	15 – 25 (25) ºC	65 – 85 (75) %
L. rubellus	0.80 g	120 – 160 días	0.2	1.0	10 – 35 (25) ºC	70 – 90 (80) %
E. eugeniae	3.00 g	50 – 70 días	0.4	2.5	15 – 35 (30) ºC	70 – 85 (80) %
P. excavatus	0.55 g	40 – 50 días	0.2	1.0	20 – 40 (35) ºC	75 – 85 (80) %
L. mauritii	0.80 g	150 días	0.3	3.2	20 – 35 (30) ºC	50 – 80 (60) %

a procesos de vermicompostaje donde se mantenga la temperatura controlada; así esta especie ha sido frecuentemente producida en Estados Unidos para ser comercializada como cebo de pesca (Domínguez, 2004).

Perionyx excavatus (Perrier, 1872) y *Perionyx sansibaricus* (Perrier, 1872)

Conocidas como lombriz azul y lombriz púrpura, estas especies de lombrices presentan una tasa de reproducción muy alta bajo condiciones óptimas de vermicompostaje, registrando hasta un 90% de éxito en la eclosión de sus capullos (Domínguez, 2004). Sin embargo, el uso de estas especies en procesos de vermicompostaje se encuentra restringido a climas tropicales y subtropicales debido a su incapacidad para resistir temperaturas bajas.

Lampito mauritii (Kinberg, 1867)

Esta especie de lombriz es endémica de la India, por lo que su uso a nivel mundial en procesos de vermicompostaje está poco extendido, aunque existen trabajos que han descrito que esta especie es capaz de vermicompostar residuos orgánicos (Parthasarathi, 2007; Tripathi & Bhardwaj, 2004). Incluso, esta lombriz se desarrolla mejor que *E. fetida* en sustratos orgánicos con menor contenido en humedad (Tripathi & Bhardwaj, 2004), por lo que podría emplease preferentemente en aquellos lugares donde la escasez de agua sea un factor limitante para el vermicompostaje. Sin embargo, la mayor duración de su ciclo biológico y su menor tasa reproductiva, en comparación con otras especies de lombrices (Tabla I.2), podrían ser responsables de que su utilización en vermicompostaje no se haya extendido fuera de la India.

I.2.3. MICROORGANISMOS IMPLICADOS EN EL VERMICOMPOSTAJE

En los procesos de vermicompostaje se encuentran involucrados un gran número de microorganismos, fundamentalmente bacterias y hongos mesófilos los cuales juegan un papel fundamental en la descomposición, mineralización y estabilización del residuo orgánico (Aira et al., 2007a, 2007b; Domínguez et al., 2010). El desarrollo y actividades de las lombrices y microorganismos se encuentran estrechamente ligados, ya que por una parte los microorganismos constituyen una parte fundamental de la dieta de las lombrices, con una importancia de menor a mayor de bacterias, algas, hongos y protozoos, y por otra las

lombrices modifican la estructura física de los residuos, fragmentando la materia orgánica y aumentando su superficie, lo cual incrementa la actividad de los microorganismos (Edwards & Fletcher, 1988). Además, el aparato digestivo de la lombriz es un sistema complejo, similar para algunos autores al rumen, en el cual existen, cohabitan e intervienen diferentes microorganismos y actividades enzimáticas, que modifican sustancialmente la composición química y microbiológica del material orgánico ingerido (Drake et al., 2006).

Los primeros estudios sobre la microbiología del vermicompostaje describieron como la acción de lombrices sobre el residuo afecta significativamente a su biomasa microbiana, la cual incrementa significativamente justo después de su paso a través del intestino y va disminuyendo confirme el residuo recién excretado por la lombriz, denominado "cast", termina de ser mineralizado por los microorganismos (Parthasarathi & Ranganathan, 1999, 2000). Por otro lado, ha sido también demostrado que la acción de las lombrices sobre el residuo no sólo modifica su biomasa microbiana, sino también la estructura y la biodiversidad de la comunidad microbiana inicialmente presente en el residuo, la cual cambia y evoluciona de una forma diferente si la comparamos con los cambios que ocurren en la microbiota del residuos cuando este mismo se degrada mesofílicamente sin lombrices (Sen & Chandra, 2009). Lores et al. (2006) vermicompostaron tres diferentes estiércoles usando independientemente tres especies distintas de lombrices para procesar cada uno de ellos, y observaron que la microbiota inicial de cada residuo es transformada tras su vermicompostaje en una nueva comunidad microbiana, la cual puede ser discriminada en base a la especie de lombriz empleada, pero que aun se relaciona con la comunidad inicialmente contenida en el residuo orgánico. Este resultado indica que 1) la acción particular de una especie concreta de lombriz sobre diferentes substratos podría favorecer el desarrollo común de ciertos grupos de microorganismos en los diferentes vermicomposts originados, y 2) algunos grupos microbianos autóctonos de cada substrato se conservan después de su vermicompostaje. A pesar de estos hallazgos, hasta ahora no existen estudios que hayan esclarecido en qué medida diferentes especies de lombrices modifican la microbiota inicialmente albergada en el residuo orgánico, y como el desarrollo de esta microbiota durante el proceso de vermicompostaje se ve influenciado por la diferentes características fisicoquímicas del cada residuo.

Aunque varios estudios previos han estudiado la biodiversidad bacteriana en vermicompost resultantes de la transformación de diferentes residuos, revelando presencia de bacterias pertenecientes a los filos bacterianos Proteobacteria, Bacteroidetes,

Verrucomicrobia, Actinobacteria, Chloroflexi,, Gemmatimonadetes y Firmicutes (Fracchia et al., 2006; Vaz-Moreira et al., 2008; Yasir et al., 2009), la información individualmente proporcionada por estos estudios resulta contradictoria ya que ha sido derivada de un único vermicompost investigado por técnicas que pueden producir resultados no comparables. Igualmente, la información disponible sobre la identidad y biodiversidad de los hongos desarrollados durante el vermicompostaje se encuentra limitada unos pocos trabajos particulares que estudiaron la comunidad fúngica de vermicomposts producido a partir de estiércoles mediante el uso de técnicas de cultivo en placa (Anastasi et al., 2004, 2005; Pizl & Novakova, 2003). Actualmente, no se ha estudiado comparativamente los diversos microorganismos contenidos en varios tipos de vermicompost producidos a partir de diferentes residuos orgánicos ni tampoco la evolución de estos durante los diferentes procesos de vermicompostaje. Por estos motivos, la identidad de los microorganismos que se encuentran comúnmente involucrados en diferentes procesos de vermicompostaje, así como aquellos que podrían considerarse característicos de los vermicompost obtenidos, es aún una incógnita. Nuevos estudios deberían ser enfocados a establecer que especies microbianas se encuentran asociadas a la mayoría de procesos de vermicompostaje, con una cierta independencia del tipo de residuo inicial y la especie de lombriz empleada. En este sentido, investigaciones comparativas sobre las diferencias/similitudes entre las comunidades microbianas presentes en distintos vermicomposts obtenidos a partir de diferentes residuos orgánicos, podrían arrojar interesante información sobre la microbiota típicamente asociada a este tipo de producto.

1.2.4. FAUNA ASOCIADA AL PROCESO DE VERMICOMPOSTAJE

En los procesos de vermicompostaje, especialmente aquellos realizados a gran escala, pueden participar muchos organismos que colonizan los residuos orgánicos para alimentarse de la materia orgánica o utilizarlo como refugio o cobijo. Un ejemplo son los organismos detritófagos como cochinillas u otros insectos que pueden competir con las lombrices por el alimento. Además, pueden encontrase asociados otros invertebrados que participan en la descomposición del sustrato orgánico como nematodos, ácaros e insectos detritófagos que compiten con la lombriz por el alimento sin causar daños directamente. Todos estos organismos se conocen como fauna asociada o acompañante. En un proceso de vermicompostaje correctamente manejado, ninguno de los organismos mencionados es capaz

de causar perjuicio a las lombrices, aunque la proliferación de algunos de ellos indicaría que el desarrollo del proceso de vermicompostaje no es adecuado (Martínez et al., 2003).

I.2.5. CARACTERÍSTICAS REQUERIDAS DE LOS RESIDUOS ORGÁNICOS PARA SU UTILIZACIÓN EN PROCESOS DE VERMICOMPOSTAJE

I.2.5.1. Humedad

La exigencia de un humedad en el residuo superior al 50% se debe a que las lombrices poseen un mecanismo de intercambio gaseoso que se realiza a través de su epidermis (Edwards & Bohlen, 1996). Esto implica que el contenido en agua del residuo orgánico a vermicompostar debe ser ajustado antes de iniciar el proceso de vermicompostaje en base a los requerimientos óptimos de la lombriz particular usada en el proceso (Tabla I.2).

I.2.5.2. Estructura física

El residuo debe tener una estructura física lo suficientemente porosa que permita no sólo el desplazamiento de las lombrices, sino también el paso del aire y el drenaje de un posible exceso de agua en el proceso. Un óptimo grado de aireación en el residuo es fundamental, ya que las lombrices requieren concentraciones de oxigeno comprendidas entre 55 y 65% (Edwards & Bohlen, 1996). En el caso de residuos cuya estructura no permita la difusión pasiva del aire, estos deben ser previamente acondicionados, mezclándolo con otro tipo de residuo que actúe como estructurante (Garg et al., 2008; Nogales et al., 2008).

I.2.5.3. pH

El valor de pH del residuo puede afectar al proceso de vermicompostaje ya que las lombrices presentan un rango de tolerancia a este factor, así como un valor de pH óptimo para en el cual logran desarrollarse y reproducirse con más eficiencia. Por ejemplo, *E. fetida* y *E. andrei* son lombrices capaces de tolerar valores de pH en el residuo orgánico en el que se desarrollan comprendidos entre 5 y 9, aunque prefieren valores cercanos a la neutralidad (Edwards & Bohlen, 1996; Nogales et al., 2008).

I.2.5.4. Contenido de carbono y nitrógeno

Varios estudios han establecido que distintas especies de lombrices se muestran más eficaces en el vermicompostaje de residuos orgánicos cuando los valores de relación C/N de estos están cercanos a los óptimos requeridos para su desarrollo (Ndegwa & Thompson, 2000; Suthar, 2007). De forma general, se acepta que residuos orgánicos con una relación C/N entre 20-30 permiten un aceptable desarrollo de la mayoría de las especies de lombrices, mientras que si la relación C/N del residuo se encuentra excesivamente fuera de este margen el crecimiento y/o la reproducción de las lombrices pueden verse afectados negativamente (Nogales et al., 2008). En estos casos, la mezcla de dos residuos con relaciones C/N complementarias permite que el substrato orgánico resultante sea más óptimo para el desarrollo de las lombrices, respecto a cada uno de los residuos por separado.

I.2.5.5. Concentración de sales

El contenido en sales de los residuos orgánicos en los que se desarrollan las lombrices durante el vermicompostaje puede desequilibrar la composición iónica de los fluidos internos de esos oligoquetos ya que las lombrices tienen una baja capacidad de osmorregular las sales que absorben con el agua a través de su piel. Por esta razón, una elevada concentración de sales en el residuo orgánico puede impedir a que éste sea procesado por las lombrices. En caso de *E. fetida* y *E. andrei*, residuo orgánicos con valores de conductividad eléctrica superiores a 8 dS m^{-1} tendrían un contenido en sales letal para estas lombrices (Edwards, 1988). En zonas con alta disponibilidad de agua dulce, el lavado previo de los residuos con alta salinidad podría ser usado una técnica de acondicionamiento para reducir su contenido en sales hasta un nivel tolerable por las lombrices, aunque esto elevaría el coste del proceso (Nogales et al., 2008).

I.2.5.6. Concentración de amoniaco y amonio

Altos niveles de amoniaco, o de su forma protonada, el ión amonio, contenidos en el residuo resultan extremadamente tóxicos para la mayoría de especies de lombrices. Concretamente en el caso de lombrices de la especie *E. fetida* o *E. andrei*, niveles de amoniaco y amonio por encima de 1 o 0.5 mg g^{-1} de sustrato, respectivamente, se consideran tóxicos para estas lombrices (Edwards, 1988). En este caso, los residuos pueden ser acondicionados,

saturándolos de agua y dejándolos airear durante varios días para facilitar la volatilización de su contenido en amoniaco (Elvira et al., 1996).

I.2.5.7. Concentración de sustancias o elementos tóxicos

En ocasiones el residuo orgánico a vermicompostar puede incluir elementos o sustancias, que aún en pequeñas cantidades son nocivas para el desarrollo de las lombrices como por ejemplo son los metales pesados, fenoles, plaguicidas, etc. Esas sustancias pueden provocar alteraciones en el metabolismo de las lombrices, afectando al crecimiento, desarrollo sexual, producción de cápsulas e incluso a la supervivencia de éstas. Una pequeña cantidad de los metales ingeridos por las mismas puede incorporarse a los tejidos a través de la absorción intestinal (Fleming & Richards, 1982), y si ésta va a ser utilizada como proteína animal o como complemento a piensos de animales es conveniente regular el contenido de metales presentes en los residuos a vermicompostar. Además, la mayoría de estos metales pasan de nuevo al medio por las deyecciones de la lombriz, lo cual afectaría negativamente a la calidad de los vermicomposts obtenidos (Elvira et al., 1995; Suthar & Singh, 2009). Los plaguicidas contenidos en algunos residuos orgánicos pueden ser, dependiendo de su composición, fácilmente acumulados por las lombrices, lo que afecta a su supervivencia (Yasmin & D'Souza, 2010), por lo que su presencia es un factor que puede limitar el desarrollo óptimo del proceso de vermicompostaje.

I.2.5.8. Actividad biológica

El agrupamiento inicial de ciertos residuos y su humectación para conseguir un contenido óptimo en agua para las lombrices puede generar una intensa proliferación microbiana, debido principalmente al alto contenido en nutrientes y en microorganismos en el residuo orgánico, lo cual conlleva una intensa actividad biológica en el residuo que inicia su degradación de forma incontrolada liberando sustancias perjudiciales para las lombrices e incluso originado un autocalentamiento excesivo del residuo (Domínguez, 2004). En este caso, el residuo debe ser precompostado inicialmente de forma que la inoculación de las lombrices debe realizase una vez que esta fase inicial de activación e intensa degradación biológica del residuo haya concluido.

I.2.6. SISTEMAS DE VERMICOMPOSTAJE

Según la forma en la que los residuos son procesados por las lombrices se puede distinguir entre los sistemas de vermicompostaje tradicionales y sistemas de vermicompostaje con alimentación continua o flujo continuo (Garg et al., 2008).

I.2.6.1. Sistemas de vermicompostaje tradicionales

En estos sistemas el residuo orgánico es inoculado con lombrices y su degradación se produce en un solo paso, de modo que una vez ésta finaliza, todo material vermicompostado es retirado de una sola vez separándolo de las lombrices. Estos tipos de sistemas pueden ser los siguientes:

a) Literas o camellones

Una litera no es más que un espacio rectangular delimitado por maderas, ladrillos, bloques de cemento o cualquier elemento que sirva de contención para el residuo que va a ser vermicompostado y que no permita el escape de las lombrices (Figura I.10).

Figura I. 10. Literas utilizadas en procesos de vermicompostaje.

El largo y ancho de las literas depende de la escala a la que se realice el vermicompostaje, mientras que su espesor suele ser como máximo de 50 cm, ya que una gran cantidad de residuo apilada puede generar calor y además de compactar el material impidiendo el movimiento de la lombriz a través de éste. Una vez formada la litera con el residuo previamente acondicionado para lombrices, éstas se introducen en el residuo, usualmente esparciéndolas uniformemente sobre toda su superficie. Otra opción es disponer

el residuo entre dos cordones de material orgánico previamente vermicompostado y con una alta densidad de lombrices, de forma que las lombrices vayan introduciéndose poco a poco en el residuo fresco desde los cordones, donde el material se encuentra agotado.

Una vez que la densidad de lombrices alcanza el límite poblacional soportado por el residuo de la litera y la reproducción se detiene, las lombrices deben ser separadas del residuo. Esto se puede conseguir añadiendo una nueva litera adyacente con nuevo residuo fresco junto a la litera ya procesada. De este modo, las lombrices se desplazan a la nueva litera en busca de nuevo alimento, mientras que el residuo ya procesado por la lombriz termina de estabilizarse durante la fase final de maduración. Otra manera de separar las lombrices, cuando la degradación del residuo ha finalizado, consiste en cubrir la litera con una malla de diámetro apropiado que permita el paso de la lombriz, disponiendo sobre ella una capa de nuevo residuo fresco. Así, las lombrices ascienden al nuevo residuo y pueden ser retiradas del antiguo con la ayuda de la malla. Aunque este método es más rápido, presenta el inconveniente que la operación debe repetirse varias veces con objeto de retirar la mayor parte de la población de lombrices del residuo ya procesado.

b) Contenedores

En estos sistemas el residuo orgánico se encuentra ubicado en el interior de un receptáculo. De esta manera, las lombrices se presentan más protegidas frente a las condiciones ambientales, en comparación con el sistema de literas. Una vez que la población de lombrices ha llegado a su límite y el residuo ha sido procesado, el paso de las lombrices hasta un nuevo receptáculo se consigue gracias a que ellos suelen presentar una malla en su parte inferior que permite el paso de las lombrices, hacia otro contenedor relleno con residuo fresco (Figura I.11).

Figura I.11. Contenedores utilizados en procesos de vermicompostaje.

I.2.6.2. Sistemas de vermicompostaje de alimentación continua

Estos sistemas permiten ir procesando cantidades parciales del residuo orgánico que son incorporadas al vermicompostaje de forma más o menos constante a la vez que parte del residuo ya estabilizado es retirado del sistema. Estos sistemas de vermicompostaje, según su modo de funcionamiento, se pueden clasificar en los siguientes tipos:

a) Vermicompostadores verticales modulares

Están formados por varias unidades cilíndricas o rectangulares desmontables, llamadas módulos, cada una de las cuales presenta un soporte inferior perforado que permite el paso de las lombrices entre los distintos módulos (Figura I.12). Estos módulos son ensamblados verticalmente unos sobre/debajo de otros partiendo de una unidad inicial donde se encuentra la población inicial de lombrices. De este modo, durante el proceso de vermicompostaje, nuevos módulos conteniendo residuo fresco se van siendo añadidos periódicamente al sistema conforme las lombrices van agotando el residuo contenido en cada unidad. Esto permite que las lombrices vayan sucesivamente degradando el residuo fresco añadido en cada nuevo módulo, a la vez que se desplazan a través del sistema de módulos dejando el material ya agotado libre de lombrices, pudiendo éste ser retirado para que los microorganismos completen el proceso de mineralización y estabilización del residuo.

Figura I.12. Vermicompostadores verticales modulares.

b) Vermicompostadores verticales mecanizados (vermirreactores)

También denominados como vermirreactores verticales, en estos sistemas de vermicompostaje se lleva a cabo una carga periódica de residuo orgánico freso sobre un contenedor elevado donde se desarrollan las lombrices, el cual presenta un suelo perforado

junto a un sistema de criba mecánico que permite descargar el residuo de las capas inferiores del contenedor una vez ha sido vermicompostado (Figura I.13). De esta forma, las lombrices van ascendiendo verticalmente para procesar el residuo fresco añadido periódicamente en la superficie del reactor, mientras que el residuo del fondo que ya ha sido agotado es descargado libre de lombrices sobre un receptáculo dispuesto debajo de vermirreactor.

Figura I.13. Vermireactor de alimentación semicontinua y descarga vertical diseñado y fabricado por el Departamento de Ecoloxia y Bioloxia Animal de la Universidad de Vigo.

Figura I.14. Vermicompostador de flujo horizontal diseñado por la Universidad de Costa Rica (Sede Atlántico, Turrialba).

c) Vermicompostadores de flujo horizontal

Consisten en largos corredores dispuestos horizontalmente en los cuales una estrecha porción en uno de los extremos se rellena inicialmente con residuo fresco donde se añaden las lombrices (Figura I.14). Adyacentemente pequeñas franjas con residuo fresco se añaden periódicamente permitiendo así que las lombrices vayan desplazándose hacia el otro extremo del vermicompostador. Conforme la población de lombrices avanza hacia el otro extremo del

vermicompostador el residuo ya procesado va siendo retirado. En este tipo de sistema se debe procurar que las capas de residuo fresco que se añaden no sean muy espesas, para conseguir que sean procesadas rápidamente por las lombrices.

I.2.7. ESCALAS DE LOS PROCESOS DE VERMICOMPOSTAJE

El vermicompostaje puede desarrollarse a diferentes escalas, en función de los fines que se persigan. Básicamente se pueden establecer cuatro escalas (Nogales et al., 2008).

a) Escala doméstica

Su objetivo es reciclar los residuos de cocina y otros desperdicios que se originan en el propio hogar convirtiéndolos en un fertilizante orgánico para en el huerto o el jardín. En estos procesos los residuos son procesados por las lombrices en contenedores sencillos como por ejemplo cajas o recipiente de madera, plástico o metal, sin un control preciso del proceso. Al finalizar el proceso la separación del producto de las lombrices suele realizarse manualmente, aunque actualmente existen en el mercado una gran variedad de vermicompostadores modulares que facilitan esta tarea.

b) Escala pequeña o de laboratorio

En este caso los procesos de vermicompostaje se desarrollan, utilizando recipientes o vermirreactores pequeños para monitorizar minuciosamente el proceso, recabándose así información sobre los fenómenos acontecidos durante el vermicompostaje. A esta escala las condiciones ambientales en las que se desarrollan suelen estar muy bien controladas, manteniéndose la humedad y temperatura del residuo bajos valores óptimos.

c) Escala mediana o piloto

A esta escala cantidades relevantes de residuos son vermicompostados por pequeñas empresas del sector agroganadero con objeto de reciclar residuos de cosecha o desechos derivados de la cría de animales como el estiércol, produciendo vermicomposts que serán utilizados en la misma empresa. En este caso, aunque el control de proceso es mayor que en el caso del vermicompostaje doméstico, las condiciones en las que se ejecuta no suelen ser monitorizadas para conseguir un óptimo desarrollo del proceso. Por otro lado, el vermicompostaje a escala piloto también se lleva a cabo en centros de investigación con el

propósito de evaluar los posibles inconvenientes de desarrollar el vermicompostaje de un residuo concreto a una escala mayor o bien para generar vermicompost en cantidad suficiente para realizar estudios que permitan validar su aplicación al campo.

d) Escala industrial

Esta escala del vermicompostaje tiene como finalidad el procesado eficiente de una gran cantidad de residuos orgánicos para la obtención y comercialización del vermicompost obtenido. Con este fin, el proceso es monitorizado tratando de optimizar el tiempo y calidad del vermicompost producido; para lo cual generalmente se requiere la mecanización del proceso junto con adecuado diseño de planta. En este sentido las plantas de vermicompostaje industrial suelen contar de las siguientes áreas (Martínez et al., 2003):

- Área de recepción y acondicionamiento del residuo: zona donde el residuo es acumulado y acondicionando según los requerimientos de la lombriz empleada.
- Área de vermicompostaje: en ésta se disponen los sistemas de vermicompostaje donde se procesa el residuo. En esta área también suelen estar presentes otras estructuras adicionales, como por ejemplo: cubiertas superficiales de cierre, sistemas de riego, sistemas de calefacción, aireación y/o de control de la humedad ambiental; las cuales permiten mantener el proceso de vermicompostaje bajo condiciones más controladas.
- Área de cría: en ella se disponen las instalaciones para mantener la reserva de lombrices disponible para los procesos de vermicompostaje que se llevan a cabo en cada época del año según la disponibilidad de residuos y la demanda de vermicompost en mercado.
- Área de almacén y procesado del producto final: es una zona ventilada y protegida de la lluvia donde el vermicompost termina de madurar, reduciéndose su humedad, normalmente mediante secado al aire. Una vez maduro el vermicompost se tamiza y se envasa.

I.2.8. MONITORIZACIÓN DE UN PROCESO DE VERMICOMPOSTAJE

La eficacia del vermicompostaje para la estabilización de residuos orgánicos está condicionada al desarrollo idóneo de la población de lombrices durante el proceso, por lo que la importancia del controlar y monitorizar los factores que se exponen a continuación ha sido descrita en importantes estudios sobre el reciclaje de diferentes residuos orgánicos mediante el vermicompostaje (Martínez et al., 2003; Nogales et al., 1995, 2008).

I.2.8.1. Alimentación

La cantidad de residuo usada en cada tipo del sistema de vermicompostaje debe ser la suficiente para permitir el desarrollo de la población de lombrices durante el proceso. En procesos de vermicompostaje de alimentación continua, donde el residuo se añade periódicamente, la cantidad del mismo debe ser adecuada para permitir una óptima tasa de crecimiento y reproducción de las lombrices, pero sin llegar a producir un exceso de nutrientes en el substrato donde se desarrollan las lombrices, ya que esto podría desencadenar una intensa actividad microbiana que afectaría negativamente a las lombrices.

I.2.8.2. Dinámica de la población de lombrices

Por este motivo, la densidad de lombrices debe ser monitorizada durante el vermicompostaje, evitando que se alcancen elevadas densidades de lombrices que conllevan una competencia entre los individuos y se ralentice el proceso. La monitorización de la densidad de lombrices es particularmente esencial durante la fase de aclimatación, ya que un descenso significativo de esta población podría de manifiesto que el residuo no es óptimo para desarrollar un proceso de vermicompostaje. Además del número de lombrices durante el proceso de vermicompostaje, la evaluación de la biomasa individual de las lombrices permite conocer si el residuo es lo suficientemente nutritivo para la lombriz, lo cual viene indicado por un aumento del peso individual de las lombrices juveniles inoculadas hasta llegar a alcanzar su máximo. Finalmente una disminución del peso de las lombrices indica que el residuo se encuentra ya agotado. Por otro lado, la determinación de la biomasa es importante para controlar la estrategia que deseamos aplicar en el sistema de vermicompostaje. Si lo que se precisa es altos rendimientos en la degradación del residuo para producir vermicompost, se necesitan valores de biomasa altos pero con muchos individuos pequeños. Si interesa producir proteína o lombrices para cebo, se precisa un cultivo con pocos individuos que puedan alcanzar el mayor tamaño posible.

I.2.8.3. Madurez sexual, tasa de reproducción y fertilidad de las lombrices

El tamaño de las lombrices cuando alcanzan su madurez sexual durante el proceso de vermicompostaje está íntimamente relacionado con la disponibilidad de nutrientes, los

factores ambientales, el ciclo biológico de la especie y la densidad de población. De esta manera, conocer dentro de la población el porcentajes de individuos clitelados (sexualmente maduros), preclitelados inmaduros y juveniles nos ofrece una información directa sobre la viabilidad del residuo para el vermicompostaje (residuos inadecuados no permiten la maduración sexual de las lombrices), así como el estado de degradación del residuo (cuando el residuo se ha agotado por las lombrices estas pierden su clitelo). Además, el número de cápsulas por día o semana que se producen durante el proceso de vermicompostaje y el número de individuos que emergen en la eclosión de los mismos, permite estimar el potencial de la lombriz para vermicompostar el residuo.

I.2.8.4. Predadores de las lombrices

La posible presencia de algunos animales como los ratones, aves y topos debe ser vigilada durante el proceso de vermicompostaje, ya que éstos pueden constituir una amenaza para las lombrices. La depredación de las lombrices puede controlarse con la implantación, sobre y debajo de las literas de lonas resistentes que impidan el paso de estos depredadores. Otros depredadores de las lombrices más pequeños, las como hormigas, ácaros, tijeretas, ciempiés, etc., son más difíciles de controlar, pudiendo llegar a establecerse en colonias de alta densidad en el sistema de vermicompostaje, ocasionando daños considerables. Por lo general estos depredadores se suelen controlar manteniendo la humedad del sustrato por encima del 80% y un pH superior a 7. En los países tropicales, la planaria (*Bipalium kewense*, Moseley) se puede considerar como la plaga de mayor incidencia en procesos de vermicompostaje. Este platelminto se adhiere a la lombriz, alimentándose de su contenido interno hasta matarla. El control de esta plaga se realiza manteniendo el pH del residuo orgánico entre 7.5-8, ya que este parásito comienza su actividad depredadora a pH ácidos.

I.2.8.5. Controles físico-químicos del residuo orgánico

Durante el proceso de vermicompostaje el estado del residuo orgánico que va siendo degradando debe controlarse, monitorizando su temperatura, su grado de humedad y aireación, y su pH, ya que las lombrices requieren unos valores óptimos de estos parámetros para crecer y reproducirse a la mayor tasa posible, procesando el residuo en el menor tiempo posible.

Con este fin, la humedad óptima del residuo que está siendo procesado suele mantenerse constante durante el vermicompostaje, recurriendo a riegos más o menos periódico, dependiendo de la estructura del residuo y su capacidad de retención de agua. Por otro lado, el exceso de humedad que puede causar el riego, o aquel debido a las precipitaciones que afectan a sistemas de vermicompostaje llevados a cabo al aire libre sin cubierta protectora, puede ser evitado depositando el residuo sobre una superficie porosa con ligera pendiente que permitan el drenaje del agua a través del residuo.

La temperatura durante el proceso es de primordial importancia puesto que afecta a la tasa metabólica de la población de lombrices, así como los patrones de distribución y actividad de las mismas, condicionando la tasa de crecimiento y reproducción de la población de lombrices durante el vermicompostaje (Edwards & Bohlen, 1996). Por lo tanto, si se desea conseguir una efectiva degradación de residuo en el menor tiempo posible, su temperatura durante el proceso de vermicompostaje debe mantenerse dentro de los rangos tolerables por cada especie de lombriz, tratando de que sea lo más cercana a la óptima para su desarrollo (Tabla I.2). En climas calidos, los riegos en los sistemas de vermicompostaje permiten regular un posible exceso de temperatura en el residuo. Por el contrario, en climas fríos, el aumento del espesor de la capa de residuo orgánico donde se desarrollan las lombrices ayuda a minimizar la pérdida de su temperatura en el residuo, aunque hay que tener en cuenta que un excesivo apilamiento de residuo orgánico puede dar lugar a un excesivo autocalentamiento y a procesos de fermentación, los cuales pueden causar la muerte de las lombrices.

Por último, las lombrices son fotofóbicas y no se desarrollarán en la superficie del residuo si este se encuentra iluminado (Edwards & Bohlen, 1996). Por lo tanto el vermicompostaje llevado a cabo bajo condiciones de oscuridad, en cámaras o en recintos cerrados permite que la biodegradación del residuo sea más eficiente. En procesos de vermicompostaje al aire libre la utilización de distintos tipos de cubiertas, como por ejemplo una simple lámina de cartón o una capa superficial de un material que permita la aireación, permite que las lombrices se distribuyan también en la capa más superficial (Martínez et al., 2003). La aplicación de una cubierta sobre la superficie del residuo también proporciona un cierto grado de aislamiento térmico, evitando además la evaporación del agua contenida en el residuo, al tiempo que la condensación en la cara inferior de la cubierta mejora las condiciones de humedad.

I.2.8.6. Control de la descomposición del residuo mediante análisis de actividades de enzimas (bioindicadores)

En los últimos años la utilización de diversas actividades enzimáticas como bioindicadores de la degradación de los residuos orgánicos durante en los procesos de vermicompostaje ha suscitado un gran interés ha suscitado. Estudios previos han evaluado en profundidad la utilidad de analizar la actividad potencial de las siguientes enzimas como bioindicadores en el vermicompostaje (Benítez et al., 1999; Yakushev & Byzov, 2009):

- Actividad deshidrogenada: las deshidrogenadas son enzimas intracelulares presentes en todos los microorganismos vivos que se encuentran implicadas en procesos metabólicos de oxidación de sustancias orgánicas bajo condiciones aeróbicas. Así, la actividad deshidrogenasa medida durante el vermicompostaje puede ser usada como una medida indirecta de la potencial actividad de los microorganismos implicados en este proceso bioxidativo.

- La actividad β-glucosidasa, que se encuentra implicada en la degradación de la materia orgánica que ocurre durante el vermicompostaje, ya que las enzimas con actividad β-glucosidasa catalizan la hidrólisis de glucósidos unidos por enlaces tipo β, el paso final en la descomposición de diferentes compuestos orgánicos.
- Actividades fosfatasa: diversos tipos de enzimas con actividad fosfatasa permiten a los microorganismos involucrados en el proceso de vermicompostaje liberar compuestos inorgánicos de fósforo a partir del los compuesto orgánicos. En este sentido, la actividad fosfatasa es indicativa de la mineralización de compuesto orgánicos organofosforados durante o tras un proceso de vermicompostaje.
- Actividad arilsulfatasa: las enzimas con actividad arilsulfatasa liberan sulfatos de compuesto orgánicos ricos en azufre, de forma que la actividad de estas enzimas es indicativa de la mineralización de este tipo de compuestos orgánicos en procesos de vermicompostaje.
- Actividades ureasa y proteasa: estas actividades pueden ser utilizadas durante un proceso de vermicompostaje como bioindicadores de la mineralización del compuesto orgánicos nitrógenados, ya que las enzimas con actividad ureasa catalizan la hidrólisis de enlaces no peptídicos mientras que los enlaces peptídicos son rotos por la acción de las enzimas con actividad proteasa.

- Actividad de hidrólisis de la fluoresceina diacetato: esta actividad se basa en la hidrólisis de diacetato de fluoresceina (FDA), un compuesto sintético que puede ser hidrolizado por de muchas enzimas no-específicas de los microorganismos. De esta manera, la hidrólisis de FDA ofrece una indicación general de capacidad degradativa de los microorganismos involucrados en la bioxidación del residuo orgánico durante el proceso de vermicompostaje.

Estas actividades enzimáticas han sido previamente monitorizadas en multitud de estudios sobre procesos de vermicompostaje de residuos diferentes (Aira et al., 2007a; Benítez et al., 2002; Nogales et al., 2005; Parthasarathi & Ranganathan, 2000; Pramanik et al., 2007; Sen & Chandra, 2009). En general, se acepta que inicialmente las distintas actividades enzimáticas aumentan durante los primeros estadios del vermicompostaje para posteriormente ir disminuyendo a medida que avanza la biodegradación de los residuos orgánicos, hasta llegar a unos valores de actividad significativamente inferiores a los registrados inicialmente en el residuo orgánico. Así, los vermicomposts originados tendrán una menor actividad que los materiales orgánicos frescos. Por otro lado, el valor comparativo de las diferentes actividades enzimáticas que se registran en el material estabilizado (vermicompost) puede ser como indicador de la funcionalidad metabólica de la comunidad microbiana que alberga estos materiales (Vivas et al., 2009). Más información sobre este tema se expone en el apartado I.3.2.2 de la presente memoria de Tesis Doctoral.

I.2.8.7. Microorganismos patógenos

Algunos residuos orgánicos (especialmente los ganaderos) suelen contener patógenos vegetales y humanos. En este sentido, diferentes estudios han demostrado que un efectivo proceso de vermicompostaje consigue la reducción de los patógenos contenidos en los residuos orgánicos dando lugar a un producto final sin riesgos para la salud (Eastman, 1999, 2001; Monroy et al., 2009; Rodríguez-Canché et al., 2010). A pesar de esto, se debe realizar una evaluación de los posibles restos de patógenos en los vermicompost.

I.2.9. VERMICOMPOSTAJE COMO ECOBIOTECNOLOGÍA PARA EL TRATAMIENTO DE RESIDUOS ORGÁNICOS

El vermicompostaje es un proceso ecobiotecnológico que permite estabilizar la materia orgánica de gran número de residuos orgánicos convirtiéndola en una materia orgánica madura, considerándose así como una ecobiotecnología limpia, sin impacto ambiental y cuyos costes de inversión, energéticos y de mantenimiento son moderadamente bajos (Martínez et al., 2003). Por otro lado, el vermicompostaje también ha sido considerado como un proceso de biorremediación ya que muchos trabajos han demostrado la eficacia de este proceso para reducir los niveles de contaminantes orgánicos medioambientalmente peligrosos contenidos en ciertos residuos (Garg et al., 2008; Srivastava et al., 2005; Suthar, 2008b).

Hasta la fecha un gran número de estudios han demostrado la capacidad del vermicompostaje para biodegradar y estabilizar residuos orgánicos de diferente naturaleza (Tabla I.3), los cuales se pueden agrupar en dos categorías:

1. Residuos convencionales para procesos de vermicompostaje: tradicionalmente los estiércoles de distinto origen animal han sido considerados como materiales orgánicos naturales y óptimos para la alimentación y desarrollo de diversas especies de lombrices, las cuales transforman este tipo de residuo orgánico en un material estabilizado y maduro.
2. Residuos no convencionales para procesos de vermicompostaje: existe un gran número de residuos orgánicos generados por diversas actividades de nuestra sociedad que, a pesar de no ser considerados óptimos para el desarrollo de las lombrices en condiciones naturales, han sido bioestabilizados eficazmente por medio del vermicompostaje. Sin embargo, la mayoría de estos residuos no convencionales requiere un acondicionamiento previo, e incluso su mezcla con otro residuo, convencional o no convencional, con el fin de conseguir que el residuo presente las características requeridas para el adecuado desarrollo de las lombrices durante el vermicompostaje (Apartado I.2.4).

Tabla I.3. Residuos orgánicos de diferente origen vermicompostados exitosamente.

Residuos orgánico	**Lombriz**	**Trabajos pioneros y más relevant**
Varios estiércoles (vaca, oveja, cabra, caballo, búfalo, cerdo, camello)	*P. excavatus*	Kale et al. (1982)
	E. fetida	Garg et al. (2005)
Purín de cerdo	*E. fetida*	Chan & Griffiths (1988)
Excretas de aves	*E. fetida*	Hamilton et al. (2008)
Lodos urbanos de depuradora	*E. fetida*	Neuhauser et al. (1980);Frank et al. (1983)
Lodos de industria láctea	*E. andrei*	Nogales et al. (1999)
Lodos industrias papeleras	*E. andrei*	Elvira et al. (1996)
Lodos de papel no reciclable	*E. fetida*	Gupta & Garg (2009)
Lodos de efluentes de piscifactorías	*E. fetida*	Marsh et al. (2005)
Lodos de la producción de biogas	*E. fetida* y/o *L. mauritii*	Tripathi & Bhardwaj (2004)
Lodos de petroquímicas	*E. fetida*	Martín-Gil et al. (2008)
Chapapote del *Pestige*	*E. eugeniae*	Rajesh Banu et al. (2005)
Residuos de jardinería	*E. andrei* y/o *L. rubellus*	Engelstad (1991)
Residuos de cultivos de cereales	*P. excavatus*	Manna et al. (1997); Reddy & Ohkura (2004)
	E. eugeniae	Suthar (2008a)
Residuos de cultivo de mostaza	*E. fetida*	Bansal & Kapoor (2000)
Residuos de producción de algodón	*E. fetida*	Albanell et al. (1988)
Residuos sólidos urbanos	*E. eugeniae*	Datar et al. (1997)
Restos de cocina	*P. excavatus*	Chaudhuri et al. (1993)
Procedentes de cafetería	*E. fetida*	Orozco et al. (1996)
Residuos de la industria textil	*E. fetida*	Kaushik & Garg (2003)
Residuo olivar (orujillo)	*E. andrei*	Nogales et al. (1998)
Residuos de olivar (alperujo)		Melgar et al. (2009)
Residuos de la industria vitivinícolas	*E. andrei*	Nogales et al. (2005)
Residuos industria caña de azúcar Residuo de prensado	*E. eugeniae* y/o *L. mauritii*	Parthasarathi & Ranganathan (1998)
Lodos del destilado de caña	*P. excavatus*	Suthar & Singh (2008a)

I.2.10. VERMICOMPOSTS COMO ENMIENDAS ORGÁNICAS REVALORIZADAS

El vermicompost en sus diferentes acepciones (humus de lombriz, biohumus, lumbricompost) es definido por la normativa española (Real Decreto 824/2005 y Real Decreto 865/2010) como un producto estabilizado obtenido a partir de materiales orgánicos, por digestión con lombrices, bajo condiciones controladas, que debe cumplir unas características mínimas para ser comercializado para su aplicación en agricultura (Tabla I.4) Estos requerimientos mínimos que exige la legislación española suelen ser cubiertos con creces cuando el proceso de vermicompostaje ha sido efectivo ya que este proceso se caracteriza por transformar los residuos orgánicos en productos orgánicos inodoros, higienizados, estables, maduros y con un apreciable contenido de nutrientes disponibles para las plantas y sustancias orgánicas complejas similares a las sustancias húmicas del suelo (Buchanam et al., 1988; Edwards & Burrows, 1988; Hait & Tare, 2011; Li et al., 2011; Orozco et al., 1996; Rodríguez-Canché et al., 2010; Romero et al., 2007; Sangwan et al., 2008).

Además, se ha descrito que durante el proceso de vermicompostaje se originan sustancias con acción fitohormonal (Tomati et al., 1988). Debido a estas propiedades, estudios comparativos frente a otras enmiendas y fertilizantes orgánicas han considerados a los vermicompost como unos materiales orgánicos con excelentes cualidades agrícolas (Chaoui et al., 2003; Saha et al., 2008, 2010a). Igualmente, existen estudios que recomiendan a los vermicompost como sustratos de cultivos, bien solos o mezclados con otros substratos (Arancon et al., 2004, 2008; Warman & AngLopez, 2010; Zaller, 2007). Además existen una gran cantidad de estudios que han descrito como la aplicación de vermicompost en agricultura mejora notablemente la fertilidad del suelo y así el rendimiento de los cultivos (Arancon et al., 2005a; Gutiérrez-Miceli et al., 2007; Manivannan et al., 2009; Vasanthi & Kumaraswamy, 1999).

Por otro lado, los vermicomposts son además productos con interesantes propiedades biológicas. En este sentido, varios estudios han demostrado que los vermicompost contienen enzimas extracelulares acomplejadas a su materia orgánica, las cuales se mantienen activas tras la incorporación de estos materiales al suelo mejorando su estatus bioquímico (Benítez et al., 2000, 2005; Pascual et al., 2002). Desde el punto de vista microbiológico, existen estudios que han revelado la presencia, e incluso la proliferación en materiales vermicompostados, de microorganismos beneficiosos capaces de incrementar la fertilidad de

Tabla I.4. Requisitos para la utilización de vermicomposts como: 1) enmienda orgánica de suelos según el Real Decreto 824/2005, modificado por la Orden APA/863/2008 y como 2) substrato orgánico sustitutivos de suelo para cultivos según el Real Decreto 865/2010 (marcado con asterisco los requerimientos específicos para este uso).

Característica evaluada	**Valores requeridos**		
Propiedades Físico-químicas			
Materia orgánica total	≥ 30%		
Humedad	≤ 40%		
Relación C/N	< 20		
N orgánico respecto al N total	≥ 85%		
Granulometría	90% de las partículas < 25mm		
Microorganismos			
Escherichia coli	< 1000 número más probable g^{-} (NMP) [1]		
Salmonella	Ausente en 25 g		
*Listeria monocytogenes**	Ausente en 1 g		
*Enterococcaceae**	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 suelos y favorecer el crecimiento de plantas (Gopal et al., 2009; Kale et al., 1992). Asimismo, debido a sus propiedades adsorbentes y microbiológicas ciertos vermicompost han sido exitosamente usados para combatir diversas plagas de cultivos (Arancon et al., 2005b, 2007; Szczech & Smolinska, 2001; Szczech, 1999; Yardim et al., 2006), así como para controlar la dinámica de plaguicidas y/o biorremediar suelos contaminados por diversos

xenobióticos (Contreras-Ramos et al., 2008; Di Gennaro et al., 2009; Moreno et al., 2009b, 2011). Además, recientemente varios estudios han conseguido aislar de vermicomposts distintos microorganismos con utilidad biotecnológica, gracias a su capacidad de resistir y degradar xenobióticos (Blaszak et al., 2011; Moreno et al., 2009a), de producir enzimas con utilidad en procesos industriales (Zambare et al., 2011a, 2011b), e incluso de evitar el desarrollo de hongos patógenos vegetales gracias a sus actividades antifúngicas (Gopalakrishnan et al., 2011; Yasir et al., 2009, 2010). De esta manera los vermicomposts deberían ser considerados, no sólo como enmiendas o fertilizantes, sino como bioproductos con características microbiológicas atractivas para la agricultura, la recuperación de suelos contaminados, así como, para la búsqueda de microorganismos con capacidades biológicas de interés. Además, recientemente existen estudios que han descrito la utilidad de los vermicomposts para otros procesos biotecnológicos, usándose para acelerar la producción de biogas en digestores anaeróbicos (Chen et al., 2010; Yang et al., 2009) o para construir sistemas de biorremediación que potencien la biodegradación de xenobióticos como son las biobarreras (Moreno et al., 2009b), biofiltros (Fu et al., 2011) y biocubiertas (Moon et al., 2010). Sin embargo, aunque los estudios mencionados han revelado las interesantes capacidades de los microorganismos presentes en ciertos vermicomposts, actualmente existe escaso conocimiento sobre las comunidades microbianas que típicamente se encuentran asociadas a los diversos vermicomposts. Un mayor conocimiento sobre las comunidades microbianas albergadas en los diversos vermicomposts producidos a partir de diferentes residuos orgánicos, así como sobre cómo estas comunidades varían según las particularidades del proceso, es esencial para poder transformar eficientemente diferentes residuos orgánicos en productos estables y maduros que muestren propiedades biológicas que permitan su valorización como materiales bioactivos. Algunas de las técnicas actualmente disponibles que podrían ser empleadas en este tipo de estudios con objeto de investigar la identidad de los microorganismos presentes en distintos procesos de vermicompostaje y en los vermicomposts obtenidos se describen a continuación en el apartado I.3 de la introducción de la memoria de la Tesis Doctoral.

I.3. ESTUDIO DE LAS COMUNIDADES MICROBIANAS EN PROCESOS DE VERMICOMPOSTAJE

La comprensión del funcionamiento de procesos biológicos complejos, tales como el vermicompostaje, exige conocer qué microorganismos se encuentran en ellos y cómo funciona la comunidad microbiana que éstos componen. De este modo, conocer la diversidad microbiana y las funciones biológicas de la microbiota implicada en el vermicompostaje es esencial para entender este proceso biotecnológico. Por este motivo, el apartado I.3 de la presente memoria de Tesis Doctoral está dedicado a describir los métodos o técnicas actualmente disponibles para evaluar la diversidad de microorganismos (Figura I.15), así como, las funciones biológicas de las comunidades microbianas implicadas en procesos de vermicompostaje (Figura I.16).

I.3.1. MÉTODOS DE ESTUDIO DE LA BIODIVERSIDAD DE COMUNIDADES MICROBIANAS

I.3.1.1. Métodos basados en el crecimiento de microorganismos en medios de cultivo

Tradicionalmente el estudio de la diversidad microbiana en muestras ambientales se ha basado en el cultivo de microorganismos en medios nutritivos especialmente formulados para ello. De esta manera, usando varios tipos de medios de cultivo se puede estimar el número total de microorganismos (principalmente bacterias y hongos) presentes en una muestra, y además, aislar microorganismos con capacidades fisiológicas concretas.

En el ámbito del vermicompostaje, Vaz-Moreira et al. (2008) se sirvió de esta metodología para estudiar la diversidad de bacterias heterótrofas presente en un vermicompost producido a partir de residuos de cocina. En este estudio se consiguió cultivar 33 cepas bacterianas diferentes, entre las cuales las pertenecientes al género *Bacillus* fueron dominantes. Igualmente gracias al uso de medios de cultivo en placa, Anastasi et al. (2005) revelaron importante información sobre la composición fúngica de un vermicompost producido a partir de estiércoles y restos de plantas. En este estudio, un total de entre 5.3 x 10^4 y 4.0 x 10^5 CFU g^{-1} de vermicompost fueron evidenciadas, dependiendo del tipo de medio de cultivo usado y la temperatura de incubación; siendo los géneros *Penicillium* y *Aspergillus* los de mayor peso en la comunidad fúngica de ese vermicompost.

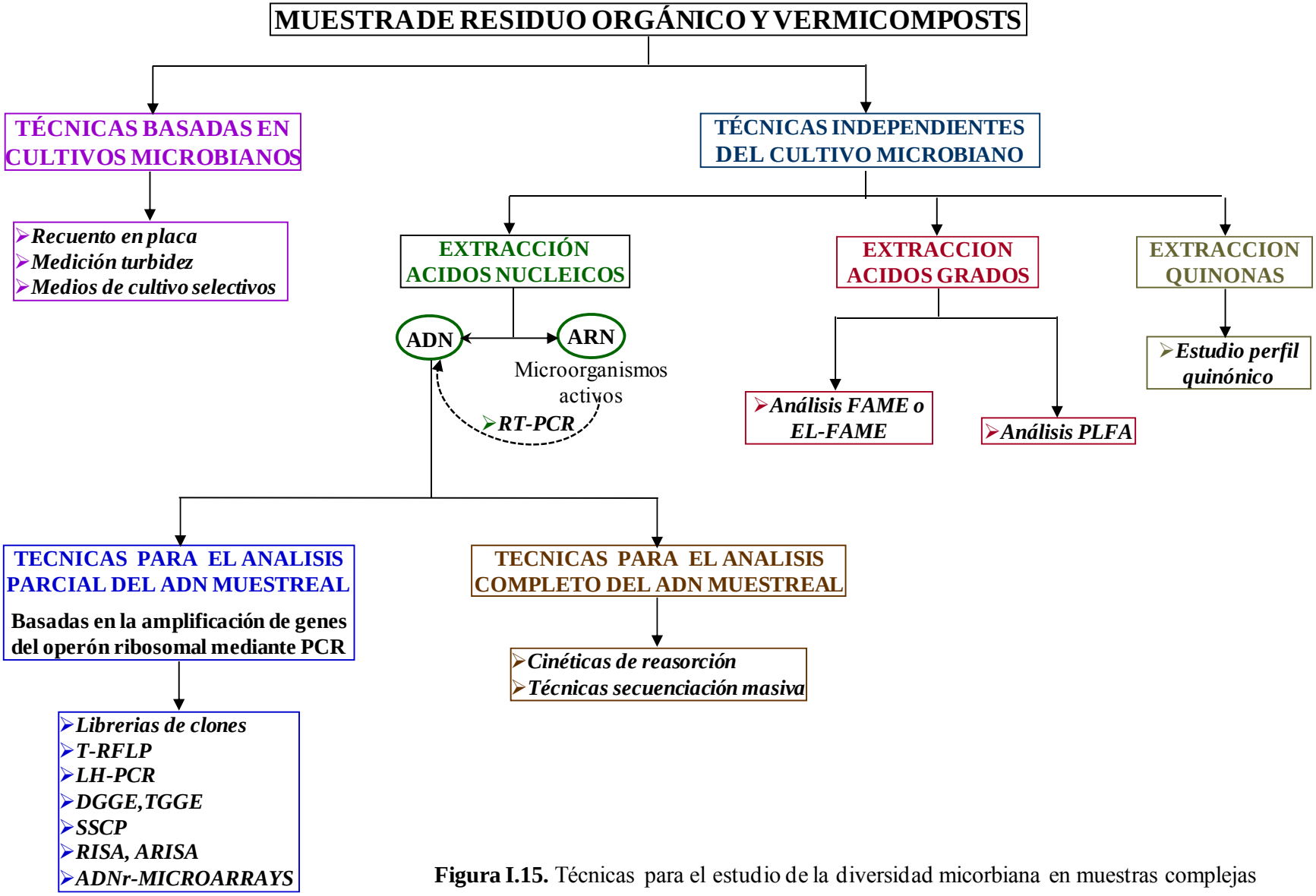


Figura I.15. Técnicas para el estudio de la diversidad micorbiana en muestras complejas

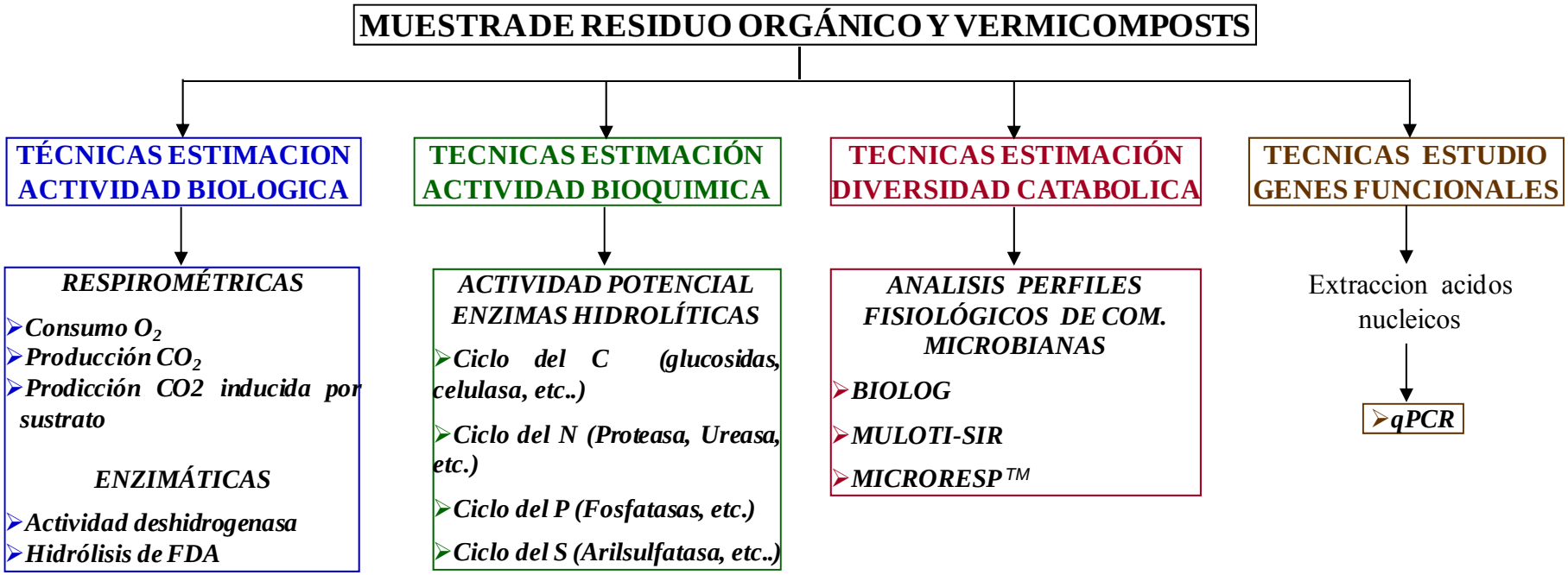


Figura I.16. Técnicas para el estudio de las funciones biológicas de comunidades microbianas en muestras complejas

A pesar de que esta metodología ha proporcionado relevante información sobre la microbiota asociada al vermicompost, los datos proporcionados por este tipo de técnicas se encuentran sesgados ya que no todos los microorganismos son capaces de crecer en medios de cultivo. En este sentido, recuentos microscópicos directos del total de bacterias viables presentes en materiales orgánicos bioestabilizados, tales como composts, revelaron que menos del 1% de los procariotas totales presentes pueden crecer en medios de cultivo (Dees & Ghiorse, 2001; Haruta et al., 2002). Excepcionalmente, Narihiro et al. (2003) consiguieron cultivar el 56 % del total de bacterias observadas microscópicamente en un compost producido a partir de desechos caseros; lo cual representa aun un importante sesgo en el total de procariotas. En el caso de residuos bioestabilizados mediante vermicompostaje, ningún estudio de este tipo ha sido desarrollado aun, aunque no sería sorprendente que, al igual que en la mayoría de composts, una importante parte de la diversidad microbiana que integra la "caja negra" que constituye la microbiota asociada al vermicompost quedara fuera del alcance de estos métodos de estudio tradicionales.

I.3.1.2. Métodos basados en el análisis de marcadores moleculares

A partir de la década de los ochenta se desarrollaron novedosas y potentes técnicas que permiten el estudio de los microorganismos presentes en diversos tipos de muestras biológicas sin necesidad de ser cultivados. Estas técnicas se basan en el análisis de marcadores moleculares: moléculas que se encuentran presentes en todos los microorganismos y cuya composición depende del grupo taxonómico al que pertenecen. Entre estas moléculas encontramos los ácidos grasos de los fosfolípidos, las quinonas y los ácidos nucleicos (ADN y ARN). Mediante este tipo de técnicas es posible obtener un conocimiento global sobre la comunidad microbiana presente en muestras biológicamente complejas, puesto que permiten: 1) Determinar la estructura de la comunidad microbiana revelando el número de taxones que la componen e incluso la abundancia relativa de cada uno de ellos, permitiendo así estimar su diversidad biológica; 2) Producir una huella identificativa (*fingerprinting*) de la comunidad microbiana presente en una muestra, permitiendo así estudiar los cambios que se producen en ella, así como, el grado de similitud respecto a otras comunidades microbianas de muestras diferentes.

I.3.1.2.1. Técnicas basadas en el análisis de ácidos grasos

Las membranas celulares de todos los microorganismos están constituidas principalmente por fosfolípidos, los cuales están formados por una región hidrofílica unida a cadena hidrófoba compuesta por ácidos grasos; excepto en el caso de los procariotas del dominio arqueas donde la región hidrófoba está constituida por moléculas de fitano o bifitano. Teniendo en cuenta que los ácidos grasos de fosfolípidos varían en función de cada especie microbiana, el patrón de los diversos ácidos grasos presentes en una muestra puede ser usado como *fingerprinting* de su comunidad microbiana, proporcionando además una buena estimación de su biomasa microbiana (Gómez-Brandón et al., 2010b). Además, ya que ciertos ácidos grasos son considerados característicos de determinados grupos de microorganismos, su cuantificación relativa permite evaluar la estructura de la comunidad microbiana en base a ellos (Tabla I.5).

Actualmente existen básicamente dos técnicas que permiten analizar los ácidos grasos de una muestra: FAME (*fatty acid methyl ester*) y PLFA (*Phospholipid fatty acid*). Ambas se diferencian entre sí según la naturaleza de los ácidos grasos estudiados. La metodología denominada como FAME hace referencia al estudio de todos los ácidos grasos contenidos en una muestra: polares, neutros y no polares. En el caso del análisis PLFA, esta técnica permite analizar solamente los ácidos grasos procedentes de fosfolípidos presentes en la muestra, puesto que tras la extracción del conjunto de ácidos grasos se realiza una separación de los ácidos grasos polares correspondientes a este tipo de lípidos. Teniendo en cuenta que los fosfolípidos que constituyen las membranas microbianas son desfosforilados muy rápidamente tras la muerte del microorganismo, el análisis PLFA proporciona información sobre los microorganismos vivos en la muestra (Zelles, 1999), mientras que el análisis FAME puede proporcionar información sobre la historia reciente de la comunidad microbiana de la muestra, al incluir otros ácidos grasos procedentes de microorganismos muertos recientemente. Sin embargo, el análisis FAME puede presentar la desventaja de que ácidos grasos procedentes de restos vegetales o asociados a ácidos húmicos pueden ser co-extractados junto con los ácidos grasos procedentes de los microorganismos, distorsionando la información sobre la microbiota presente en la muestra (Marschner, 2007). No obstante, Schutter & Dick (2000) solventaron este inconveniente mediante un método de extracción basado en una metilación alcalina moderada capaz de extraer sólo los ácidos grasos unidos al enlace éter, procedentes de células microbianas, sin extraer los demás ácidos grasos libres en

la muestra. Independientemente de la técnica de extracción empleada, la identificación y cuantificación de los ácidos grasos extraídos se realiza mediante cromatografía de gases (GC) y espectrometría de masas (MS) tras su conversión a ésteres metílicos.

Tabla I.5. Ácidos grasos usados como marcadores de grupos microbianos (Marschner, 2007; Moore-Kucera & Dick, 2008).

Grupo Taxonómico	Acido graso indicador
Bacterias (común)	14:0
	15:0
	17:0
Bacterias Gram-positivas	i15:0
	a15:0
	i16:0
	i17:0
	a17:0
Bacterias Gram-negativas	16:1ω5
	16:1ω7
	17:1ω9
	18:1ω5
	18:1ω7
	cy17:0
	cy19:0
Actinomicetos	10Me16:0
	10Me17:0
	10Me18:0
Hongos	18:1ω9c
	18:2ω6
	18:3ω3
Protozoos	20:2ω6
	20:4ω6

Ambas técnicas han sido frecuentemente empleadas para investigar como la comunidad microbiana de los residuos orgánicos es transformada por un proceso de vemicompostaje (Domínguez et al., 2010; Gómez-Brandón et al., 2010a, 2011a, 2011b; Lores et al., 2006; Verkhovtseva et al., 2002). Entre estos estudios, destaca el realizado por Lores et al., 2006), quienes mediante análisis FAME, revelaron que la estructura de la comunidad microbiana desarrollada en un vermicompost depende del tipo de residuo vermicompostado, así como de la especie de lombriz empleada en el proceso, según ha sido previamente mencionado en el apartado I.2.3. En los capítulos 4 y 9 de la presente memoria de Tesis Doctoral se ha empleado el análisis PLFA y FAME, respectivamente, para examinar la transformación microbiana causada por un proceso de vermicompostaje, así como, para evaluar el efecto que tuvo la aplicación de composts y vermicomposts sobre la estructura de la comunidad microbiana de un suelo agrícola.

I.3.1.2.2. Técnicas basadas en el análisis de quinonas

Las quinonas son también moléculas presentes en todos los microorganismos, entre los cuales exhiben una marcada variación química dependiendo del grupo microbiano al que pertenezcan. Hiraishi (1999) describió como el análisis de las quinonas permite conocer la abundancia de los principales grupos microbianos presentes en varios tipos de muestras biológicas, aunque sin llegar a niveles taxonómicos bajos. Además, este autor estableció que la concentración de quinonas de una muestra puede ser considerada como un fiel indicador de su biomasa, mientras que el perfil de los distintos tipos de quinonas encontrados de una muestra puede ser consideraros como un *fingerprinting* de su comunidad microbiana. Gracias a toda la información proporcionada por estas moléculas, el perfil de quinonas contenidas en composts han sido investigado con objeto de estudiar la microbiota implicada en este proceso de bioestabilización de residuos (Hu et al., 1994; Huang et al., 2010; Tang et al., 2004, 2006). En cuanto al proceso de vermicompostaje, las comunidades microbianas de vermicomposts han sido escasamente estudiadas mediante esta técnica (Hiraishi, 2002).

I.3.1.2.3. Técnicas basadas en el análisis de ácidos nucleicos

Las técnicas basadas en el análisis del ADN y/o ARN contenido en una muestra se han impuesto rápidamente a los métodos previamente mencionados debido a la mayor cantidad

de información que proporcionan. Dentro de estas técnicas podemos diferenciar las que analizan de manera global la totalidad del ADN y/o ARN de la muestra y las técnicas que se centran en análisis de ciertos genes concretos (Ranjard et al., 2000) (Figura I.15). Ambos grupos de técnicas pueden estar centradas en el análisis del ADN de la muestra, o bien en su contenido en el ARN. El análisis del ADN contenido en una muestra permite estudiar la diversidad biológica de todos los microorganismos presentes en la misma: activos, aletargados o esporulados, mientras que el análisis de su contenido en ARN limita el estudio a aquellos microorganismos con actividad metabólica significativa en la muestra (Molin & Givskov, 1999). Por este motivo, en el caso de estudios enfocados a evaluar la biodiversidad de muestras ambientales, el análisis de su contenido ADN ha sido el más utilizado. A continuación se describen las diferentes técnicas que permitirían analizar, de forma parcial o completa el ADN contenido en muestras procedentes de procesos de vermicompostaje.

I.3.1.2.3.1. Técnicas basadas en el análisis parcial del contenido en ADN

Entre los genes que forman parte del genoma de todos los microorganismos el gen que codifica el ARN ribosómico (ARNr) 16S en procariotas (gen ADNr 16S) y el gen para el ARNr 18S (gen ADNr 18S) de eucariotas son los fragmentos genómicos más empleados para evaluar la diversidad microbiana en muestras ambientales (Head et al., 1998). Estos genes presentan una serie de características que los hacen idóneos como marcadores moleculares: 1) están presentes en todos los organismos y tienen la misma función en todos ellos, presentando así un tamaño similar en todos los organismos; 2) la secuencia de nucleótidos que compone estos genes incluye regiones altamente conservadas, es decir constantes en los distintos microorganismos, junto a regiones con distinto grado de variabilidad, la cuales permiten identificar con diferente nivel de resolución taxonómica el taxón microbiano al que pertenece un gen ADNr determinado; 3) su transmisión no está sujeta a transferencia génica horizontal entre microorganismos, siendo principalmente vertical, por lo que el análisis de sus secuencias nos permiten establecer relaciones filogenéticas entre los microorganismos; 4) su expresión en los microorganismos es constitutiva, por lo que el estudio del ARNr que codifican permite identificar a los organismos activos de la comunidad. De esta manera identificando los diferente genes ADNr 16S o 18S contenidos en una muestra podemos conocer su biodiversidad procariótica o eucariótica (Amann et al., 1995).

Para este fin, la reacción en cadena de la polimerasa (PCR: *Polymerase Chain Reaction*) es una técnica que juega un papel fundamental, ya que permite obtener un gran número de copias de un fragmento de ADN concreto, es decir, amplificarlo. La PCR se basa en la utilización de dos pequeños oligonucleótidos iniciadores o *primers* (aprox. 20 nucleótidos) que pueden unirse a los extremos del fragmento de ADN que va a ser amplificado gracias a la acción de una ADN-polimerasa termoestable. La selección de *primers* capaces de unirse a las regiones conservadas del los genes ADNr permite amplificar los genes ADNr presentes en el ADN extraído de una muestra, aumentando la concentración de estos fragmentos de ADN hasta niveles que permitan su posterior estudio. De esta forma, el producto de esta PCR contiene un conjunto de copias diferentes (amplicones) producidas a partir de los diversos genes ADNr que proceden de los microorganismos presentes en la muestra. Entonces, determinando el número de amplicones del ADNr que son diferentes y su abundancia relativa se puede estimar la diversidad de microorganismos de la muestra a partir de la cual se originaron estos amplicones. Esto es posible ya que los amplicones procedentes de microorganismos diferentes tienen una longitud y/o secuencia distinta como consecuencia de las regiones hipervariables presentes en los genes ADNr, que son características de cada especie microbiana. Así, el análisis de la secuencia de los diversos amplicones de los genes ADNr permite identificar qué taxones están presentes en la muestra. A continuación se describe brevemente el fundamento de algunas técnicas que permiten estudiar la comunidad microbiana de una muestra mediante el análisis de los genes ADNr presentes en la muestra tras ser amplificados por PCR.

Librerías de clones

Esta técnica se fundamenta en aislar las diversas copias de los genes ADNr contenidas en un producto de PCR introduciéndolas en el interior de un microorganismo huésped, principalmente una bacteria o levadura, mediante el uso de vectores genéticos (plásmido, cósmido, etc.). De esta manera se obtienen un conjunto de clones del microorganismo huésped cada uno de los cuales contiene un sólo gen ADNr. Mediante secuenciación de los genes ADNr introducidos en los diversos clones se puede identificar los microorganismos de los que provienen esos genes comparando cada una de sus secuencias con aquellas depositadas en bases de datos online (NCBI, Ribosomal data base, etc.). Esta técnica permite así conocer que microorganismos están presentes en la muestra así como su abundancia

relativa. Frecuentemente, como paso previo a la secuenciación de los clones de la librería se realiza un análisis ARDRA (*amplified rDNA restriction analysis/ análisis de restricción de un fragmento amplificado del DNA ribosómico*), que consiste en digerir con "enzimas de restricción" el gen ADNr contenido en cada clon, de forma que éste es cortado de una forma concreta según su secuencia. Entonces, comparando los patrones de digestión de los genes contenidos en los diversos clones se puede determinar que número de clones son los que contienen genes ADNr distintos, secuenciándose sólo un clon representante de cada gen ADNr con diferente patrón de corte. De esta manera se abarata el coste de secuenciación.

Recientemente, esta técnica ha sido empleada para investigar la comunidad bacteriana asociada al intestino de la lombriz *Lumbricus rubellus* (Knapp et al., 2009; Singleton et al., 2003), usada en procesos de vermicompostaje (apartado I.2.2). Estos estudios han revelando que la comunidad bacteriana asociada a la pared intestinal difiere de la encontrada en las deyecciones de la lombriz, la cual está influenciada por el tipo de substrato consumido por la lombriz. Sin embargo, existen pocos trabajos que hayan usado esta técnica para estudiar la microbiota desarrollada en un residuo orgánico tras su vermicompostaje. Yasir et al. (2009) construyó librerías de clones para el gen ADNr 16S con objeto de comparar la comunidad bacteriana presente en una mezcla de lodos lácteos y de papel, antes y después de ser vermicompostado por *E. fetida*. Así los autores describieron la aparición tras el vermicompostaje de bacterias pertenecientes a los grupos Actinobacteria y Planctomycetes, junto con un aumento del número de Gamma-proteobacterias y Bacteroidetes respecto a la comunidad microbiana del residuo inicial.

T-RFLP (*Terminal Restriction Fragment Length Polimorphism* / Polimorfismo de la longitud del fragmento de restricción terminal)

En esta técnica, la amplificación de los genes ADNr se realiza usando *primers* para regiones conservadas, uno de los cuales contiene un fluoróforo. Esto produce que las copias del gen ADNr queden marcadas en su extremo 5'. Entonces, todos estos amplicones se cortan usando enzimas de restricción, de forma que genes con secuencia diferentes son cortados por lugares distintos produciendo fragmentos terminales 5' (unidos al fluoróforo) de longitud distinta. Estos fragmentos se separan según su longitud mediante electroforesis, cuantificando la intensidad de fluorescencia emitida por cada tipo de fragmento. Así se obtiene un cromatograma dónde cada pico representa un fragmento de un tamaño

determinado con una intensidad de fluorescencia proporcional a su abundancia en la muestra (Figura I.17).

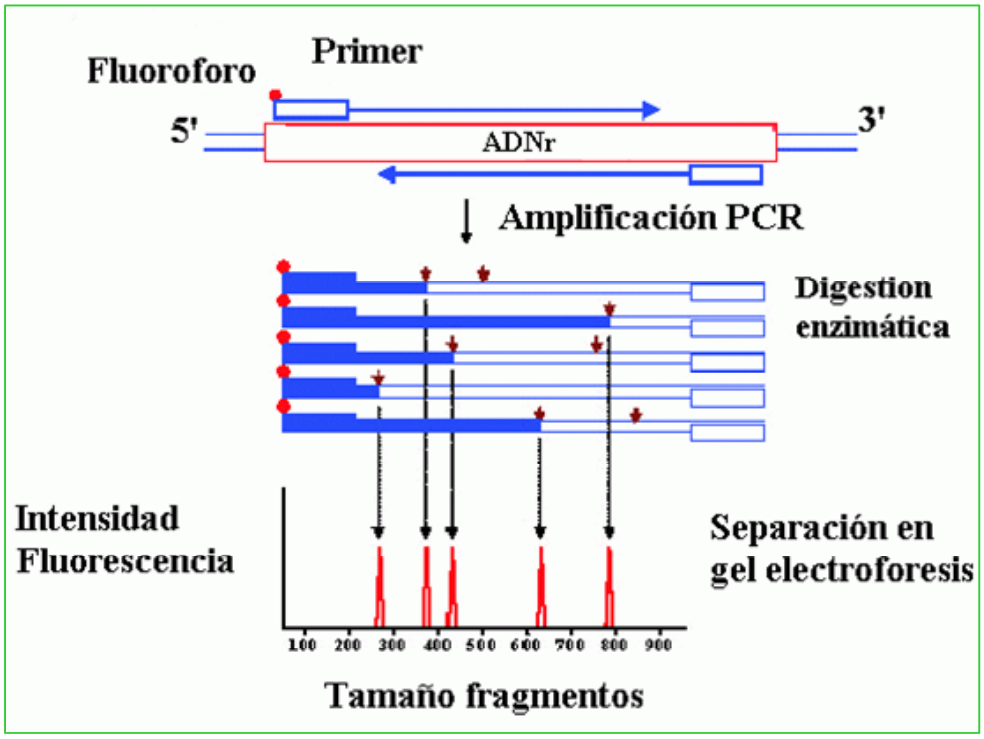


Figura I.17. Análisis de amplicones de genes ADNr 16S mediante T-RFLP.

Este método permite generar perfiles genéticos de la comunidad (*fingerprinting*) que pueden ser usados para evaluar el grado de similitud entre comunidades microbianas de diversas muestras. Igualmente permite estimar la diversidad microbiana de una muestra puesto que el número de diferentes fragmentos correspondería con el número de genes ADNr distintos procedentes de los diversos taxones microbianos presentes en la muestra. Aunque esta estimación debe interpretarse con cautela ya que se conoce que genes de especies microbianas diferentes pueden presentar el mismo sitio de corte a pesar de contener diferente secuencia, subestimándose entonces la biodiversidad presente en la muestra (Dunbar et al., 2001). Salvando este inconveniente, T-RFLP análisis ha sido exitosamente usado para estudiar la sucesión de la comunidades bacterianas y fúngicas durante el compostaje (Tiquia, 2005). Sin embargo, la microbiología de procesos de vermicompostaje aun no ha sido examinada mediante esta técnica.

LH-PCR (*Length Heterogeneity PCR* / Heterogeneidad en la longitud de genes amplificados por PCR)

Al igual que para el T-RFLP esta técnica requiere que uno de los *primers* usados para amplificar el gen ADNr incluya un fluoróforo. En este caso no se digieren los productos de PCR, puesto en esta técnica la PCR está dirigida a amplificar fragmentos pequeños del gen ADNr (300-600 bp), los cuales debido a las inserciones y delecciones naturales presentes en sus regiones hipervariables varían en longitud dependiendo del microorganismo de cual que proviene el gen ADNr amplificado. Como en el T-RFLP, los distintos fragmentos se separan según longitud y su abundancia se cuantifica según su intensidad de fluorescencia, determinando así la diversidad de genes ADNr amplificados a partir del ADN de la muestra. No obstante, esta medida de diversidad no debe ser usada como una estimación de la diversidad de especies presentes en la muestras, si no que más bien corresponden a nivel de taxones más altos, ya que frecuentemente genes ADNr pertenecientes a microorganismos de distintas especies originan amplicones del mismo tamaño si esas especies microbiana se encuentra próximas filogenéticamente (Suzuki et al., 1998). Dejando a un lado esta consideración, esta técnica es considerada más sencilla que las anteriores y ha sido empleada para estimar la diversidad microbiana en muestras de ambientes acuáticos y suelos (Ritchie et al., 2000; Suzuki et al., 1998). Sin embargo, en el caso de muestras procedentes de procesos de vermicompostaje u otros procesos semejantes esta técnica aun no ha sido empleada.

DGGE (*Denaturing Gradient Gel Electrophoresis* / Electroforesis en gel con gradiente desnaturalizante) y TGGE (*Temperature Gradient Gel Electrophoresis* / Electroforesis en gel con gradiente térmico)

Estas técnicas permiten separar los diferentes amplicones de los genes ADNr procedentes de los diversos microorganismos de una muestra según su secuencia de nucleótidos. Ambas técnicas se basan en la amplificación de los genes ADNr mediante una reacción de PCR que adiciona a cada amplicón una secuencia extra de nucleóticos compuesta por enlaces guanina-citocina, denominada cola GC. Gracias a esta cola, cuando los amplicones del gen ADNr se desplazan en un gel de electroforesis en el que existe un gradiente químico (DGGE) o térmico (TGGE), éstos se desnaturalizan separándose las dos hebras, quedando unidas las

hebras por la cola GC añadida que permanece sin desnaturalizar. De esta forma, los diferentes amplicones de ADNr se van desnaturalizando de forma diferente según su contenido en enlaces guanina-citosina a la vez que van migrando en el gel hasta que, cuando las hebras quedan completamente abiertas, el amplicón se detiene en un punto del gel donde originan una banda (Muyzer et al., 1993) (Figura I.18).

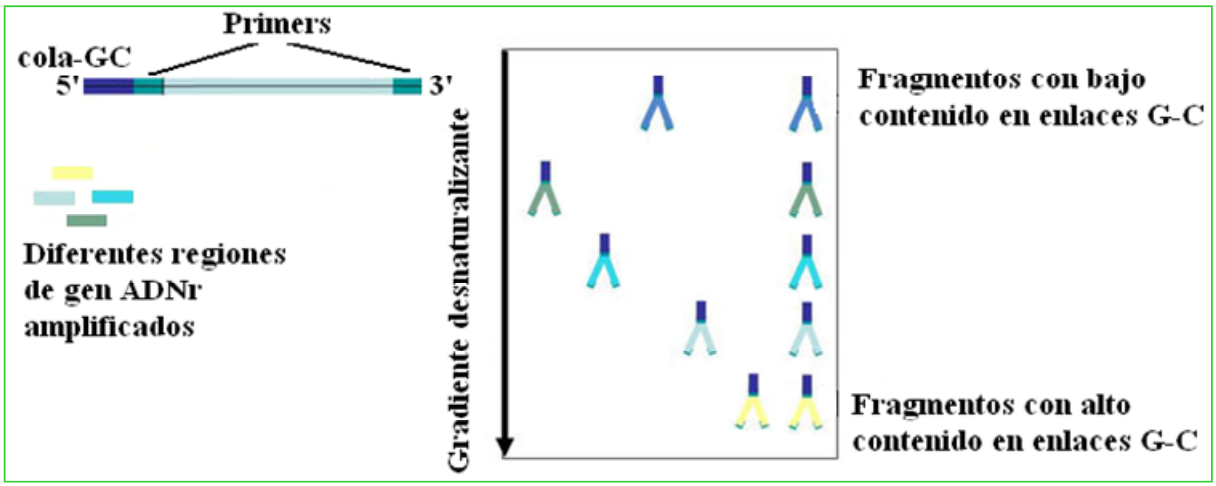


Figura I.18. Separación de diferentes amplicones de genes ADNr 16S mediante DGGE o TGGE.

De este modo, cada banda en el gel está constituida por amplicones con la misma secuencia, los cuales han sido producidos a partir del un gen ADNr perteneciente a una especie microbiana concreta. Así, las diversas bandas observadas en el gel indicaría el número de especies microbianas distintas en la comunidad y la intensidad de las bandas es tomada como un reflejo de su abundancia. La escisión y secuenciación de las diferentes bandas permite además identificar a los microorganismos a los que corresponden el gen ADNr contenido en la banda. Sin embargo, la medida de diversidad microbiana basada en el número de bandas distintas detectadas no es completamente fiable debido a que es posible que amplicones del ADNr con diferentes secuencias generen una misma banda, es decir, una misma banda corresponde a más de un microorganismo (Sekiguchi et al., 2001). Por otro lado, existen más de una copia para cada gen ADNr dentro del genoma de cada microorganismo (ej.1-15 para el gen ADNr 16S en bacterias, dependiendo de la especie) entre las cuales se ha detectado un cierto grado de polimorfismo (Tourova, 2003), lo que implica que un único microorganismo puede poseer varios genes ADNr con distinta

secuencia que en la DGGE o la TGGE originan más de una banda. Además de ello, la capacidad de resolución de bandas en este tipo de geles es limitada. Estos fenómenos son los responsables de que el número de bandas que pueden resolverse en un gel desnaturalizante sea de entorno a 35 bandas, que no incrementan a pesar de aumentar el número de genes ADNr distintos en la muestra (Loisel et al., 2006).

Debido a estos inconvenientes habría que considerar el patrón de bandas generados en este tipo de geles como un *fingerprinting* de la estructura de comunidad microbiana y no como una medida de su biodiversidad. En este sentido, Vivas et al., 2009) se sirvieron del DGGE para evaluar como el *fingerprinting* de la comunidad de bacterias presentes en el alperujo fue transformado de forma diferente dependiendo de si este residuo fue compostado o vermicompostado. Sen & Chandra (2009) también describieron como la comunidad bacteriana presente en residuos de caña de azúcar fue divergentemente modificada cuando este residuo fue procesado con y sin lombrices, originándose entonces productos orgánicos que mostraron *fingerprintings* diferentes entre sí. En los capítulos 1, 2, 6 y 7 de la presente memoria de Tesis Doctoral se describen y se ha utilizado la técnica de la DGGE para determinar el *fingerprinting* de la estructura de comunidades bacterianas y/o fúngicas presentes en muestras procedentes de procesos de vermicompostaje así como en vermicompost maduros.

SSCP (*Single-strand conformation polymorphism* / Polimorfismo de conformación de cadena única)

En esta técnica el producto de PCR que contiene los distintos genes ADNr amplificados es sometido a una digestión con una exonucleasa degradando una de las hebras de estos amplicones, de forma que cada copia del gen ADNr queda constituida por una sola cadena de nucleótidos. Cada monohebra que constituye entonces los genes ADNr adopta un plegamiento sobre sí misma dependiendo de la secuencia de nucleótidos de la cadena, por lo que genes ADNr con diferente secuencia suelen plegarse de forma distinta adoptando una conformación de un tamaño diferente. De esta manera, genes ADNr con secuencia distinta se pueden separar en un gel de electroforesis, originando un patrón de bandas característico de la comunidad microbiana, donde las diversas bandas corresponden a los genes ADNr procedentes de los distintos microorganismos presentes en la muestra (Lee et al., 1996; Schwieger & Tebbe, 1998). Al igual que en el caso de la técnica anterior, cada una de las

bandas de un gel SSCP puede contener más de un gen procedente de distintos microorganismos, ya que en ocasiones genes ADNr diferentes pueden adoptar un plegamiento parecido deteniéndose en el mismo lugar del gel.

Fracchia et al. (2006) fueron los primeros en usar esta técnica para examinar la comunidad bacteriana presente en un vermicompost producido a partir de una mezcla de estiércoles animales procesados por *E. fetida*. Estos autores caracterizaron este tipo de vermicompost obteniendo un perfil de bandas correspondientes al gen bacteriano ADNr 16S; en este perfil de bandas, nueve fueron consideradas características, siendo escindidas, reamplificadas y clonadas, identificando así 23 secuencias diferentes del gen ADNr 16S a partir de esas bandas. Estas secuencias correspondieron a bacterias pertenecientes a los filos Proteobacteria (6 secuencias), Chloroflexi (5), Bacteroidetes (3), Gemmatimonadetes (3), Acidobacteria (2), Actinobacteria (2) y Firmicutes (2), siendo el 83% de ellas no cultivables. Más recientemente, Sen et al. (2008) también se sirvieron de esta técnica para estudiar como la estructura de la comunidad bacteria presente en el residuo de caña de azúcar fue transformada durante el vermicompostaje. Así, estos autores encontraron que el *fingerprinting* de la comunidad bacteriana implicada en ese proceso de vermicompostaje fue independiente de la transformación química del residuo mientras que, en cambio, estuvo influenciado por la población de lombrices desarrollada durante el proceso.

RISA (*Ribosomal Intergenic Spacer Analysis* / Análisis del espacio intergenico ribosomal)

A diferencia de las técnicas mencionadas anteriormente que estudian la diversidad de secuencias que codifican los genes ADNr (principalmente el ADNr 16S o ADNr 18S), ésta se basa en amplificar el fragmento de ADN que se separa dentro del operon ribosomal los genes codificantes del ARNr 16S y 23S, en el caso de los organismos procariotas, o los ARNr 18S y 28S, en el caso de los eucariotas. Estas regiones se denominan IGS (*intergenic spacers/espacios intergénicos*) y se caracterizan porque presentan una variabilidad en secuencia y en longitud dependiendo del microorganismo al que pertenecen, existiendo, al igual que para los genes ADNr, bases de datos de IGS que permiten identificar a que microorganismos pertenece un IGS detectado en una muestra. Los IGS presentes en una muestra pueden ser amplificados usando las secuencias conservadas adyacentes de los extremos de los genes ADNr. De esta forma, RISA es una técnica electroforética similar al

DGGE que permite separar, en base a su diferente tamaño, los diferentes IGS amplificados a partir de los microorganismos presentes en la muestra. Así esta técnica permite igualmente obtener un patrón de bandas considerado un *fingerprinting* de la comunidad microbiana presente en esa muestra, donde cada banda correspondería a un taxón diferente.

Una variante de esta técnica es la denominada ARISA (*Automatic Ribosomal Intergenic Spacer Analysis/análisis automático del espacio intergenico ribosomal),* la cual puede ser desarrollada añadiendo un fluoróforo al IGS amplificado mediante un *primer* marcado; esto permite entonces la detección automática y cuantificación de IGS de diferente tamaño separados durante la electroforesis. Esta técnica ha sido mostrada útil para estudiar las comunidades microbianas implicadas en el compostaje (Schloss et al., 2003), aunque todavía no ha sido aplicada para estudiar la microbiología de procesos de vermicompostaje.

***ADNr-Microarray* / Chips de ADNr**

Los microarrays, o chips de ADN, permiten la detección de una amplia variedad de microorganismos presentes en una muestra. Estos chips están constituidos por fragmentos de genes ADNr característicos de microorganismos que permiten detectar la presencia de esos ADNr diana en los productos PCR obtenidos a partir de la amplificación de los genes ADNr de microorganismos presentes una muestra. Estos microarrays-ADNr suelen contener más de un fragmento del ADNr específico para cada especie de microorganismo buscado, así como fragmentos ADNr que son específicos para varios microorganismos clasificados dentro del mismo grupo de taxonómico. De esta forma, en un mismo chip pueden estar incluidas hasta miles de secuencias para detectar en los productos de PCR la presencia de genes ADNr de diversos microorganismos, identificándolos a diferente nivel taxonómico (Kirk et al., 2004). Estos fragmentos de genes ADNr buscados o sondas, se encuentran anclados en los microarrays-ADNr en forma de monohebra de ADN, de forma que, cuando sobre estos chips se aplica el producto de PCR de los distintos genes ADNr amplificados de una muestra usando un *primers* marcado con un fluoróforo (este producto debe ser previamente digeridos para que los amplicones del gen ADNr se presenten en forma de monohebras), se pueden producir entonces hibridaciones entre las sondas del chip y los genes ADNr diana amplificados con secuencia complementarias, desencadenando la aparición de señales fluorescentes en distintas zonas del microarray donde se localizan las sondas para los genes

ADNr buscados, indicado así la presencia estos microorganismos diana en la muestra (Figura I.19).

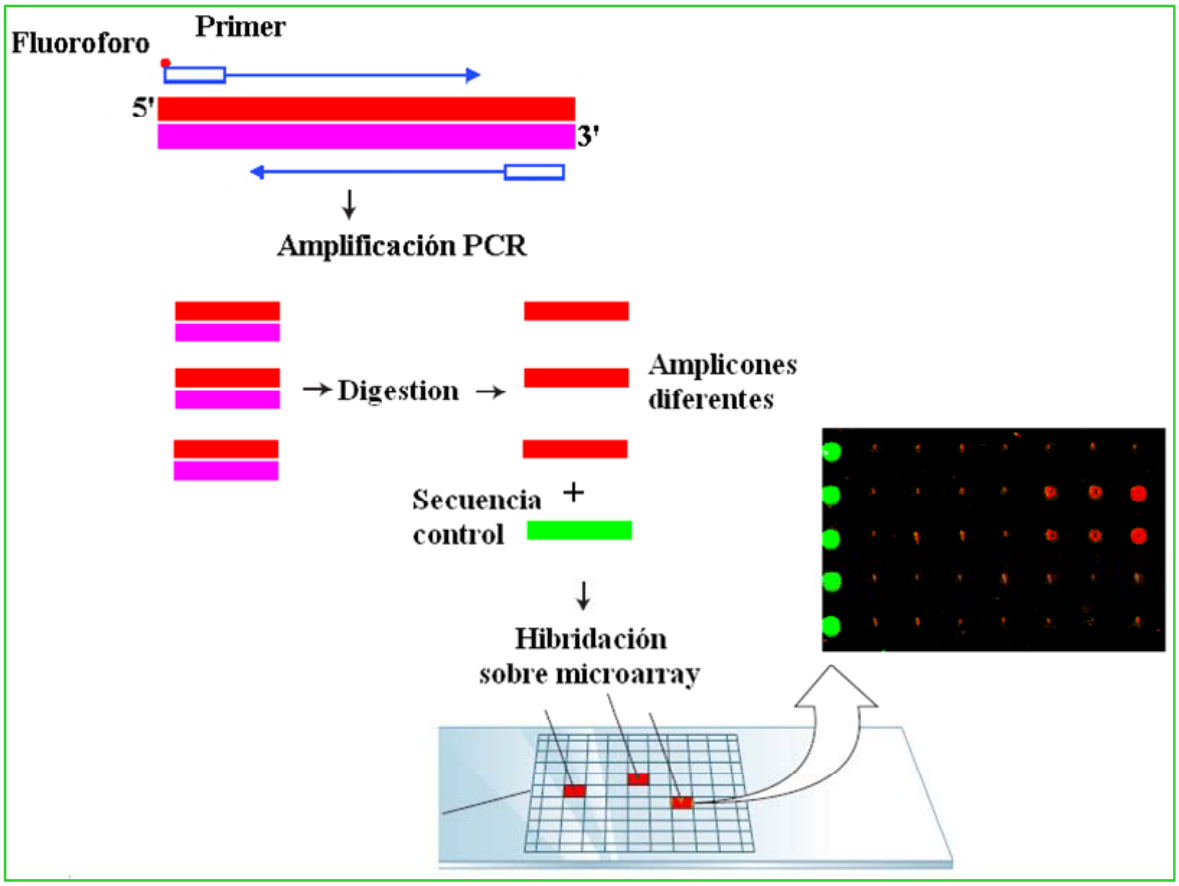


Figura I.19. Análisis de amplicones de genes ADNr 16S mediante ADNr-Microarrrays.

Los microarrays-ADNr permiten la detección simultánea de un gran número de microorganismos en una muestra sin necesidad de secuenciar los amplicones de los genes ADNr producidos por PCR. Sin embargo, su aplicación no es adecuada para estimar toda la diversidad de microorganismos presentes en una muestra puesto que obviamente los chips no pueden contener sondas específicas para los microorganismos desconocidos que se puedan encontrar en la muestra. Por otro lado, en ocasiones sucede que, a pesar de que el chip dispone de una sonda específica para detectar un microorganismo concreto, la presencia de éste pasa inadvertida debido a la baja afinidad de la sonda para su ADNr o a causa del bajo número de amplicones originados a partir del gen ADNr del microorganismo. A pesar de estas limitaciones los microarrays constituyen potentes herramientas para estudiar la diversidad de microorganismos que presuntamente se pueden encontrar en una muestra. En este sentido dos novedosos microarrays fueron desarrollados para estudiar la microbiota en

procesos de bioestabilizadores de residuos orgánicos: el Compochip, que incluye varias sondas para detectar la presencia de microorganismos frecuentemente implicados en procesos de compostaje (Franke-Whittle et al., 2005, 2009a), y el Anaerobiochip, para detectar diversas especies de microorganismos metanógenos que pueden aparecer en los digestores anaeróbicos (Franke-Whittle et al., 2009b). Sin embargo, hasta ahora no se ha diseñado ningún microarray que incluya genes diana para microorganismos frecuentemente encontrados en muestras de vermicomposts, tal vez debido al escaso conocimiento existente sobre los microorganismos típicos de este proceso. En el capítulo 6 de la presente memoria de Tesis Doctoral se describe y se ha utilizado el Compochip para el análisis de las comunidades bacterianas presente en cuatro vermicomposts de diferente origen.

I.3.1.2.3.2. Técnicas basadas en el análisis completo del contenido en ADN

El ADN de una comunidad microbiana es una mezcla de genomas de diferentes especies presentes en distinta proporción. La forma más simple de estudiar la diversidad de estos genomas es mediante el estudio de la cinética de reasociación del ADN total de la comunidad. Esta técnica consiste en desnaturalizar con calor el ADN extraído de la muestra y mediante un espectrofotómetro determinar la proporción de ADN que se reasocia en función del tiempo, gracias a que la monohebra de ADN y el ADN de doble cadena tienen picos de absorbancia a longitudes de onda diferentes. Se considera que la curva de reasociación del ADN de una comunidad es proporcional al número de genomas diferentes y a su tamaño. De esta manera el tiempo transcurrido para que el 50% del ADN se haya reasociado es un índice de diversidad biológica que se ha aplicado para caracterizar la comunidad microbiana de muestras de suelo (Torsvik et al., 1990). Este índice proporciona una estimación del número de diferentes genomas presentes en la muestra, aunque no permite conocer la abundancia relativa de los diferentes genomas individuales. A pesar de la simplicidad de esta técnica, su aplicación al estudio de la diversidad de microorganismos en muestras ambientales ha caído en desuso, debido principalmente a las enormes ventajas que ofrecen las nuevas técnicas desarrolladas recientemente para analizar conjuntamente todo el ADN contenidos en muestras ambientales, como son las diferente técnicas de secuenciación masiva. En estas técnicas, tras la fragmentación del ADN total, es posible determinar la secuencia de estos fragmentos gracias a secuenciadores automáticos basados en la PCR junto con nucleótidos fluorescentes.

Actualmente existen diversas técnicas de secuenciación masiva clasificadas como: métodos de secuenciación de 1ª generación o shotgun secuencing, 2ª generación o secuenciación masiva en paralelo y 3ª generación o *single molecule real time sequencing*. Hasta ahora, la secuenciación masiva del ADN contenido en muestras procedentes de reactores de biogas ha proporcionado una útil información no sólo sobre la comunidad microbiana desarrollada en este proceso, sino también sobre la presencia de genes funcionales importantes para la producción de metano durante este proceso (Krause et al., 2008; Schlüter et al., 2008). Aunque estas técnicas de secuenciación masiva aun no han sido empleadas en procesos de vermicompostaje, su aplicación podría llegar a ser una de las metodologías más prometedoras que existen en la actualidad para determinar los microorganismos típicamente implicados en este proceso.

I.3.2. MÉTODOS DE ESTUDIO DE LAS FUNCIONES BIOLÓGICAS EN COMUNIDADES MICROBIANAS

Debido a que una comunidad microbiana está constituida por un conjunto de diversos microorganismos capaces de realizar diversas funciones, esta puede considerase como una unidad funcional caracterizada por la sumatoria de sus propiedades metabólicas. La conexión entre la funcionalidad de una comunidad microbiana y la diversidad microbiológica que encierra es aún un tema de discusión abierto. Un análisis más profundo de las propiedades funcionales de la microbiota responsable del vermicompostaje se debería llevar a cabo mediante la determinación de parámetros que reflejen su funcionalidad, a la vez que permitan su caracterización. A continuación se mencionan brevemente las aproximaciones disponibles para explorar la funcionalidad de sistemas biológicos complejos tales como el vermicompostaje.

I.3.2.1. Determinación de la actividad biológica de comunidades microbianas

La actividad biológica desarrollada por todos microorganismos presentes en muestras de vermicompost es el reflejo de todos procesos metabólicos implicados en el funcionamiento de la comunidad microbiana alojada en este tipo de muestras (Benítez et al., 1999; Vivas et al., 2009). Uno de los parámetros que permiten conocer la actividad biológica de una muestra es su respiración basal, la cual se puede determinar midiendo el desprendimiento de

CO_2, o bien el O_2 consumido, en una muestra incubada en recipientes herméticos bajo unas condiciones constante de temperatura y humedad durante un cierto periodo de tiempo (Alef & Nannipieri, 1995). Varios trabajos han estimado la respiración basal durante el vermicompostaje con objeto de estimar la estabilidad del substrato degradado (Aira & Domínguez, 2008; Gómez-Brandón et al., 2011a, 2011b). En este sentido se puede considerar que un substrato ha sido satisfactoriamente vermicompostado cuando desarrolla una comunidad microbiana estable, lo cual se refleja en un consumo O_2 o producción de CO_2 que no varía significativamente a lo largo del tiempo.

Otros trabajos han estimado la actividad biológica de la comunidad microbiana de una muestra basándose en la respiración inducida por substrato (SIR), la cual es una medida indirecta que viene definida por la respiración resultante tras la adición de un substrato orgánico exógeno a la muestra, usualmente glucosa (Aira et al., 2007b; Lazcano et al., 2008).

Otra medida indirecta de la actividad biológica de los microorganismos que componen una comunidad microbiana se puede obtener determinando la actividad potencial de las enzimas deshidrogenasas presente en la muestra. Las dehidrogenasas son enzimas intracelulares presentes en todos los microorganismos vivos que vivos, en los cuales catalizan la transferencia de hidrógeno y electrones de un compuesto a otro. Consecuentemente, la medida de la actividad deshidrogenasa total de una muestra refleja las actividades oxidativas de la microbiota contenida en una muestra ofreciendo una estimación indirecta de su actividad biológica (García et al., 1997; von Mersi & Schinner, 1991). La actividad deshidrogenasa total se puede determinar mediante la adición de substratos sintéticos (TTC: cloruro de trifeniltetrazolio; o INT: 2-ρ-iodofenil-3-ρ-nitrofenil-5-feniltetrazolio) a una muestra, los cuales son reducidos por la acción de las deshidrogenadas de los microorganismos activos (Alef & Nannipieri, 1995).

La actividad biológica de los microorganismos presentes en una muestra también puede ser estimada midiendo la hidrólisis de diacetato de fluoresceina (FDA). Este es un compuesto apolar incapaz de penetrar en el interior de microorganismos puesto que no puede atravesar la membrana celular. Cuando la FDA es hidrolizada por las diversas lipasasas, esterasas y proteasas de los microorganismos activos de una muestra se libera fluoresceína, que al ser polar, se incorpora al interior de microorganismos activos. De esta manera midiendo la fluorescencia resultante de la hidrólisis de FDA ocurrida en muestras incubada bajo condiciones óptimas de pH y temperatura, se obtiene un índice de la potencial actividad biológica de los microorganismos presentes en la muestra (Schnürer & Rosswall, 1982).

Tanto la actividad deshidrogenasa como la hidrólisis de FDA son dos determinaciones empleadas en estudios recientes para estimar la actividad de la comunidad microbiana presente en muestras de vermicompost. Sen & Chandra (2009) monitorizaron la actividad deshidrogenasa durante el vermicompostaje de residuos de caña de azúcar y encontraron que esa actividad estuvo positivamente correlacionada con el número de unidades formadoras de colonias determinado en cultivo en placa, indicando así que la actividad biológica responsable del vermicompostaje está condicionada por la población de microorganismos presentes en el substrato. Asimismo, Yakushev & Byzov (2009) evaluaron la hidrólisis de FDA en diferentes tipos de composts y vermicomposts, recomendando la determinación de esta actividad para el control del proceso de vermicompostaje.

I.3.2.2. Medida de las actividades bioquímicas en comunidades microbianas

La evaluación de ciertas actividades enzimáticas durante un proceso de biodegradación proporciona información sobre la capacidad potencial de la comunidad microbiana residente en una muestra para llevar a cabo reacciones específicas responsables de los procesos de transformación de los compuesto orgánicos en este proceso (Mondini et al., 2004). Especial importancia en este ámbito tienen las enzimas hidrolasas implicadas en la degradación de compuestos que son usados como fuente de energía y nutrientes por los microorganismos durante el proceso de vermicompostaje. Así, el funcionamiento de la comunidad microbiana durante el vermicompostaje no se puede entender correctamente sin la participación de procesos enzimáticos (Benítez et al., 1999).

Por este motivo, la evaluación de la actividad de ciertas enzimas en vermicomposts ha sido considerada útil para estudiar la funcionalidad metabólica de su comunidad microbiana (Vivas et al., 2008). De esta forma, el análisis de la actividad de diversos tipos de enzimas hidrolíticas, como por ejemplo: β-glucosidasas, celulasas, ureasas, proteasas, fosfatasas, etc., ha sido determinada en multitud de estudios para evaluar la biodegradación y estabilización de residuos orgánicos durante procesos de vermicompostaje tanto a escala laboratorio como a escala piloto (Aira et al., 2006, 2007a; Benítez et al., 2002; Melgar et al., 2009; Nogales et al., 2005; Pramanik et al., 2007; Sen & Chandra, 2009). Al igual que en el caso de la actividad deshidrogenada, cada tipo de actividad hidrolítica se mide mediante la adición de un substrato sintético específico a una muestra de vermicompost incubada bajo condiciones

optimas de pH y temperatura, produciéndose entonces la hidrólisis del substrato por el tipo concreto de hidrolasas estudiadas.

Valiéndose de esta metodología, Benítez et al. (1999) describió como la disminución de la actividades hidrolíticas medidas durante el vermicompostaje es el resultado de que la comunidad microbiana desarrollada ha llegado a estabilizarse después de degradar los compuesto orgánicos disponibles en el residuo vermicompostado. Asimismo, Sen & Chandra (2009) establecieron que en un vermicompost el valor de actividad para las diferentes enzimas hidrolíticas depende de los grupos funcionales de microorganismos presentes en su comunidad microbiana. En este sentido, la evaluación de la actividad de las enzimas hidrolíticas en diversos productos orgánicos bioestabilizados ha sido llevada a cabo con objeto de comparar la diversidad funcionalidad de las comunidades microbianas contenidos en cada uno de ellos (Vivas et al., 2009). No obstante, se debe señalar que el valor de estas actividades enzimáticas, analizadas mediante métodos de incubación en presencia de un sustrato sintético bajo condiciones óptimas, no corresponde con la actividad biológica desarrollada *in situ* por los microorganismos existentes en la muestra, sino que se trata de una estimación de su actividad potencial, la cual permite comparar comunidades microbianas de muestras con diferentes características fisicoquímicas bajo las mismas condiciones de incubación.

I.3.2.3. Estimación de la diversidad catabólica de comunidades microbianas

La funcionalidad de las comunidades microbianas presente en muestras de vermicomposts puede evaluarse basándose en la habilidad de los microorganismos de la comunidad para utilizar diversas fuentes de carbono (Garland & Mills, 1991). De esta forma, el grado de utilización de diversos substratos aplicados a una muestra ofrece información sobre las distintas capacidades fisiológicas de los microorganismos que la componen, representando así el perfil fisiológico a nivel de comunidad o CLPP (*Community Level Physiological Profile*) (Degens & Harris, 1997). Actualmente, existe varios tipos de sistemas comerciales que permiten determinar el CLPP de la comunidad microbiana de una muestra: Biolog, Multi-SIR o Microresp.

Biolog es un sistema automatizado que consiste en microplacas de 96 pocillos, cada uno de ellos conteniendo medio de cultivo suplementado con una única fuente de carbono distinta, además de otros nutrientes básicos y un indicador colorimétrico, el cual señala el

grado de consumo de cada substrato orgánico en el medio de cultivo (Garland & Mills, 1991). Este sistema proporciona un patrón de utilización de los diferentes substratos orgánicos *in vitro* cuando cada pocillo de la microplaca se inocula una alícuota de una dilución acuosa de la muestra. Este sistema presenta la problemática derivada de las técnicas microbiológicas de cultivo, implicando que la diversidad catabólica medida en la muestra mediante este sistema es un reflejo sólo de algunos microorganismos aeróbicos que han sido capaces de crecer en el medio de cultivo, con lo cual la representatividad de la capacidad catabólica propia de toda la microbiota contenida en la muestra es cuestionables. A pesar de estas limitaciones, el sistema de microplacas Biolog ha sido frecuentemente utilizado en estudios previos para evaluar la diversidad funcional de las comunidades microbianas presentes en el vermicompostaje (Aira et al., 2006, 2007b; Gómez-Brandón et al., 2010a; Sen & Chandra, 2009). En estos estudios, la aplicación de esta técnica reveló como la presencia de *E. fetida* favorece que la microbiota desarrollada en el sustrato vermicompostado fuera capaz de degradar mayor cantidad de substratos así comparada con un substrato compostado sin lombrices.

De forma opuesta al Biolog, otros métodos como el Multi-SIR o el Microresp no requieren de la obtención de un cultivo microbiano a partir de la muestra, permitiendo así evaluar la diversidad catabólica de toda la comunidad microbiana en la misma muestra. El método Multi-SIR fue inicialmente descrito por Degens & Harris (1997) para evaluar la diversidad catabólica de muestras de suelos, y se basa en diluir la muestra estudiada en una solución acuosa tamponada (relación muestra: solución, 1:2) que contiene una fuente única de carbono, de forma que tras un tiempo de incubación en un frasco McCartney, el CO_2 producido es recuperado y cuantificado por medio de cromatografía de gases u otro método equivalente. Esta técnica aunque permite evaluar la actividad de todos microorganismos contenidos en la muestra, impide detectar las actividades catabólicas asociadas a los consorcios microbianos, debido a que la desagregación física de la muestra en las distintas soluciones acuosas con los substratos orgánicos impide que los microorganismos integrantes del consorcio se encuentre en las condiciones originales adecuadas para el desarrollo su actividad (Chapman et al., 2007). Quizás este inconveniente, junto con el hecho de que la aplicación de esta técnica es mas laboriosa en comparación con el sistema Biolog, son responsables que ella no haya sido aun usada para determinar el CLPP en vermicompostaje.

MicroResp^{TM} es un sistema comercial patentado, desarrollado en Campbell et al. (2003), al que se le atribuye la virtud de estimar la diversidad catabólica de muestras ambientales sin

alterar demasiado las condiciones *in situ* de la misma (Chapman et al., 2007), evitando la laboriosa metodología de medida requerida por el sistema Multi-SIR ya que, al igual que el Biolog, MicroResp™ consiste en una microplaca de 96 pocillos (Figura I.20). Estos pocillos están dispuestos en dos placas superpuestas, una placa inferior o base con pocillos profundos, de una capacidad de 1, 2 mL, en los cuales se depositará los distintos sustratos que actúa como fuente de carbono junto con la muestra; y una la placa superior que contiene pocillos poco profundos rellenos de una solución de agar indicadora del pH, mediante la cual se detecta el CO_2 producido en cada pocillo profundo correspondiente. Aunque este sistema ha sido usado satisfactoriamente para estudiar como la enmienda suelos con composts afecta a su CLPP (Cordovil et al., 2011), hasta ahora este sistema no ha sido usado para evaluar el perfil fisiológico de las comunidades microbianas implicadas en el vermicompostaje. En el capítulo 7 de la memoria de Tesis Doctoral se describe la aplicación del sistema MicroResp™ para determinar el CLPP de cuatro vemicomposts producidos a partir de residuos orgánicos de distinto origen.

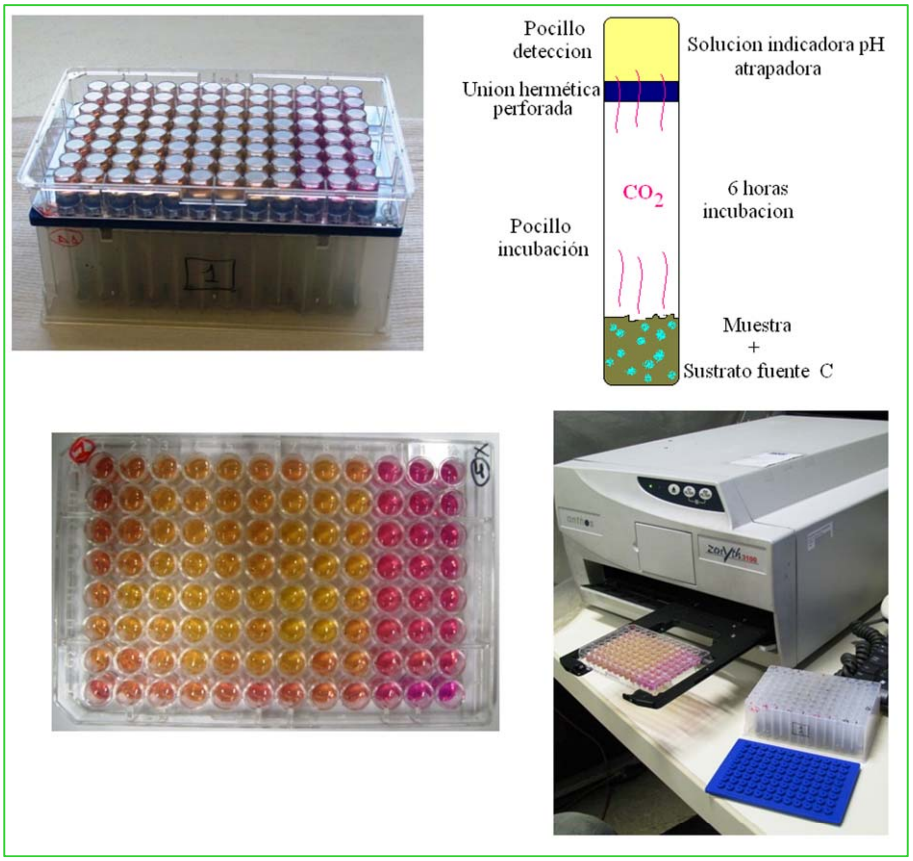


Figura I.20. Uso del sistema de micropocillos MicroResp™ para determinar la diversidad catabólica de comunidades microbianas.

I.3.2.4. Análisis de la funcionalidad genética de comunidades microbianas

El análisis de la presencia de algunos genes funcionales dentro de la comunidad microbiana de un vermicompost puede permitir conocer la capacidad de sus microorganismos para participar en diversos procesos biológicos. En este sentido, el estudio de genes implicados en el desarrollo de procesos biológicos tales como: desnitrificación, nitrificación, fijación de nitrógeno, oxidación de metano, presentes en el metagenoma de la comunidad microbiana de vermicomposts proporciona información sobre la capacidad de esa comunidad para desarrollar esas funciones.

La metodología aplicada para estudiar la diversidad de estos genes funcionales es análoga a la referida en el caso de los métodos de estudio de diversidad de los genes ADNr, aunque con la diferencia de que se requiere la utilización de *primers* apropiados para amplificar estos genes por PCR. Además, en el caso de los genes funcionales es muy interesante la cuantificación del número de copias de un tipo de gen concreto presente en la comunidad microbiana de un vermicompost, ya que así sería posible conocer el potencial de esta comunidad en el desarrollo del proceso en el cual se encuentra implicado el gen.

Con este objetivo, la aplicación de una variante de la PCR conocida como PCR cuantitativa (qPCR) o a tiempo real es una técnica que nos permite evaluar el número de copias un gen concreto presentes en una muestra (Wong & Medrano, 2005). En la qPCR mientras el gen de estudio va siendo amplificado, el número de copias generadas en cada ciclo de amplificación, que depende de la cantidad de copias inicial de ese gen en la muestra, es determinado usando distintos sistemas de marcaje (ej. agentes intercalantes o sondas específicas para el gen marcadas con fluorocromos) que generalmente emiten una señal fluorescente equivalente a la cantidad de ADN amplificada durante la reacción. Entonces, el número de copias del gen estudiado presentes en una muestra se puede estimar comparando la fluorescencia observada en la qPCR de la muestra con la fluorescencia que se observa en la qPCR realizada paralelamente usando unos controles externos con concentraciones conocidas y crecientes del gen diana (curva patrón). Aunque la qPCR permitiría determinar el número de ciertos genes funcionales presentes en la comunidad microbiana de un vermicompost, conocer la expresión de estos genes es indispensable para revelar la participación en el proceso estudiado de los microorganismos de la comunidad que los poseen. Para el estudio de expresión de genes funcionales es necesario la detección del ARN mensajero (ARNm) entre todo el ARN extraído de la muestra. Esto se puede conseguir

usando la qPCR, se requiere previamente el paso previo de síntesis del ADN complementario (ADNc) a partir del ARN extraído de la muestra. Esta síntesis de ADNc se puede realizar mediante una reacción que utiliza una transcriptasa inversa (Ausubel et al., 2002). De esta manera, analizando el ADNc de una muestra mediante qPCR con los *primers* adecuados para amplificar un gen funcional concreto es posible conocer la expresión de este gen en la muestra (Wong & Medrano, 2005). De este modo, usando el ADN y el ARN (ADNc) de muestras del suelo rizosférico de leguminosas, Sharma et al. (2005) consiguieron determinar el número de copias de los diferentes de genes *nirK* y *nirS*, los cuales codifican nitrito reductasas responsables la desnitrificación microbiana, así como su expresión en estos ambientes. Sin embargo, este tipo de estudios, aun no se realizado para evaluar la presencia y expresión de genes funcionales durante un proceso de vermicompostaje.

OBJETO DEL TRABAJO

La gran productividad y rentabilidad de los cultivos bajo plástico y/o en invernaderos ha favorecido que esta intensiva práctica agrícola tenga un gran auge a nivel mundial, a pesar de la enorme cantidad de residuos vegetales que este tipo de cultivos produce, principalmente matas de plantas desechadas tras la cosecha y frutos no comercializados (destríos). En España, 62505 hectáreas están dedicadas a cultivos de invernadero, concentrándose el 70% de esos cultivos en la Comunidad Autónoma Andaluza, particularmente en las cercanías de la costa almeriense-granadina. En áreas con alta densidad de este tipo de cultivos, los residuos vegetales de invernadero se generan en cantidades demasiado grandes para ser gestionadas eficientemente por los sistemas de tratamiento de residuos actuales, llegando a suponer un problema ambiental. Idealmente, el uso de tecnologías económicas de bajo impacto ambiental que permitan el reciclaje de estos residuos vegetales en materiales orgánicos estables, maduros, ricos en nutrientes, y con propiedades biológicas adecuadas para su valorización como enmienda orgánica de suelos o como productos con interés agrícola, constituye la estrategia de gestión más apropiada desde el punto de vista ambiental, ya que así se podría recuperar parte de los nutrientes empleados en los cultivos de invernadero para el crecimiento vegetal, cerrando además el ciclo de la materia orgánica.

A pesar de que el vermicompostaje es considerado un proceso biotecnológico de bajo coste medioambientalmente sostenible que ha sido extensamente empleado para biodegradar y bioestabilizar una enorme variedad de residuos orgánicos de diferente naturaleza, la posibilidad de reciclar residuos vegetales de cultivos bajo plástico y/o invernadero en materiales orgánicos de valor mediante el desarrollo de esos procesos no sido evaluada hasta la fecha. Los residuos vegetales de invernadero presentan particularidades específicas, debido a los sistemas intensivos de producción agrícola aplicados, como es la posible presencia de residuos de plaguicidas que puedan afectar negativamente al desarrollo de las lombrices inoculadas al material orgánico. El vermicompostaje de los residuos vegetales de invernadero (matas de plantas y destríos de frutos no comercializables) así como el de los residuos generados por supermercados (destríos de frutos comercializados) permitiría su revalorización en productos orgánicos maduros y con cualidades físico-químicas adecuadas para su utilización en agricultura y recuperación de suelos; que además podrían ser empleados como materiales bioactivos para diversos fines según sus propiedades biológicas. En este sentido, las comunidades microbianas desarrolladas en diferentes procesos de vermicompostaje como consecuencia de la actividad de las lombrices sobre distintos residuos no sólo aceleran la descomposición y estabilización de los mismos sino que además son responsables de las propiedades biológicas particulares de los vermicomposts

producidos. Sin embargo, en la actualidad no se conoce en profundidad como evolucionan las comunidades microbianas de los distintos residuos orgánicos conforme estos son vermicompostados, permaneciendo aun sin definir la identidad de los microorganismos típicamente implicados en los procesos de vermicompostaje. Igualmente, existe aun escasa información sobre como las diferentes comunidades microbianas alojadas en vermicomposts maduros difieren en estructura, composición y diversidad funcional. La aplicación de técnicas de biología molecular para desarrollar nuevas investigaciones enfocadas a aclarar la microbiología del proceso de vermicompostaje puede ayudar, por una parte, a mejorar el proceso, y por otra a prever los microorganismos desarrollados en diferentes procesos de vermicompostaje, ayudando a predecir la utilidad de los vermicomposts como productos bioactivos.

El **objetivo general** del presente trabajo de Tesis Doctoral conecta con las ideas que se han descrito y está dirigido a evaluar el proceso de vermicompostaje de residuos vegetales (matas de plantas y destríos) generados por los cultivos bajo plástico y/o invernadero como método biotecnológico que permita reciclar estos residuos, en materiales orgánicos estables, maduros, ricos en nutrientes, y con propiedades biológicas que permitan su valorización como productos con interés agrícola, enmienda orgánica para suelos, o materiales bioactivos con diversas utilidades. Con ello se persigue aumentar y diversificar las vías de reutilización, reciclado y valorización de esos residuos así como de otros de características similares como son los producidos por los comercios y supermercados. La investigación desarrollada ha tratado de proporcionar una información útil que cubra el vacío existente sobre este tema; pero al mismo tiempo ha estado orientada hacia una serie de objetivos específicos, pioneros y vanguardistas que han permitido adquirir conocimientos innovadores sobre la temática que nos ocupa. Este objetivo general se concretó en los siguientes objetivos específicos:

- **Objetivo 1.** Evaluar la viabilidad de las lombrices *E. andrei* y/o *E. fetida* para vermicompostar los residuos vegetales de invernadero, diseñando y optimizando el proceso según las características de los principales tipos de residuos: matas de plantas y frutos de destríos. Este objetivo se desarrolló en los capítulos 1, 2, 3 y 4 / *chapters 1, 2, 3 and 4.*
- **Objetivo 2.** Investigar los cambios en las propiedades químicas, bioquímicas (actividades enzimáticas) y microbiológicas que los residuos vegetales de invernadero experimentan debido a su vermicompostaje con el fin de evaluar su biodegradación y

bioconversión en productos orgánicos estables y maduros. Este objetivo se desarrolló en los capítulos 1, 2, 3 y 4 / *chapters 1, 2, 3 and 4*.

- **Objetivo 3.** Estudiar el efecto de un insecticida frecuentemente aplicado en cultivos de invernadero (imidacloprid) sobre la actividad microbiana del residuo y el desarrollo de las lombrices durante su vermicompostaje. Este fue el objetivo del capítulo 5 / *chapters 5.*
- **Objetivo 4.** Comparar las comunidades microbianas de los vermicomposts de residuos de invernadero frente a la de los vermicomposts de otros residuos orgánicos diferentes para valorar su calidad desde un punto de vista microbiológico. Este objetivo se trató en los capítulos 6 y 7 / *chapters 6 and 7*.
- **Objetivo 5.** Investigar el potencial del vermicompost de residuo de invernadero junto con el de otros vermicomposts para ser utilizados como materiales bioactivos en la biodegradación de plaguicidas. Este fue el objetivo del capítulo 7 / *chapters 7*.
- **Objetivo 6.** Evaluar el potencial de dos vermicomposts procedentes de destríos de frutos de supermercado y de invernadero, respectivamente, como enmiendas orgánicas de suelos agrícolas o de suelos contaminados por metales pesados. Este objetivo se desarrolló en los capítulos 8 y 9 / *chapters 8 and 9.*

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

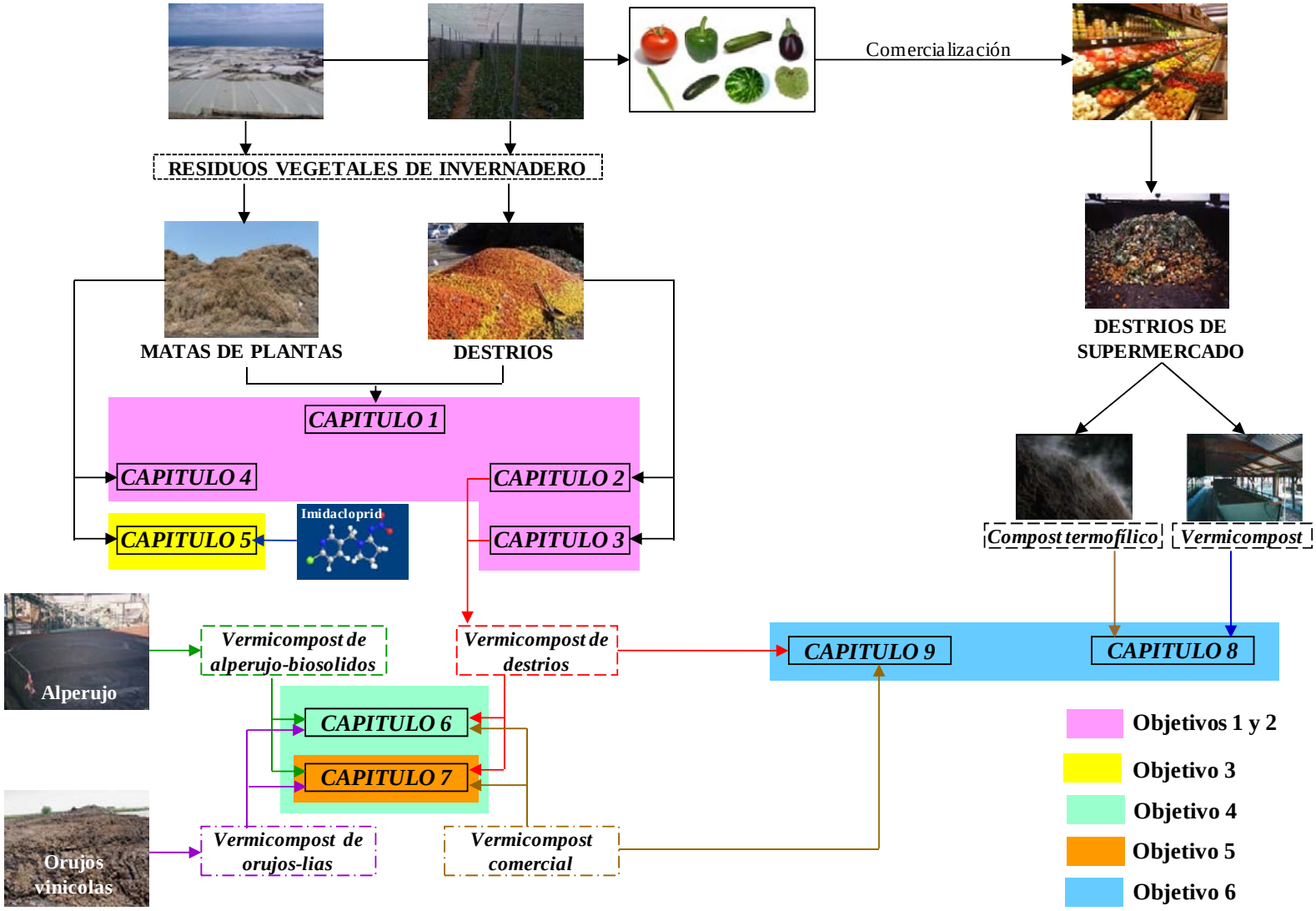


Figura O.1. Objetivos y capítulos que conforman la memoria de Tesis Doctoral

MATERIALES

Para la realización de los estudios que constituyen la Tesis Doctoral, que se recogen entre los capítulos 1 y 9 de la presente memoria, se han utilizado diferentes especies de lombrices epigeas, residuos orgánicos, vermicompost y composts, plaguicidas (Imidacloprid, Diuron y Metalaxyl) y suelos. Un resumen de los materiales utilizados se expone a continuación:

M.1. LOMBRICES EPIGEAS UTILIZADAS

Las lombrices utilizadas en los procesos de vermicompostaje desarrollados pertenen a las siguientes especies de la familia Lumbricidae, orden de los Haplotáxidos y subclase de los oligoquetos:

- ***Eisenia andrei*** (Bouché, 1972): utilizada en el proceso de vermicompostaje desarrollado en el capitulo 1, procedieron del stock mantenido en la Estación Experimental del Zaidín (CSIC), Granada.
- ***Eisenia fetida*** (Savigny, 1826): utilizada en los procesos de vermicompostaje desarrollados en los capítulos 2, 3, 4 y 5, fueron suministradas por la empresa Empresa Lombricor S.C.A (Algallarin, Córdoba)

M.2. RESIDUOS VEGETALES DE INVERNADERO SELECCIONADOS

Los residuos vegetales procedieron de plantas cultivadas en invernadero y/o bajo plastico localizados en el Campo de Dalias (El Ejido, Almeria) (utilizados en el capitulo 1) y en el área de Carchuna (Motril, Granada) (Capitulos 2, 3, 4 y 5) (Figura M.1).

- **Campo de Dalias**: representa un área de cultivos en invernaderos y/o bajo plástico que ocupa una extensión aproximada de 22000 Ha en el poniente Almeriense. En el año 2003 se generaron más de 650000 Tm de residuos vegetales (matas de plantas y destríos de frutos hortícolas), principalmente procedentes del cultivo de pimiento, que es el predominante en esta región. Otros cultivos desarrollados en invernadero en esta región que generaron una gran cantidad de residuos vegetales fueron los cultivos de tomate, melón y pepino, aunque según su extensión en superficie no siga el mismo orden la producción de residuos vegetales depende del periodo de cultivo y del porte vegetativo de la planta cultivada. (Figura M.2).

Area de Carchuna: representa un área de cultivos en invernaderos y/o bajo plástico con una extensión aproximada de 521 Ha en la costa granadina. El cultivo mayoritario es el de tomate de la variedad “cherry”, seguido a gran distancia por los cultivos de calabacín y pepinos, y dependiendo de la época del año, aguacates, chirimoyas y sandias.

Figura M.1. Cultivos en invernadero desarrollados en el área de Carchuna (Motril, Granada) y en el Campo de Dalias (El Ejido, Almeria).

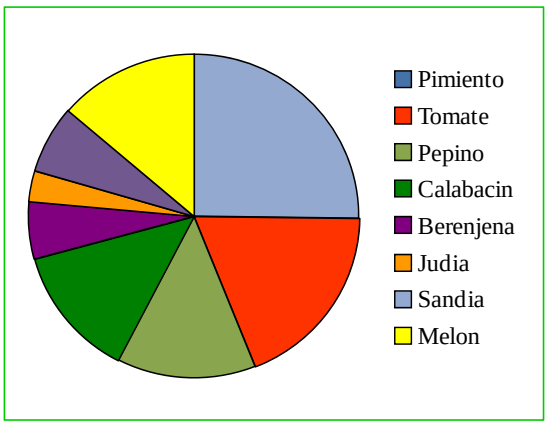


Figura M.2. Porcentaje de residuos vegetales de invernadero generados por los cultivos en el Campo de Dalias.

Entre los años 2007-2009 la producción total de estos residuos vegetales, constituidos principalmente por destrios y matas de plantas, varió entre 22000 y 25500 Tm año-^{1} (Figura M.3). La proporción de destríos de frutos respecto a las matas de cultivo fue bastante

similar en esos años, con un valor medio de destríos de frutos del 19% respecto al total de residuos vegetales de invernadero (Figura M.3). Respecto a los tipos de destríos, el 80% de los frutos fueron tomates "cherry", 11 % de calabacines y en menor porcentaje el resto de los otros cultivos (Figura M.4).

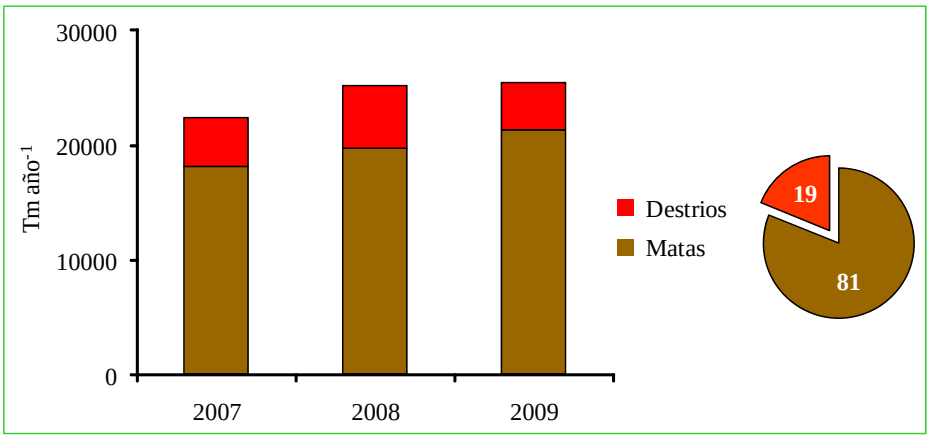


Figura M.3. Producción de residuos vegetales de invernadero en el área de Carchuna (Motril, Granada): porcentajes de destríos de frutos y matas de plantas entre 2007 y 2009.

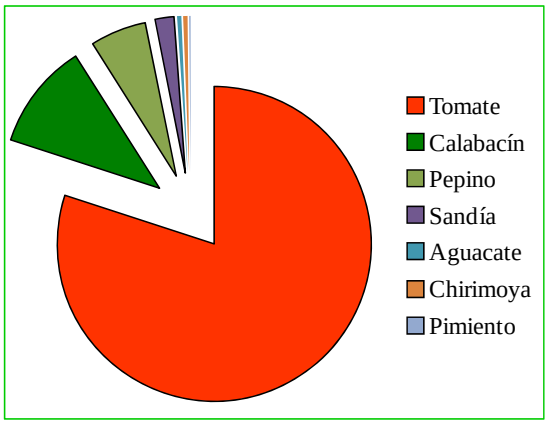


Figura M.4. Porcentaje de destrios de frutos procedentes de diversos cultivos de invernadero del área de Carchuna entre 2007 y 2009 (Motril, Granada)**.**

Los residuos vegetales de invernadero seleccionados en los procesos de vermicompostaje desarrollados en la Tesis Doctoral se exponen a continuación.

- **Residuos vegetales de diferentes** plantas **/ Heterogeneous plant wastes (HP):** constituidos por matas de plantas de diferentes cultivos de invernadero (Figura M.5), fueron utilizados en el proceso de vermicompostaje desarrollado en el capitulo 1 / chapter 1.

- **Residuos vegetales de plantas de tomate / Tomato-plant waste (P):** constituidos por matas de plantas de tomate cultivadas en invernadero (Figura M.6), fueron utilizados en los procesos de vermicompostaje desarollados en los capítulos 1, 4 y 5 / Chapter 1, 4 and 5.
- **Destrios de tomates / Damaged tomato-fruits or tomato-fruit wastes (T):** son frutos de tomates descartados o retirados del comercio (Figura M.7). Estos residuos fueron utilizados secos en el proceso de vermicompostaje desarollados en los capítulos 1 / chapter 1, y frescos en el vermicompostaje desarrollado en los capitulos 2 y 3 / Chapters 2 and 3.

Figura M.4. Residuos de matas de plantas de diferentes cultivos de invernadero

Figura M.5. Residuos de matas de plantas de cultivos de tomate de invernadero

Figura M.7. Destrios de frutos de tomates cultivados en invernaderos.

La tabla M.1 recoge algunas características de los residuos vegetales de invernadero utilizados en los procesos de vermicompostaje desarrollados en la Tesis Doctoral. Mayor información sobre las características de los residuos y las metodologías analíticas utilizadas se exponen en los correspondientes capítulos de esta memoria de Tesis Doctoral.

Tabla M.1. Características de los residuos vegetales de invernadero utilizados en los procesos de vermicompostaje desarrollados.

Capitulo	Residuo Vegetal de Invernadero	pH	EC (dS m^{-1})	COT (g kg^{-1})	N (g kg^{-1})	C/N
	Residuos vegetales heterogéneos de plantas					
1	Heterogeneous plant wastes, HP	8.5	17	151	22	7
	Residuos vegetales de plantas de tomate					
1	Tomato-plant waste, P (Dalías)	7.6	12	315	35	9
4 y 5	Tomato-plant waste, P (Carchuna)	7.2	10	386	17	22
	Destrios de tomates					
1	Damaged tomato-fruits, T (dried)	8.3	5.0	460	23	20
2 y 3	Tomato-fruit wastes (fresh)	3.9	1.5	459	23	20

M.3. RESIDUOS ORGÁNICOS CONTROL O UTILIZADOS COMO ACONDICIONANTES EN LOS PROCESOS DE VERMICOMPOSTAJE DESARROLLADOS

Con el fin de evaluar los procesos de vermicompostaje desarrollados se utilizaron otros residuos orgánicos, que fueron empleados como controles frente a los residuos vegetales de invernadero o bien como acondicionantes que son mezcla de éstos residuos a fin de optimizar y/o mejorar el proceso.

- **Paja de cereal** / Straw (S): suministrada por la Finca “La Parra”, Deifontes, Granada, fue utilizada como acondicionante de los residuos vegetales de invernadero en el proceso de vermicompostaje desarrollado en el capítulo 1 / Chapter 1.
- **Estiércol de vaca maduro / Cow dung (D) or cattle manure:** suministrado por una granja de vacas lecheras localizada en las cercanías de la ciudad de Granada, fue utilizado en los procesos de vermicompostaje desarrollados como material acondicionante de los residuos vegetales de invernadero (capítulo 1 / chapter 1) así como control del proceso (en los capítulos 1, 4 y 5 / chapters 1, 4 and 5), ya que se considera un sustrato óptimo para ser vermicompostado por *E. andrei y E. fetida*. **Estiércol de oveja** / Sheep manure**:** suministrado por el Grupo de Investigación de Pequeños Rumiantes (IFNA, EEZ-CSIC) fue empleado como sustrato para la inoculación de las lombrices en los procesos de vermicompostaje de alimentación continua desarrollados en los capítulos 2 y 3 / chapters 2 and 3.

- **Lodo de papelera / Paper-mill sludge (S)**: procedente de la planta de aguas residuales de la Empresa Torras-PAPEL (Motril, Granada), fue utilizado solo o como material acondicionante de los residuos de plantas de tomate cultivadas en invernadero en los procesos de vermicompostaje desarrollados en los capítulos 4 y 5 / chapters 4 and 5.

La tabla M.2 recoge algunas características de los residuos orgánicos utilizados como controles o como materiales acondicionantes en los procesos de vermicompostaje desarrollados. Mayor información sobre las características de esos residuos y las metodologías analíticas utilizadas se exponen en sus correspondientes capítulos de esta memoria de Tesis Doctoral.

Tabla M.2. Características de los residuos orgánicos control o utilizados como materiales acondicionantes en los procesos de vermicompostaje desarrollados.

Capitulo	Residuo orgánico	pH	EC (dS m^{-1})	COT (g kg^{-1})	N (g kg^{-1})	C/N
1	Straw, S	7.3	2.4	571	1.9	294
1	Cow dung, D	9.3	9.2	401	15	27
5	Cattle manure					
4	Cow dung, D	9	2.8	237	16	15
2 y 3	Sheep manure	8.6	1.8	138	9.6	14
4 y 5	Paper-mill sludge, S	8.2	0.6	175	7.3	24

M.4. VERMICOMPOSTS UTILIZADOS PARA EL ESTUDIO DE LAS COMUNIDADES MICROBIANAS

Los siguientes vermicomposts de diferente origen y naturaleza fueron utilizados en los estudios sobre la estructura, diversidad y funcionalidad bacteriana y fungica de esos materiales:

- **Vermicompost de destríos de tomate / Vermicompost from damaged tomato fruits (DT)**: obtenido a partir de destríos de tomate mediante procesos de vermicompostaje de alimentación continua desarrollados en los capítulos 2 y 3 / chapters 2 and 3 (Figura M.8), su microbiota fue estudiada en los, capitulos 6 y 7 / chapter 6 and7.
- **Vermicompost de residuos oleícolas y biosólidos / Vermicompost from olive-mill waste and biosolids (OB):** obtenido a partir de una mezcla de alperujo y biosólidos municipales en proporcion 8:1 (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 biosólido, colocándose a ambos lados un cordón de amortiguación consistente en vermicompost de estiércol (Figura M.9). En este cordón se introdujo una biomasa total de lombrices (*E. fetida*) equivalente al 10% del peso seco de la mezcla de residuos. Durante el periodo de vermicompostaje (6 meses) la humedad se mantuvo entre 80-85% con riegos periódicos por aspersión. Transcurrido ese periodo, las lombrices fueron separadas manualmente, y el vermicompost obtenido fue madurado durante 2 meses. Este vermicompost fue estudiado en los capítulos capitulos 6 y 7 / Chapters 6 and 7.

Figura M.8. Vermireactor utilizado para el proceso de vermicompostaje de destrios de tomate

Figura M.9. Litera utilizada para el proceso de vermicompostaje de residuos oleicolas y vitivinicolas

- **Vermicompost de residuos vitivinícolas / Vermicompost from winery wastes (WW):** obtenido a partir de una mezcla orujos vinícolas extractados mezclados con lias en proporción 1:1 (ps:ps) que fue vermicompostada en literas de madera siguiendo la misma metodología que en el vermicompostaje de los residuos oleícolas y biosólidos. El vermicompost de residuos vitivinícolas (WW) se investigó en los capitulos 6 y 7 / Chapters 6 and 7.
- **Vermicompost de estiércol de vaca / Vermicompost from cattle manure (CM):** vermicompost producido a escala industrial en la la Empresa Lombricor S.C.A (Algallarin, Córdoba) a partir de estiércol de vaca, el cual es sometido a un proceso de biodegradación utilizando lombrices de la especie *E- fetida*. El proceso se lleva a cabo en cordones de 20 cm de altura durante 4 meses. Posteriormente y previa separación de las lombrices es madurado durante 1 mes, secado y tamizado. Este vermicompost se estudió en los capitulos 6 y 7 / Chapters 6 and 7.

- **Vermicompost comercial / Commercial vermicompost (CV):** vermicompost producido por la Empresa Humus-Fertil (La Roda, Albacete) a partir de una mezcla de diversos estiércoles animales vermicompostada mediante *E. fetida*, se utilizó como material referencial para evaluar los grupos microbianos de un vermicompost mediante el análisis su contenido en PLFA (Phospholipid fatty acids) que se realizó en el capitulo 4 / chapter 4.

La tabla M.3 recoge algunas características de los vermicomposts utilizados en los estudios sobre comunidades microbianas. Mayor información sobre las características de esos vermicomposts y las metodologías analíticas utilizadas se exponen en sus correspondientes capítulos de esta memoria de Tesis Doctoral

Tabla M.3. Características de los vermicomposts utilizados en los estudios sobre comunidades microbianas.

Capitulo	Vermicompost from	pH	EC (dS m^{-1})	COT (g kg^{-1})	N (g kg^{-1})	C/N
4	Commercial vermicompost, CV	8.9	3.3	228	20	12
6 y 7	Damaged tomato fruits, DT	10.4	4.4	156	15.8	10
6 y 7	Olive-mill waste with biosolids, OB	7.4	1.3	342	17.8	19
6 y 7	Winery wastes, WW	8.3	1.4	422	22.5	19
6 y 7	Cattle manure, CM	7.5	1.4	98	10.1	10

M.5. VERMICOMPOSTS Y COMPOSTS ENSAYADOS COMO ENMIENDAS DE SUELOS.

Para los estudios de la aplicación de los vermicomposts tanto para mejorar la fertilidad de un suelo agrícola como para recuperar un suelo contaminado por metales pesados se utilizaron los siguientes:

- **Vermicompost de residuos vegetales de supermercado / Vermicompost from grocery-food wastes (V):** es un vermicompost de uso comercial que fue producido por la Empresa Oregon Soil Corporation (Portland, Oregon, USA) a partir de residuos vegetales (frutas y verduras) generados por supermercados, los cuales fueron vermicompostados mediante lombrices de la especie *E. fetida* (Figura M.10). El proceso de vermicompostaje se desarrolló empleando un reactor automatico de alimentación

continua (Figura M.10) sobre el que diariamente se depositaron capas finas de residuos vegetales mientras que el material vermicompostado (60 días después aproximadamente) es recogido en la parte inferior del reactor. Este vermicompost fue ensayado como enmienda orgánica de un suelo agrícola en el capitulo 8 / chapter 8.

- **Compost termofilico de residuos vegetales de supermercado / Thermophilic-compost from grocery-food wastes (C)**: se trata de un compost comercial producido por la Empresa Natures Needs® a partir de residuos vegetales (frutas y verduras) generados por supermercados que fueron compostados en pilas de 2 m de altura donde se alcanzó una fase termófila (55-70 ºC) de al menos 72 horas (Figura M.10). Finalizada la fase termófila, la pila se enfrió y el material orgánico se maduró durante un 1 mes. El vermicompost fue usado como enmienda orgánica en el capitulo 8 / chapter 8.

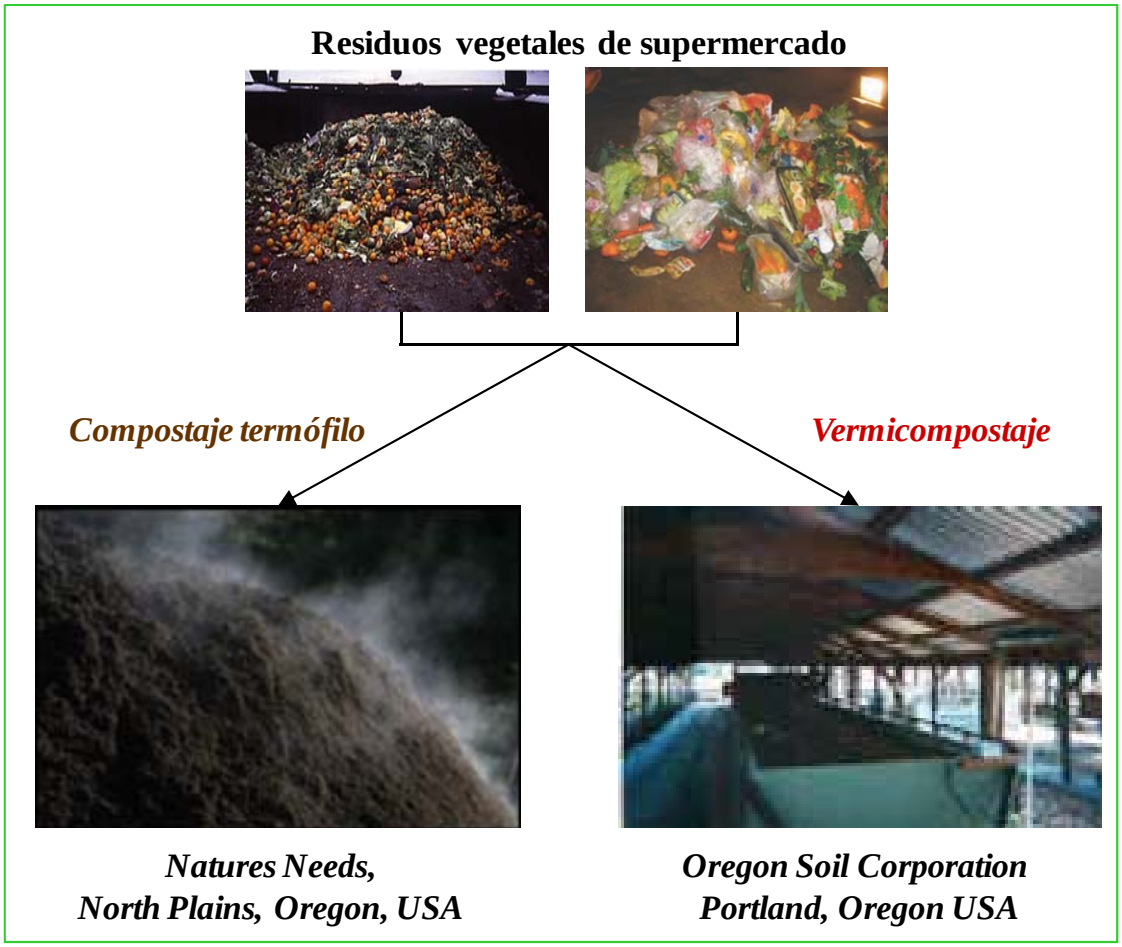


Figura M.10. Obtención de compost y vermicomposts de residuos vegetales de supermercado.

La tabla M.4 recoge algunas características del compost y vermicompost obtenidos de residuos vegetales de supermercados. Mayor información sobre las características de esos vermicomposts y las metodologías analíticas utilizadas se exponen en sus correspondiente capítulo de esta memoria de Tesis Doctoral.

Tabla M.4. Características del compost y vermicompost de residuos vegetales de invernadero utilizados en el estudio de la aplicación al suelo.

Capitulo	Vermicompost from	pH	EC (dS m^{-1})	COT (g kg^{-1})	NKT (g kg^{-1})	C/N
9	Vermicompost, V	7.3	1.5	155	11.4	14
9	Thermophilic-compost, C	6.9	2.2	123	9.9	12

- **Vermicompost de destríos de tomate / Vermicompost from damaged tomato fruits (DT):** sus características se exponen en el apartado M.4. y en la tabla M.3. Este vermicompost fue también usado en el capitulo 9 / chapter 9.
- **Vermicompost de estiércol de vaca / Commercial vermicompost (CV):** Su producción y algunas de sus características se exponen en el apartado M.4. y en la tabla M.3. Este vermicompost fue también usado en el capitulo 9 / chapter 9

M.6. PLAGUICIDA ENSAYADOS

- **Insecticida: Imidacloprid** (IMD) (Figura M.11) fue utilizado en el capitulo 5 / chapter 5 a fin de conocer su efecto sobre lombrices de la especie *E. fetida* durante un proceso de vermicompostaje así como en el estudio sobre la respuesta respiratoria microbiana a la presencia de plaguicidas en vermicomposts (Capitulo 7 / Chapter 7).

El imidacloprid es un insecticida nicotínico de acción sistémica por la raíz y con una notable acción por contacto y por ingestión (Zang y col., 2000). Es aplicado en suelo, sobre semilla y foliarmente en diversos cultivos (algodonero, arroz, cereales, patatas, hortalizas, frutas pomáceas, pecanas y prados) para el control de insectos chupadores, moscas blancas, termitas y escarabajos de patata de Colorado, con largo control residual. Además, es España ha sido utilizado desde 1992 en diferentes cultivos desarrollados en invernadero o bajo plástico, como los de tomates, pimientos, calabacín, etc. (Hernández et al., 1999). Las propiedades físico químicas de este insecticida se exponen en la tabla M.5.

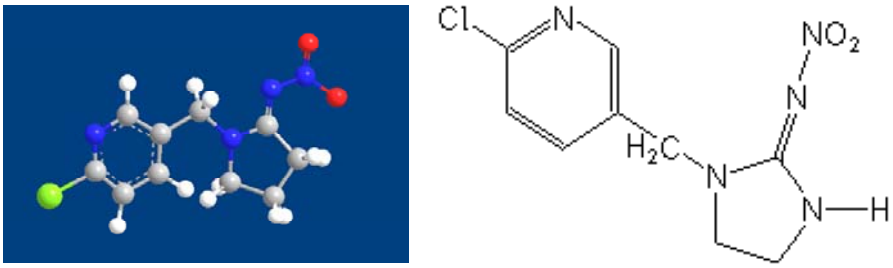


Figura M.11. Molécula del imidacloprid

Según la Organización Mundial de la Salud (OMS), el imidacloprid es moderadamente tóxico, Clase II (WHO, 2004). Su toxicidad es más evidente cuando es asimilado por ingestión que por vía cutánea o por inhalación. Su DL_{50} oral es de 450 mg kg^{-1} de peso corporal en ratas. No existen evidencias de que cause cáncer en animales de laboratorio a largo plazo ni humanos, por ello la EPA lo clasifica como de clase E. También produce daños ecológicos ya que es tóxico para especies beneficiosas como las abejas, depredadores del escarabajo de la patata y parasitoides de la mosca blanca.

Tabla M.5. Propiedades físico-químicas del imidacloprid (Tomlin, 2003).

Nombre químico (CAS)	1-[(6-chloro-3-pyridinyl)-methyl]-N-nitro-2-imidazolidinamine	**Punto de fusión**	144ºC
CAS RN	138261-41-3	**Solubilidad en Agua**	510 mg L^{-1} (20ºC)
Fórmula estequiométrica	$C_9H_{10}Cl\ N_5O_2$	**K_{oa}**	3.72 (21ºC)
Presión de vapor	$4{\cdot}10^{-10}$ Pa (20ºC)	**Masa molecular**	255.7 g mol^{-1}

- **Herbicida: Diuron (**Figura M.12) fue utilizado estudio sobre la respuesta respiratoria microbiana a la presencia de plaguicidas en vermicomposts (Capitulo 7 / Chapter 7)

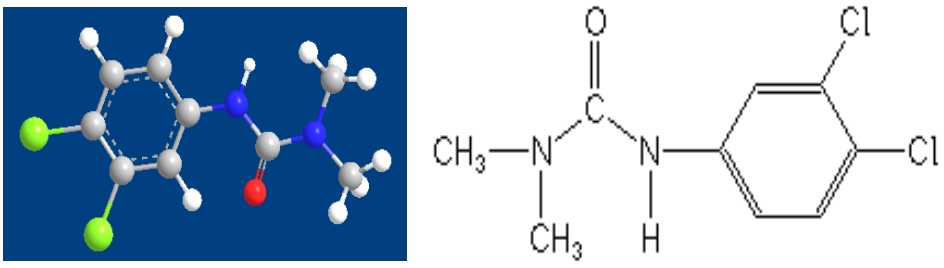


Figura M.12. Molécula de diuron

El diurón (Figura M.12) es un herbicida sistémico absorbido principalmente por la raíz que se transloca por el xilema inhibiendo la fotosíntesis. Bloquea la transferencia de electrones a nivel del fotosistema II. Se usa para el control total de musgos y malas hierbas

en zonas no cultivadas a dosis de 3 kg $ha^{-1}año^{-1}$. Además lleva a cabo un control selectivo de malas hierbas en múltiples cultivos como, espárrago, árboles frutales, viñedos, cultivos de olivos, algodón, caña de azúcar, cereales, soja, maíz (Tomlin, 2003), a dosis de 1,8 kg ha^{-1} $año^{-1}$. Las propiedades físico químicas de este herbicida se exponen en la tabla M.6.

Tabla M.6. Propiedades físico-químicas del diuron (Tomlin, 2003).

Nombre químico (CAS) N´-(3,4-diclorofenil) -N,N-dimetilurea		**Punto de fusión**	158-159 ºC
CAS RN	3330-54-1	**Solubilidad Agua**	42 mg L^{-1} (25ºC)
Fórmula estequiométrica	$C_9H_{10}Cl_2N_2O$	**K_{oa}**	700 ± 50 (25ºC)
Presión de vapor	$1{,}1 \cdot 10^{-6}$ Pa (20ºC)	**Masa molecular**	233.1 g mol^{-1}

La EPA lo clasifica toxicológicamente como clase III, sustancia tóxica y ligeramente peligrosa (Malato y col., 2002; Tomlin, 2003) y se incluyó como sustancia carcinogénica en 1997. Actualmente es considerado por la Unión Europea como sustancia peligrosa prioritaria dentro del marco de la prevención de contaminación de aguas. Por ello, su uso se suprimirá en Europa durante los próximos 20 años (Directiva 2000/60/CE). Este herbicida es ligeramente tóxico en aves e invertebrados acuáticos y moderadamente en peces, su DL_{50} oral en ratas es de 3400 mg kg^{-1} (Giacomazzi y Cochet, 2004). Por esta baja toxicidad la OMS lo clasifica toxicológicamente como clase U (WHO, 2004).

- **Fungicida: Metalaxil** (Figura M.13.) fué utilizado en el estudio sobre la respuesta respiratoria microbiana a la presencia de plaguicidas en vermicomposts (capitulo 7 / Chapter 7).

El metalaxil es una mezcla en proporción 1:1 de dos moléculas racemicas, D y L. El producto técnico esta formado por un 97% del enantiomero D (que es el más activo) y un 3 % del L. Estos dos compuestos son usados como fungicidas en agricultura, horticultura y en tratamientos forestales porque inhibe el desarrollo del micelio y la formación de esporas. El metalaxil actúa inhibiendo la síntesis de proteinas y de ergosterol específicamente en la incorporación de uridina por la ARN polimerasa I. Por eso es considerado como un fungicida de acción sistémica frente a diferentes hongos patógenos (Sukul y Spiteller, 2000) especialmente, contra Oomycetos, en particular los del orden Peronosporales tales como Phytophthora spp., Pseudoperonospora spp., Peronospora spp., Sclerospora spp., Bremia spp., Pythium spp., y otras especies patógenas de raíces, tallos y frutos (Schwinn et al., 1977;

Urech et al., 1977). Es ampliamente usado ya que es un compuesto estable, resistente en un amplio rango de pH, temperatura y luminosidad (Singh y Tripathi, 1982). Además, debido a su amplio espectro de actividad, se ha registrado a nivel mundial en varios países, incluyendo Estados Unidos, Europa, Australia e India, sobre múltiples cultivos de frutas y vegetales (Sukul y Spiteller, 2001). Las propiedades físico químicas de este fungicida se exponen en la tabla M.7.

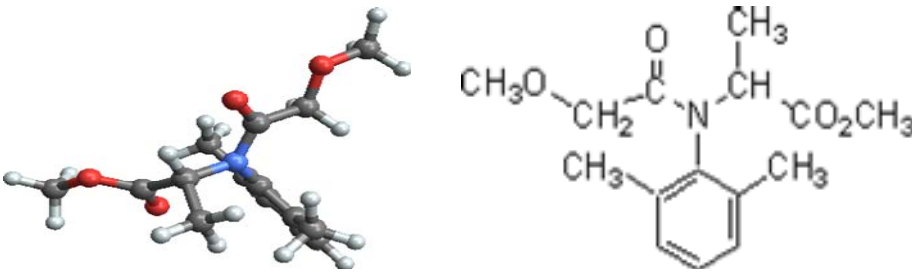


Figura M.13. Molécula de metalaxil

Tabla M.7. Propiedades físico-químicas del metalaxil (Tomlin, 2003).

Nombre químico (CAS) methyl N –(2,6-dimethilfenil)-N-(metoxyacetil)-DL-alanina		**Punto de fusión**	71,8-72,3 ºC
CAS RN	57837-19-1	**K_{oa}**	56,23 (25 ºC)
Fórmula estequiométrica	$C_{15}H_{21}NO_4$	**Presión de vapor**	0,75x 10^{-3}Pa
Masa molecular	279,3 g mol^{-1}	**Solubilidad en Agua**	8,4 g l^{-1}(22 ºC)

Según la OMS, el metalaxil es ligeramente peligroso (Clase III). La DL_{50} descrita para el enantiomero activo ó metalaxil-M es de 380 mg kg^{-1} de peso corporal en ratas y de 670 mg kg^{-1} para el compuesto racemico (WHO, 2004). Metalaxil en general presenta una baja toxicidad aguda para los humanos, pero es un irritante para los ojos. Se ha clasificado como un carcinógeno del Grupo E, es decir, una sustancia química que no muestra la evidencia de carcinogenicidad para los humanos (EPA, 1994).

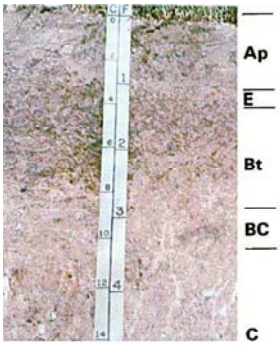
M.7 SUELOS

Para el estudio de los efectos de la aplicación al suelo de los vermicomposts producidos a partir de residuos vegetales se utilizaron dos suelos: uno agrícola y otro degradado por contaminación con metales pesados.

- **Suelo agrícola** / Soil (S):

Se utilizó la capa arable (0-10 cm) de un suelo agrícola "Crosby silt loam" localizado en la granja Waterman de la Universidad del Estado de Ohio (Columbus, Ohio, USA). El suelo clasificado como Luvisol estagnico (FAO, 2006) y como fine, mixed, active, mesic Aeric Epiaqualfs (Soil Survey Staff, 2010) se investigó en el capítulo 8 / chapter 8. Algunas carateristicas texturales y químicas del suelo agrícola se expone en la tabla M.8. Mayor información sobre las características del suelo y las metodologías analíticas utilizadas se exponen en su correspondiente capítulo de esta memoria de Tesis Doctoral.

Tabla M.8. Perfil y análisis químico y textural del suelo agrícola.



Arena	**Limo**	**Arcilla**	**pH**
g kg^{-1}	g kg^{-1}	g kg^{-1}	
290	490	220	7.6
CO_3Ca	**COT**	**NKT**	**CIC**
g kg^{-1}	g kg^{-1}	g kg^{-1}	$cmol_c$ kg^{-1}
22	8.8	1.3	14.7

- **Suelo contaminado por metales pesados / HM-contaminated soil (S):**

Figura M.14. Localización y perfil del suelo contaminado por metales pesados

En el capitulo 9 / chapter 9 se utilizó la capa superior (0-20 cm) de un suelo localizado en El Gorguel (La Unión Almería) que se encontraba contaminado por metales pesados, debido a la extracción minera de hierro, plomo y zinc (Figura M.14). El análisis químico y textural de la capa superior del suelo clasificado como Tecnosol espolico (FAO, 2006) se expone en

la tabla M.9. Mayor información sobre las características del suelo y las metodologías analíticas utilizadas se exponen en su el capítulo 9 de esta memoria de Tesis Doctoral.

Tabla M.9. Análisis químico y textural del suelo contaminado por metales pesados

Arena	**Limo**	**Arcilla**	**pH**	**CE**	**COT**	**NKT**	**CO_3Ca**
g kg^{-1}	g kg^{-1}	g kg^{-1}		dS m^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
49	218	731	7.7	8	4	0.22	4
CIC	**Fe**	**Mn**	**Cu**	**Zn**	**Cd**	**Ni**	**Pb**
$cmol_c$ kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
9.4	139	8300	165	46825	50	34	8331

CAPÍTULO 1
CHAPTER 1

VIABILIDAD DEL VERMICOMPOSTAJE PARA EL RECICLAJE DE RESIDUOS VEGETALES DE INVERNADERO

FEASIBILITY OF VERMICOMPOSTING FOR VEGETABLE GREENHOUSE WASTE RECYCLING

Fernández-Gómez, M.J., Romero, E., Nogales, R. (2010). Feasibility of vermicomposting for vegetable greenhouse waste recycling. Bioresource Technology 101, 9654-9660.

RESUMEN

El estudio se realizó con el objetivo de evaluar la viabilidad de *Eisenia andrei* para vermicompostar residuos vegetales heterogéneos de plantas (HP), de la planta de tomate (P) y de destríos de tomates (T) procedentes de invernaderos. El crecimiento de las lombrices y su reproducción se examinó a lo largo de un periodo de 12 semanas, y se evaluaron los cambios en parámetros químicos, actividad enzimática, test de fitotoxicidad y huella identificativa genética de las comunidades bacterianas. Mientras que los altos índices de salinidad en HP y P (>10 dS m^{-1}) impidieron la supervivencia de las lombrices, T fue vermicompostado registrándose un crecimiento de las lombrices y una producción de cápsulas aceptable. Este último residuo se estabilizó con éxito como indicó la significativa disminución de su contenido en COT (~13-26%) y de su relación C:N (~16-36%), y sus altos índices de germinación (~39-72%). La semajantes niveles de actividades enzimáticas y huellas identificativas de las comunidades bacterianas registradas en los diversos vermicomposts obtenidos a partir del residuo T indican que este tipo de residuo favoreció la existencia de comunidades bacterianas análogas que fueron responsables del alto grado de estabilización y madurez detectado.

Palabras clave: *Eisenia andrei*, actividad enzimática, test de fitotoxicidad, huella genética identificativa, PCR-DGGE.

ABSTRACT

This study was conducted in order to evaluate the feasibility of *Eisenia andrei* for vermicomposting heterogeneous plant (HP), tomato-plant (P), and damaged tomato-fruit (T) greenhouse vegetable wastes. Earthworm growth and reproduction were monitored over a 12-week period, and variations in chemical parameters, enzyme activity, phytotoxicity test, and genetic fingerprinting of bacterial communities were evaluated. While high rates of salinity prevented earthworm survival in HP and P (>10 dS m^{-1}), T was vermicomposted recording an adequate earthworm growth and cocoon production. The latter waste was successfully stabilized, as indicated by the significant decrease in its TOC content (~13-26%) and C:N ratio (~16-36%) and its high germination indices (~39-72%). The similar enzyme activities levels and bacterial community fingerprintings recorded in diverse vermicomposts obtained from T waste indicate that this type of waste favoured the existence of analogous bacterial communities responsible for the high degree of stabilization and maturity detected.

Keywords: *Eisenia andrei*, enzyme activity, phytotoxicity test, genetic fingerprinting, PCR-DGGE.

1.1. INTRODUCTION

The use of greenhouses is expanding worldwide, currently covering over 1.5 million hectares (Espi et al., 2006), as these crop systems improve food production and protect crops from adverse meteorological conditions and pests. The Mediterranean basin has the largest area occupied by greenhouses in Europe approximately 200,000 (Pardossi et al., 2004). Mediterranean greenhouse systems enable high-value vegetables to be produced from autumn through spring (Castilla and Hernández, 2005). However, these intensive agricultural systems generate large amounts of waste, creating an unsustainable environment that adversely affects ecological integrity and human health due to they cause agricultural pest, riverbeds pollution and uncontrolled burning, among other things (Parra et al., 2008). Vegetable waste, which can be classified into plant residues and rejected and/or damaged fruit-waste, accounts for most of these residues. Plant wastes consist of stems, twigs and roots, which are discarded after the harvesting. Fruit-waste consists of unmarketable fruit due to bruising, inappropriate size, rotting, or insect attack, as well as marketable fruits that are discarded in order to control market prices, accounting for around 25% of total greenhouse vegetable waste. Plant and fruit wastes are often placed in special containers and air-dried in order to reduce volumes.

The enormous quantities of biomass from greenhouse vegetable waste need to be recycled into nutrient-enriched organic products, which could be used for agricultural and land restoration purposes, as the low organic content (≤ 2%) of 75% of southern European topsoil (Zdruli et al., 2004) is a major problem that could be solved by adding organic amendments to the degraded soils. Low-cost and environmentally appropriate technologies, such as composting or vermicomposting, should therefore be favoured over other disposal methods. Nevertheless, due to the agricultural management procedures used in these intensive agricultural systems based on chemical fertilizers and high-yield crops, greenhouse vegetable wastes are characterized by specific features that hinder the use of these biological technologies. Unlike crop waste from conventional farming, greenhouse vegetable wastes are hardly ever bioconverted due to its low C/N, high salinity and ammonia content and may also contain large amounts of pollutants such as pesticides. Compared with conventional crop wastes, very little study has been carried out on the biostabilization of greenhouse vegetable waste.

Vermicomposting biotechnologies involve the bio-oxidation and stabilization of organic matter through the joint action of earthworms and microorganisms under aerobic and

mesophilic conditions. Vermicomposting has greater mass-reduction capacity than composting over a shorter processing time and generates products with higher humus content and significantly lower phytotoxicity (Lorimor et al., 2001). Vermicompost is also more marketable than compost due to its more attractive appearance and higher nutrient content and microbial activity (Nogales et al., 2008). It is well known that vermicomposting is effective for managing of crop wastes from conventional farming systems (Bansal and Kapoor, 2000; Suthar, 2008) through various epigeic earthworms such as *Eisenia fetida, Eisenia andrei*, *Perionyx excavatus*, and *Eudrilus eugeniae*. However, an extensive survey of the literature has led us to conclude that no study of greenhouse vegetable waste vermicomposting has been carried out. The *Eisenia fetida* and *Eisenia andrei* earthworm species, due to their high tolerance in relation to environmental variables such as pH, moisture, and temperature (Nogales et al., 2008), could represent the best option in developing a vermicomposting system for recycling organic waste in temperate climates.

The study of enzyme activity has proved helpful for assessing the stability of vermicomposted end products (Benítez et al., 1999). A decrease in dehydrogenase and overall hydrolytic activity has thus been associated with the stabilization of organic matter during the vermicomposting process. Molecular tools based on PCR amplification of the 16S rRNA gene, such as clone libraries, fluorescence in situ hybridation (FISH), restriction fragment length polymorphism (T-RFLP), quantitative PCR and microarrays, have been widely used to study bacterial communities investigating different aspects i.e. identification and/or in-situ detection of certain species, species richness, bacterial population size and genetic community structure. Among all DNA-based methods, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA is a useful tool for characterizing the genetic structure of bacterial communities in composts, as this technique can be used without subsequent sequencing and provides the genetic fingerprinting characteristics of each bacterial community. Recently, Sen and Chandra (2009) have reported that this fingerprinting method is essential for improving composting and vermicomposting technology. They showed that DGGE is capable of evaluating the relationship between changes in physico-chemical and microbial community structures occurring during the vermicomposting process due to the presence of earthworms.

In view of the above, the principal objective of this study was to assess the feasibility of using *E. andrei* to vermicompost the enormous quantities of heterogeneous-plant, tomato-plant, and damaged tomato-fruit vegetable waste produced by Mediterranean greenhouse crops. Chemical parameters, enzyme activity, and germination indices were determined in

order to evaluate chemical and biochemical changes caused by vermicomposting. DGGE of PCR-amplified 16S rDNA was carried out to genetically fingerprint bacterial communities in greenhouse vegetable waste and the resulting vermicompost in order to show the impact of earthworms on the bacterial genetic structure of mature vermicompost.

1.2. MATERIALS AND METHODS

1.2.1. Earthworms and organic-waste collection

Non-clitellated earthworms (*E. andrei*) were selected from a culture bank at the Estación Experimental del Zaidín (CSIC), Granada, Spain.

Heterogeneous plant (HP), tomato-plant (P), and damaged tomato-fruit (T) wastes, three of the most abundant greenhouse vegetable residues in Andalusia (Southern Spain), where the study was carried out, were tested. HP waste is made up of a mixture of stems, twigs, and roots from different types of greenhouse crops; P waste, consisting of stems, twigs, and roots, comes from greenhouse tomato crops; T waste consists of unsold damaged tomatoes which are unloaded into ponds after harvesting. All these waste were colleted from an organic waste-treatment plant located in El Egido, Almería (Spain) where they were being accumulated and air dried. The chemical characteristics of these greenhouse wastes, analyzed as described below, are shown in Table 1.1.

Table 1.1. Chemical composition of the organic waste used.

Organic wastes [a]	TOC (g kg $^{-1}$)	TKN (g kg $^{-1}$)	C:N ratio	pH	EC (dS m $^{-1}$)
HP	151 ± 2.4	22 ± 0.9	7 ± 0.2	8.5 ± 0.02	17.0 ± 0.10
P	315 ± 4.1	35 ± 0.8	9 ± 0.1	7.6 ± 0.02	12.0 ± 0.01
T	460 ± 1.5	23 ± 1.2	20 ± 0.9	8.3 ± 0.02	5.0 ± 0.03
S	571 ± 3.2	1.9 ± 0.1	294 ± 9.0	7.3 ± 0.01	2.4 ± 0.01
D	401 ± 2.0	15 ± 0.5	27 ± 1.0	9.3 ± 0.02	9.2 ± 0.02

TOC: total organic carbon; TKN: total Kjeldahl nitrogen; EC: electrical conductivity.
[a] HP: heterogenic plant wastes; P: tomato-plant waste; T: tomato-fruit waste; D: cow dung; S: straw.

1.2.2. Experimental design

The wastes were over-dried at 25ºC and chopped. For the purposes of optimum vermicomposting, they were mixed with cow dung (D) or straw (S) to boost their low C:N ratios to >20. The greenhouse wastes selected were therefore assayed on their own (HP, P, T) and also mixed with either cow dung (HP/D, P/D, T/D) or straw (HP/S, P/S, T/S) in 2:1 and 4:1 ratios, meaning that a total of 15 waste mixtures were tested for vermicomposting. Cow dung, regarded as an excellent material for *E. andrei* development (Nogales et al., 2008), was assayed on its own as control.

Fifty grams (dw) samples of each material were placed in triplicate in 500 ml glass pots and aerated for a week to eliminate substances that are toxic to the earthworms, as reported by Elvira et al. (1996). Moisture content was adjusted to 80–85% and five non-clitellated earthworms weighing between 0.17-0.31 g were inoculated. Samples were kept in darkness at 24 ºC for 12 weeks maintaining the constant moisture conditions by periodical watering.

Mortality rates, earthworm biomass, and cocoon population were evaluated weekly by hand. At the end of the process, the earthworms were removed. Samples from both the initial materials and resulting vermicompost were homogenized and divided into two subsamples, one of which was dried and finely ground for chemical analysis and germination testing, while the other was stored at -20°C for enzyme activity and DNA analysis.

1.2.3. Chemical analysis

The pH and electrical conductivity (EC) were measured in a 1:10 sample:water (w/v) ratio. Total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) were determined using the dichromate oxidation and Kjeldahl methods, respectively (M.A.P.A., 1986). Water-soluble carbon (WSC) was extracted at 60 ºC for 1 h with distilled water (1:10 sample:water w/v) and determined using the dichromate oxidation method. Total P was measured using the nitrovanadomolybdate method, total K and Na using photometry, and total Ca, Mg and micronutrients (Fe, Mn, Cu, and Zn) using atomic-absorption spectrometry after a digestion of the samples with HNO_3:$HClO_4$ (Williams, 1984).

1.2.4. Enzyme activity analysis

Total enzyme activities were determined in triplicate, with each reaction tube containing 0.2 g sample. Dehydrogenase activity was determined according to the von Mersi and Schinner (1991) method modified by García et al. (1997). The urease, acid-phosphatase, and β-glucosidase hydrolytic enzymes were determinated according to the methods reported by Nannipieri et al. (1980) and Tabatabai (1982).

1.2.5. Germination index

To assess the maturity and phytotoxicity levels of the initial materials and final vermicomposts, th germination index (GI) was calculated according to the technique describe by Zucconi et al. (1981), which involve incubating cress seeds (*Lepidium sativum*) with 1:10 sample:water (w/v) extracts in darkness at 25ºC for 24 h.

1.2.6. DNA extraction and bacterial community structure analysis

Total DNA was extracted from 250 mg of the sample using the MoBio UltraClean Soil DNA Isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA), following the manufacturer's protocol, although the second step (inhibitor removal solution) was repeated.

The V3 hypervariable region of 16S rRNA gene was amplified using the 338F (Lane, 1991) and 518R-GC clamp (Muyzer et al., 1993) primers. PCR was performed in a personal Mastercycler® (Eppendorf AG, Hamburg, Germany) using 2 µl DNA with a total volume of 25 µl containing 0.2 µM primers, 1 X reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20], 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} BSA, and 0.025 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany). Non-template controls were included in parallel. PCR began with denaturing at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 ºC for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were verified by electrophoresis on 1.5% agarose gels, and their concentrations were measured.

DGGE was carried out by loading 100 ng of PCR products into 8% (w:v) polyacrylamide gels containing a gradient of denaturants ranging from 40% to 65% (100% denaturant consisting of 40% [v/v] formamide and 7 M urea) (Muyzer et al., 1993). Gels were run in an INGENYphorU System (Ingeny International BV, The Netherlands) at 60°C

for 16 h at 100 V. The gels were stained with silver nitrate using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Germany) and photographed for subsequent image analysis, as describe below.

1.2.7. Data analysis

One-way ANOVA was used to analyze the significant difference between treatments with mean separation based on Tukey's test. A paired-sample *t*-test was also performed to assess the difference between parameters measured in the initial material and the final vermicompost for each treatment. Statistical analyses were conducted at >95% confidence level ($P < 0.05$) using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

A comparison of DGGE banding patterns was made with the GelCompar II software (Applied Maths, Kortrijk, Belgium). After conversion of the scanned gels, the bands were normalized and a reference position defined to align the patterns for accurate comparison after associating the bands with the standard pattern. Pattern similarity values were calculated by comparing the densitometric curves using the Pearson correlation similarity coefficient. The Dendrograms were calculated through the use of the unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm. A position tolerance of 1% was set for band matching.

1.3. RESULTS AND DISCUSSION

1.3.1. Chemical parameters

All earthworms died in the initial materials containing heterogeneous-plant or tomato-plant wastes after 24 h. This can be attributed to the high EC of these wastes (Table 1.1), as the earthworms were able to develop in substrates containing mixtures of tomato-fruit waste (T; T/S 2:1; T/S 4:1; T/D 2:1 and T/D 4:1) and cow dung (D), which had lower salinity levels. Gunadi and Edwards (2003) reported that *E. fetida* died in vegetable wastes (i.e. lettuce, pea, celery, etc.) due to their high EC (12.8±1.7 dS m^{-1}). They also reported that these worms survived for only three weeks in a vermibed composed of supermarket fruit wastes (i.e. cucumber, pear, apple, etc.).

In the viable substrates, the vermicomposting significantly altered the chemical properties of the initial materials (Table 1.2). The pH levels significantly increased after the

Table 1.2. Chemical analyses of initial materials (I) and final vermicomposts (F).

	pH			EC (dS m^{-1})			TOC (g kg^{-1})			WSC (g kg^{-1})			C:N ratio		
Treatments[a]	I	F	t-Test	I	F	t-Test	I	F	t-Test	I	F	t-Test	I	F	t-Test
D	9.3a	9.4	0.68	9.2a	8.7a	0.20	401a	319a	0.00*	29.1a	31.8a	0.11	26.9c	22.7a	0.02*
T	8.3bc	9.7	0.00*	5.0d	5.3c	0.22	460c	338ab	0.01*	82.3e	20.3de	0.00*	20.2a	16.6b	0.03*
T/S (2:1)	7.9c	9.4	0.01*	5.0d	5.9bc	0.00*	458c	357b	0.01*	72.8d	28.2b	0.00*	24.4b	15.6b	0.03*
T/S (4:1)	8.4bc	9.2	0.04*	5.4cd	5.5c	0.26	433bc	340ab	0.01*	68.2c	22.3cd	0.00*	24.1ab	15.6b	0.01*
T/D (2:1)	8.8ab	9.7	0.03*	5.6c	6.7b	0.03*	384a	335ab	0.03*	41.6b	23.2c	0.00*	21.4ab	16.5b	0.01*
T/D (4:1)	8.3bc	9.6	0.01*	6.2b	5.5c	0.02*	430b	337ab	0.02*	66.4c	18.9e	0.00*	21.4ab	17.4b	0.01*

TOC: total organic carbon; WSC: water soluble carbon; EC: electrical conductivity. Means in the same column followed by same letters are not significantly different from each other. t-test: *P* values of paired-sample t-Test, *: significant difference between the initial material and final vermicomposts.

[a]Refer to text, for explanation of treatment abbreviations.

Table 1.3. Total macronutrients and sodium content (g kg^{-1}) of initial materials (I) and final vermicomposts (F).

	TKN			P			K			Ca			Mg			Na		
Treatment$^{\delta}$	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test
D	15.0a	14.1a	0.30	5.1bcd	6.9a	0.06	28.4a	34.1a	0.01*	15.8a	20.6b	0.02	8.5a	10.8a	0.04*	2304a	2829a	0.03*
T	22.8b	20.4bc	0.11	4.9bc	9.7c	0.00*	23.6b	28.8b	0.02*	7.7c	16.7cd	0.00*	3.3c	6.1c	0.00*	798c	1522cd	0.00*
T/S (2:1)	18.9a	23.0c	0.17	3.9a	8.1ab	0.01*	21.8b	30.3b	0.00*	6.5c	14.7de	0.01*	2.4c	5.1d	0.01*	556d	1371d	0.00*
T/S (4:1)	18.0a	21.8bc	0.02*	4.4ab	7.9ab	0.00*	21.7b	28.6b	0.00*	7.0c	14.2e	0.00*	2.8c	5.3cd	0.00*	663cd	1198d	0.00*
T/D (2:1)	18.0a	20.3bc	0.06	5.8d	8.4b	0.00*	24.7b	33.5a	0.01*	11.6b	18.6bc	0.00*	5.4b	8.5b	0.00*	1363b	1864c	0.04*
T/D (4:1)	20.1ab	19.4b	0.61	5.6cd	8.2b	0.01*	24.2b	34.8a	0.01*	11.6b	23.1a	0.01*	5.2b	10.6a	0.00*	1164b	2344b	0.00*

TKN: total Kjeldahl nitrogen. Means in the same column followed by same letters are not significantly different from each other (P <0.05). t-test: P values of paired-sample t-Test, * significant difference between the initial material and final vermicompost.

$^{\delta}$Refer to text, for explanation of treatment abbreviations.

vermicomposting process, except in the case of the control treatment (D). These higher pH values could be explained by the disappearance of organic acids in tomato-fruit waste and/or to the mineralization of proteins generating ammonium. In all treatments, TOC content diminished by between 13% and 26%, with treatment T recording the largest TOC reduction. Although earlier studies have shown that earthworms mineralize cattle dung more easily than other organic wastes (Suthar, 2009), the tomato-fruit waste may give rise to a specific microbial community responsible for the high C mineralization rate. This hypothesis was partly confirmed by the significant reduction in WSC values in all vermicomposting treatments except in the case of cow dung (D). In treatment D, this organic fraction, which represents the most easily metabolisable organic matter, could be degraded prior to the addition of earthworms during the previous cow dung maturation stage.

The C:N ratio decreased by between 16% and 23% for treatments T, T/D 2:1, T/D 4:1, and D, and by over 35% for T/S 2:1 and T/S 4:1. This ratio has been widely used as an index of maturity and stability, since a decline from the initial C:N value to a final value <20 usually indicates an high degree of organic matter stabilization (Senesi, 1989), as evidenced by the C:N ratio of under 20 required by Spanish legislation for fertilizer product (Government of Spain, 2005). All the vermicomposts produced met this requirement except in the case of the vermicompost from cow dung. This could be due to the lower initial TKN content in this cow dung (Table 1.3) compared with the other substrates.

Regarding the changes in macronutrients caused by vermicomposting (Table 1.3), TKN concentration significantly changed only in treatment T/S 4:1. Earlier studies have reported that vermicomposting may enrich the N-content of vermicompost due to N additions by earthworms in the form of mucus, enzymes or nitrogenous excretory substances (Tripathi and Bhardwaj, 2004) and as a consequence of nitrogen transformation mediated by the vermicompost's microbiota through organic matter mineralization and microbial nitrogen-fixing (Bhattacharya and Chattopadhyay, 2004). On the other hand, reductions in N concentrations have been reported in vermicomposting due to in-vivo denitrification within the worm's digestive tract (Hobson et al., 2005). Part of the N-content in the initial substrate is also transformed into earthworm protein. In this study, N-concentration in the end product could be explained by the N-content in the initial substrate, earthworm growth, and decomposition efficiency during vermicomposting.

Total concentrations of K, Ca, Mg, Na (Table 1.3), and micronutrients (Table 1.4) significantly increased in all substrates after the vermicomposting processes. Higher percentage increases were recorded in vermicompost containing tomato-fruit waste

Table 1.4. Total micronutrients content (mg kg^{-1}) of initial materials (I) and final vermicomposts (F).

	Fe			Mn			Cu			Zn		
Treatments[a]	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test
D	1861a	3050a	0.00*	82a	126a	0.01*	19a	29a	0.01*	59a	80a	0.02*
T	1887a	6183d	0.00*	45c	182c	0.00*	24b	68c	0.00*	48ab	136d	0.00*
T/S (2:1)	1432b	4683b	0.00*	40c	150b	0.00*	20a	58b	0.00*	50ab	111b	0.00*
T/S (4:1)	1572b	5590d	0.00*	42c	150b	0.00*	23ab	61b	0.00*	46b	126c	0.00*
T/D (2:1)	1922a	5199c	0.00*	69b	155b	0.00*	26b	57b	0.00*	59a	114b	0.00*
T/D (4:1)	1990a	4989bc	0.00*	64b	172c	0.00*	26b	79d	0.00*	59a	110b	0.01*

Means in the same column followed by same letters are not significantly different from each other. t-test: *P* values of paired-sample t-test, * significant difference between the initial material and final vermicompost.
[δ]Refer to text, for explanation of treatment abbreviations.

Table 1.5. Growth and reproduction parameters of *E. andrei* in the feasible tomato-fruit material and control cow dung.

	Treatments [a]					
	D	T	T/S (2:1)	T/S (4:1)	T/D (2:1)	T/D (4:1)
Mean initial biomass [A] (mg worm^{-1})	224	248	200	228	220	250
Mean final biomass (mg worm^{-1})	532 a	412 abc	368 c	414 c	498 ab	406 bc
Week of maximum individual biomass	4	4	4	4	3	3
Maximum individual biomass [B](mg worm^{-1})	934 a	726 b	790 ab	838 ab	782 ab	796 ab
Maximum growth rate (mg worm^{-1} week^{-1})	178 a	120 b	148 ab	153 ab	187 a	182 a
Net biomass gained [B]–[A] (mg worm^{-1})	710 a	478 b	590 ab	610 a	562 ab	546 ab
Starting cocoon production (week)	4	3	3	3	3	3
Total cocoon production (number)	59 d	222 ab	229 ab	265 a	138 c	168 bc

Means in the same row followed by same letters are not significantly different from each other.
[a] Refer to text, for explanation of treatment abbreviations

compare with the vermicompost from cow dung. This could be explained by cow dung's lower mineralization rates, as indicated by the higher WSC content and C:N ratio recorded for this vermicompost as compared to other types (Table 1.2). The nutrient concentration increased in the vermicomposts obtained, as the earthworms reduced waste mass by enhancing organic matter mineralization. This increase in total nutrients concentration caused by *E. andrei* was previously described by Elvira et al. (1996) and recently corroborated by other authors (Garg et al., 2006; Suthar, 2007). By contrast, Garg and Kaushik (2005) reported a decrease in total potassium, total calcium, and heavy metal concentrations (Fe, Zn, Pb and Cd) after vermicomposting and concluded that this could be due to leaching of these cations caused by excess water drainage. This factor can be ruled out in our study, as the substrates were placed in pots with sealed bottoms in order to prevent leaching. This vermicomposting system avoided nutrient loss and the vermicompost obtained was rich in plant nutrients.

1.3.2. Earthworm growth and reproduction

Despite differences in the chemical characteristics of each viable mixture used for developing *E. andrei,* earthworm growth showed similar patterns, although some significant variations were observed (Figure 1.1, Table 1.5). Maximum individual biomass was recorded after 4 weeks of vermicomposting in treatments T, T/S 2:1, T/S 4:1, and D, while T/D 2:1 and 4:1, peaked a week earlier. As expected, the highest values for individual biomass and net biomass were recorded by control treatment D, whereas T showed the lowest values. On the other hand, cocoon production (Table 1.5) started one week earlier in the substrate containing tomato-fruit waste than in control treatment D. Total cocoon production in treatment D was more than half that for treatments containing tomato-fruit waste. The highest level of cocoon production was recorded in treatment T/S 4:1. This difference in cocoon production rates is related to the quality of the waste material used (Nogales et al., 2008). Tomato-fruit waste, which can be improved through small additions of straw, would therefore be an appropriate greenhouse waste for effective earthworm development.

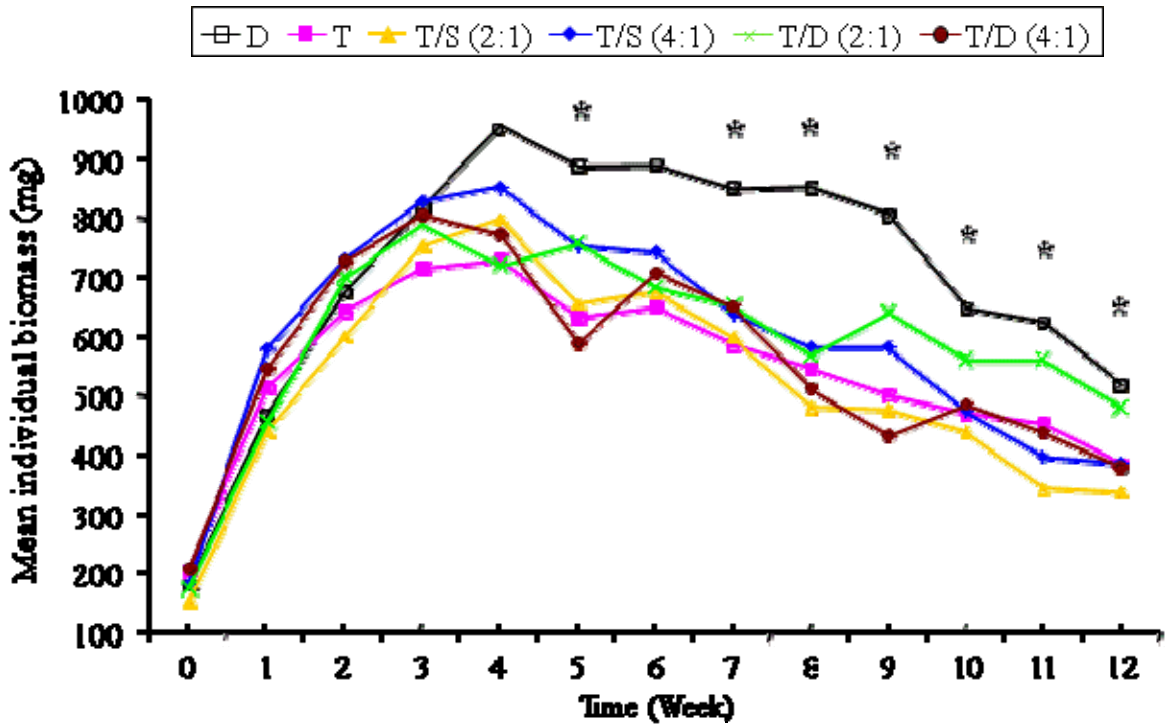


Figure 1.1. Earthworm growth during the vermicomposting process of greenhouse vegetable wastes. The asterisk (*) denotes a significant difference between treatments at a given time.

1.3.3. Enzyme activity

Dehydrogenases, which are intracellular enzymes involved in oxidative phosphorylation, can be used to assess overall microbial activity (García et al., 1997). Dehydrogenase activity values recorded in final tomato-fruit waste vermicomposts were significantly lower than those for the control treatment D (Figure 1.2a). This could be related to the higher WSC content in the vermicompost from cow dung (D) as compared to other vermicomposts (1.2). The lower dehydrogenase activity recorded in vermicomposts from tomato-fruit waste could mean that the organic matter present in this material was metabolised faster than the organic matter from cow dung. Benítez et al. (1999) have demonstrated a close correlation between WSC and dehydrogenase activity in vermicomposting, showing that the dehydrogenase activity provides a clear indication of the dynamics of organic-matter degradation which is useful in characterizing the status of vermicomposts.

As with dehydrogenase activity, hydrolytic enzyme levels analyzed in this study were lower in end products than in the initial materials (Figure 1.2b). β-glucosidases, which catalyze the hydrolysis of cellobiose and other disaccharides, play a major role in the decomposition of organic C compounds. The β-glucosidase activity in tomato-fruit waste (T)

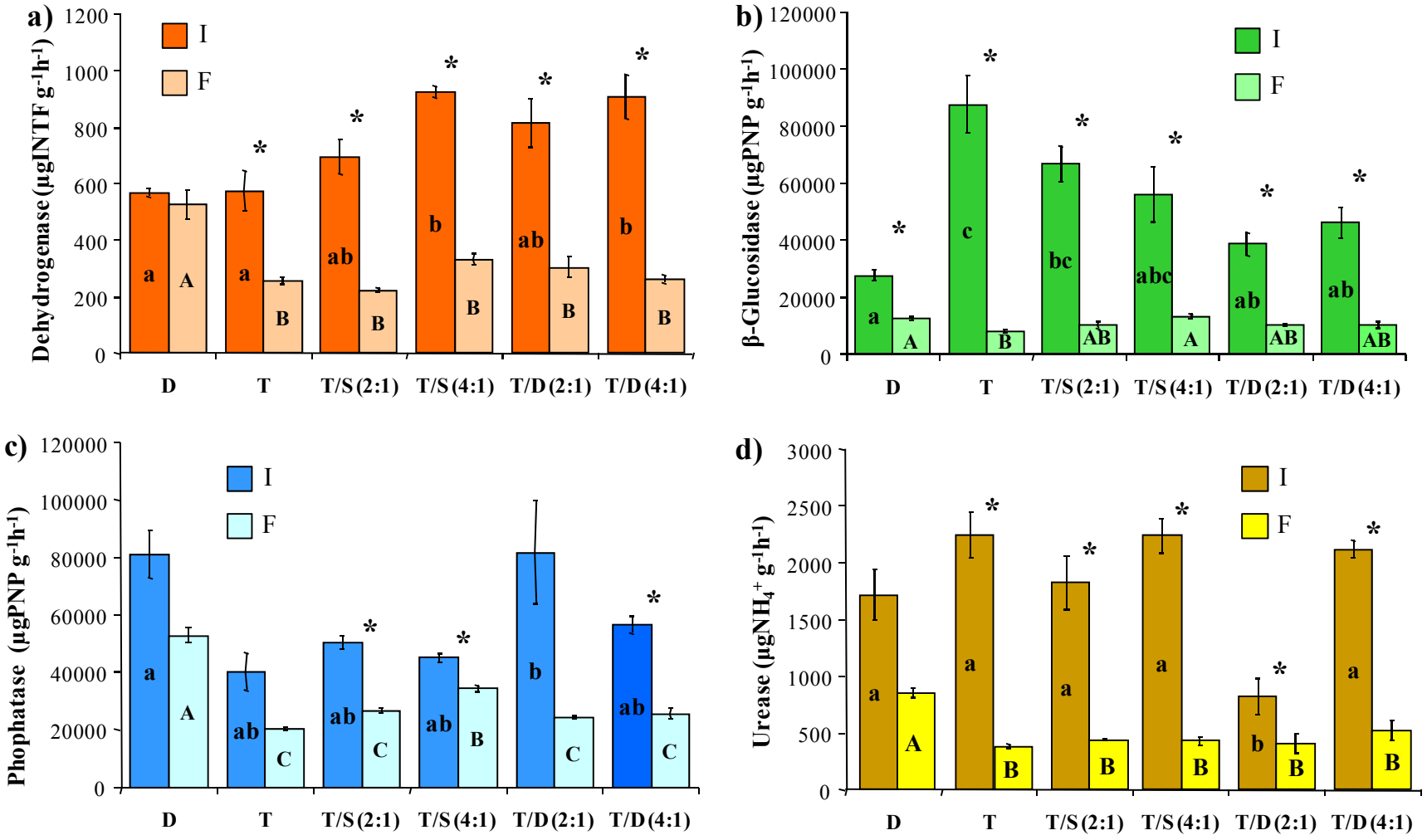


Figure 1.2. Enzyme activities in initial materials (I) and final vermicomposts (F). Error bars represent the mean standard error. Lowercase or capital letters indicate significant differences among initial materials or final vermicomposts, respectively. The asterisk (*) indicates a significant difference between the initial material and final vermicompost from each treatment.

recorded the highest level of initial activity, possibly due to the fact that it also recorded the highest WSC content (T) was the highest, and it could be related to its highest WSC content (Table 1.2). Significant decreases showed by this enzyme activity after vermicomposting indicated that this process was capable of degrading the large labile organic C fraction in greenhouse waste T. Acid-phosphatases, which catalyse the hydrolysis of organic phosphomonoester to an inorganic phosphate form, behaved in a similar way to β-glucosidases, although phosphatase activity fell significantly only in treatments T/D 4:1, T/S 2:1, and T/S 4:1 (Figure 1.2c). This could be due to residual amounts of organic-phosphate compounds, which may act as enzyme-synthesis-inducing substrates. Urease activity decreased significantly after vermicomposting in all treatments except in the case of the control treatment D (Figure 1.2d). This was possibly due to the higher level of recalcitrant ureic compound in cow dung. Both phosphatase and urease activity recorded in the end product from cow dung was significantly higher than in the other vermicomposts. According to Pramanik et al. (2007), these activities detected in vermicompost vary depending on the type of organic wastes used. These researchers found higher levels of phosphatase and urease activity in vermicompost from cow dung than from other organic wastes.

1.3.4. Germination index

Germination index (GI) values were close to zero in the initial materials containing tomato-fruit wastes (Figure 1.3). After vermicomposting, GI values significantly increased by over 50% in all treatments except in the case of the treatments D and T/D 2:1. According to Zucconi et al., (1981), composts which have a GI value higher than 50% are considered no-phytotoxic and stable for agricultural application. The lower GI values could be explained by the higher EC values recorded in treatments D and T/D 2:1. According to Iannotti et al. (1994), cress seed germination was greatly inhibited mainly due to high salinity levels in compost. Benítez et al. (2002) also reported low GI percentages in vermicompost from cattle manure as a consequence of its high EC level (6.6 dS m^{-1}). These findings indicate that vermicomposting is an effective system for eliminating the phytotoxic effects of unprocessed tomato-fruit waste from greenhouse crops.

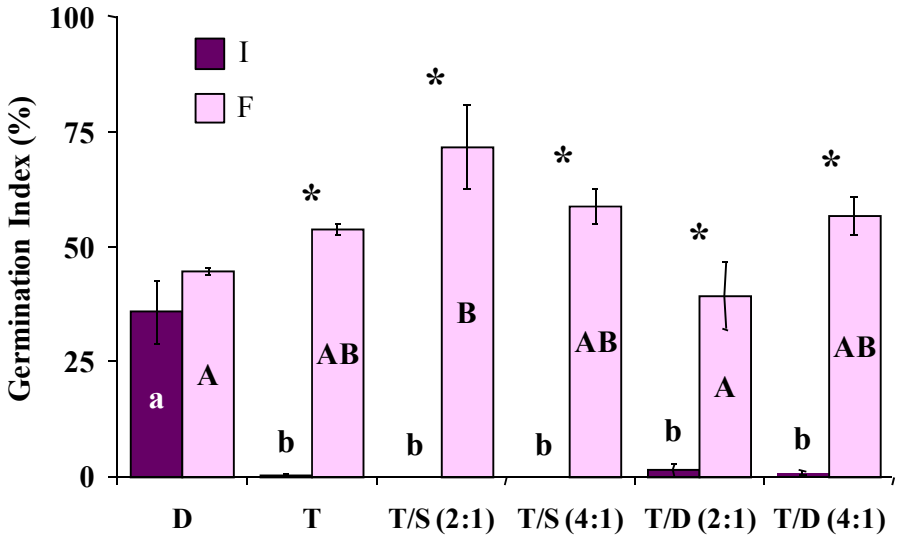


Figure 1.3. Germination indices in initial materials (I) and final vermicomposts (F). Error bars represent the mean standard error. Lowercase or capital letters indicate significant differences among the initial materials or final vermicomposts, respectively. The asterisk (*) indicates a significant difference between initial material and final vermicompost from each treatment.

1.3.5. Bacterial community

The bacterial community was genetically fingerprinted in each initial substrate and resulting vermicompost. An initial analysis of DGGE banding patterns from replicate samplings revealed a similarity coefficient >95% (data not shown). To compare all the samples on the one gel, a single sample was therefore run on the final gel. UPGMA analysis of this DGGE showed that all vermicomposts clustered into a clear group, whereas the initial materials showed no grouping (Figure 1.4). Within this cluster, the vermicomposts from cow dung (D_F) and from tomato-fruit waste (T_F) showed the lowest similarity value (Table 1.6). On the other hand, the bacterial community fingerprinting showed by the different vermicomposts containing tomato-fruit waste were highly similar, with average similarity coefficient >71%. It is clear from our findings that *E. andrei* greatly transformed the different bacterial communities of each substrate producing vermicomposts characterized by analogous bacterial communities. The impact of earthworms on bacterial community have recently been described by Sen and Chandra (2009), who reported that analysis of DGGE fingerprinting showed divergent bacterial community development in compost and vermicompost obtained from the same initial material. They also reported that the dissimilar

genetic fingerprintings observed in compost and vermicompost were due to differences in the functional responses of microbial communities in terms of enzyme activity and community-level physiological profiles. In line with this finding, the lower similarity value observed in the fingerprinting of vermicompost from cow dung could explain the chemical and biochemical differences previously reported between this vermicompost and those containing tomato-fruit waste. The high similarity values recorded on the basis of fingerprintings from vermicompost containing tomato-fruit waste may also indicate that this type of waste favoured the existence of specialized bacterial communities, thus explaining its higher rates of organic matter mineralization and stabilization. The DGGE technique is therefore useful for assessing the degree of maturity of end products. It also generates a characteristic fingerprinting that show the major changes in bacterial structure caused by the vermicomposting of tomato-fruit waste using *E. andrei*. This is consistent with previous research based on other fingerprinting methods. Using FAME analysis, Lores et al. (2006) reported that the specific fingerprintings of microbial communities in vermicompost depend on the type of substrate and earthworm species used.

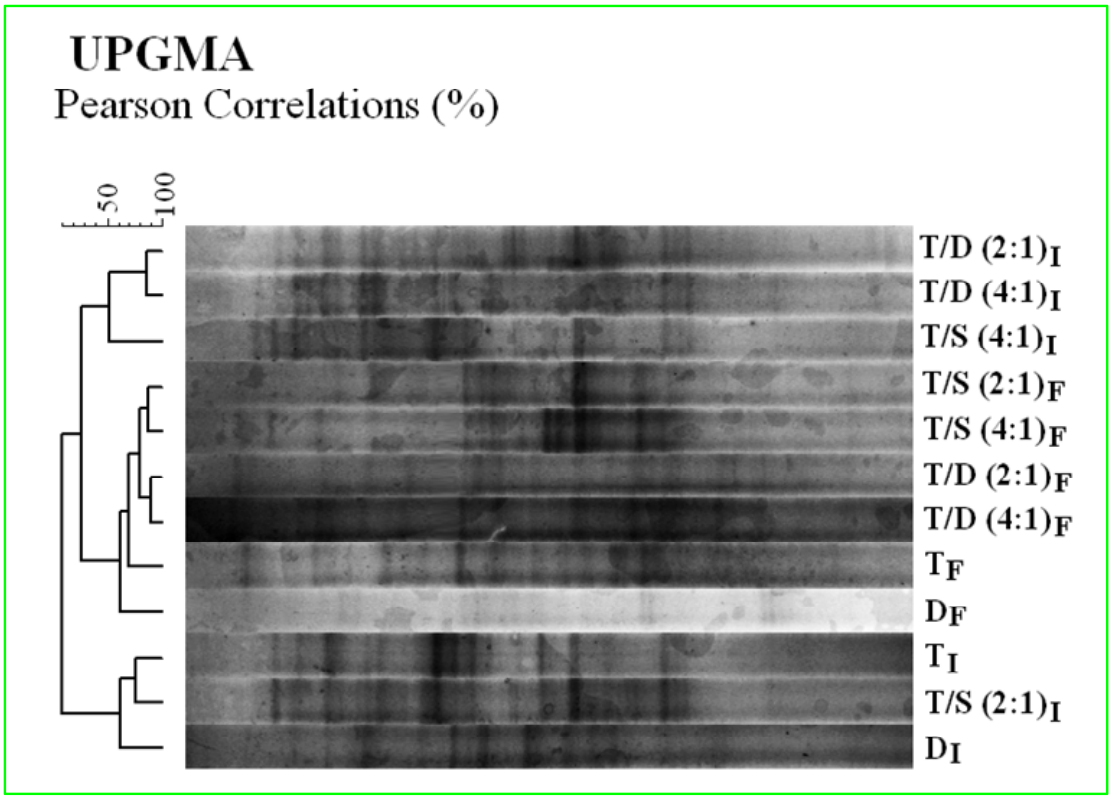


Figure 1.4. Dendrogram from cluster analysis of bacterial community structure from initial material (I) and final vermicompost (F) samples based on DGGE patterns of amplified partial 16S rRNA genes.

Table 1.6. Similarity value among DGGE patterns from initial materials (I) and final vermicomposts (F).

Treatment [a]	I						F					
	D	T	T/S (2:1)	T/S (4:1)	T/D (2:1)	T/D (4:1)	D	T	T/S (2:1)	T/S (4:1)	T/D (2:1)	T/D (4:1)
I												
D	100											
T	68.1	100										
T/S (2:1)	58.6	76.7	100									
T/S (4:1)	17.3	38.9	59.9	100								
T/D (2:1)	15.3	4.9	51.2	44.3	100							
T/D (4:1)	7.6	0	44.1	61.1	87.5	100						
F												
D	0	0	0	39.3	13.7	32.9	100					
T	0	0	0	0	31.9	29.5	30.8	100				
T/S (2:1)	0	0	0	18.9	39.3	49.7	72.2	68.9	100			
T/S (4:1)	1.7	0	0	2.6	43.9	44.7	52.4	76.6	88.9	100		
T/D (2:1)	0	0	0	16.6	38.9	31.3	81.5	69.4	85.9	74.6	100	
T/D (4:1)	0	0	0	0.9	17.4	31.3	75.9	71.2	84.2	75.2	90.5	100

All values are % Pearson correlation similarity coefficient.
[a] Refer to text, for explanation of treatment abbreviations.

1.4. CONCLUSIONS

These findings represent a sound basis for applying the vermicomposting process to the recycling of greenhouse vegetable wastes, where salt content was the main factor restricting earthworm development. Our study shows that vermicomposting is a feasible means of recycling damaged tomato-fruit waste, where, although *E. andrei* recorded lower growth than in the cow dung control treatment, cocoon production was higher. Chemical and enzymatic analyses and germination indices showed that vermicomposting was effective in biostabilizing this waste. We also show that DGGE technique generates genetic fingerprintings that accurately reflect the degree of stabilization in vermicompost

ACKNOWLEDGEMENTS

This study was financed by "Junta de Andalucía" project P05-AGR-00408. Manuel J. Fernández Gómez thanks the Science and Innovation Ministry for their FPU doctoral grant (AP2006-03452). The authors also thank Mª Angeles Delgado for technical support.

REFERENCES

Bansal, S., Kapoor, K.K., 2000. Vermicomposting of crop residues and cattle dung with *Eisenia foetida*. Bioresour. Technol. 73, 95-98.

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol. 67, 297-303.

Benítez, E., Sainz, H., Melgar, R., Nogales, R., 2002. Vermicomposting of a lignocellulosic waste from olive oil industry: A pilot scale study. Waste Manage. Res. 20, 134-142.

Bhattacharya, S.S., Chattopadhyay, G.N., 2004. Transformation of nitrogen during vermicomposting of fly ash. Waste Manage. Res. 22, 488-491.

Castilla, N., Hernández, J., 2005. The plastic greenhouse industry of Spain. Chron. Hort. 45, 15-20.

Elvira, C., Goicoechea, M., Sampedro, L., Mato, S., Nogales, R., 1996. Bioconversion of solid paper-pulp mill sludge by earthworms. Bioresour. Technol. 57, 173-177.

Espi, E., Salmeron, A., Fontecha, A., García, Y., Real, A.I., 2006. Plastic films for agricultural applications. J. Plastic Film Sheet. 22, 85-102.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan. 28, 123-134.

Garg, P., Gupta, A., Satya, S., 2006. Vermicomposting of different types of waste using *Eisenia foetida*: A comparative study. Bioresour. Technol. 97, 391-395.

Garg, V.K., Kaushik, P., 2005. Vermistabilization of textile mill sludge spiked with poultry droppings by an epigeic earthworm Eisenia foetida. Bioresour. Technol. 96, 1063-1071.

Government of Spain, 2005. Real Decreto 824/2005 sobre productos fertilizantes, in: BOE. Government of Spain, Madrid, pp. 25602-25669.

Gunadi, B., Edwards, C.A., 2003. The effects of multiple applications of different organic wastes on the growth, fecundity and survival of *Eisenia fetida* (Savigny) (Lumbricidae). Pedobiologia 47, 321-329.

Hobson, A.M., Frederickson, J., Dise, N.B., 2005. CH_4 and N_2O from mechanically turned windrow and vermicomposting systems following in-vessel pre-treatment. Waste Management 25, 345-352.

Iannotti, D.A., Grebus, M.E., Toth, B.L., Madden, L.V., Hoitink, H.A.J., 1994. Oxygen respirometry to assess stability and maturity of composted municipal solid waste. J. Environ. Qual. 23, 1177-1183.

Lane, D.J., 1991. 16S/23S rRNA sequencing, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New York, pp. 115-175.

Lores, M., Gómez-Brandón, M., Pérez-Díaz, D., Domínguez, J., 2006. Using FAME profiles for the characterization of animal wastes and vermicomposts. Soil Biol. Biochem. 38, 2993-2996.

Lorimor, J., Fulhage, C., Zhang, R., Funk, T., Sheffield, R., Sheppard, C., Newton, G.L., 2001. Manure management strategies/technologies. In: White Paper on Animal Agriculture and the Environment for National Center for Manure and Animal Waste Management, Ames, USA, 52-

M.A.P.A., 1986. Official methods of analysis. Volume III. Plants, organic products, fertilizers, soils, waters and organic fertilizers. Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación, Madrid.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011-1016.

Nogales, R., Domínguez, J., Mato, S., 2008. Vermicompostaje, in: Moreno, J., Moral, R. (Eds.), Compostaje. Ediciones Mundi Prensa, Madrid, pp. 187-208.

Pardossi, A., Tognoni, F., Incrocci, L., 2004. Mediterranean Greenhouse Technology. Chron. Hort. 44, 28-34.

Parra, S., Aguilar, F.J., Calatrava, J., 2008. Decision modelling for environmental protection: The contingent valuation method applied to greenhouse waste management. Biosystems Engineering 99, 469-477.

Pramanik, P., Ghosh, G.K., Ghosal, P.K., Banik, P., 2007. Changes in organic - C, N, P and K and enzyme activities in vermicompost of biodegradable organic wastes under liming and microbial inoculants. Bioresour. Technol. 98, 2485-2494.

Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresour. Technol. 100, 804-811.

Senesi, N., 1989. Composted materials as organic fertilizers. The Science of the Total Environment 81-82, 521-542.

Suthar, S., 2007. Vermicomposting potential of *Perionyx sansibaricus* (Perrier) in different waste materials. Bioresour. Technol. 98, 1231-1237.

Suthar, S., 2008. Bioconversion of post harvest crop residues and cattle shed manure into value-added products using earthworm *Eudrilus eugeniae* Kinberg. Ecol. Eng. 32, 206-214.

Suthar, S., 2009. Vermicomposting of vegetable-market solid waste using *Eisenia fetida*: Impact of bulking material on earthworm growth and decomposition rate. Ecol. Eng. 35, 914-920.

Tabatabai, M.A., 1982. Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. Soil Enzymes 907-943.

Tripathi, G., Bhardwaj, P., 2004. Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg). Bioresour. Technol. 92, 275-283.

von Mersi, W., Schinner, F., 1991. An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol. Fertl. Soils 11, 216-220.

Williams, S., 1984. Official methods of analysis of the Association of Official Analytical Chemists., 14th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Zdruli, P., Jones, R.J.A., Montanarella, L., 2004. Organic matter in the soils of southern Europe, in: European Soil Bureau Research Report, EUR 21083 EN. Office for Official Publications of the European Communities, Luxembourg, pp. 17.

Zucconi, F., Forte, M., Monaco, A., De Bertoldi, M., 1981. Biological evaluation of compost maturity. BioCycle 22, 27-29.

CAPÍTULO 2
CHAPTER 2

VERMICOMPOSTAJE DE ALIMENTACIÓN CONTINUA COMO UN MÉTODO DE RECICLAJE PARA VALORIZAR DESTRÍOS DE FRUTOS DE TOMATE PROCEDENTES DE CULTIVOS EN INVERNADERO

CONTINUOUS-FEEDING VERMICOMPOSTING AS A RECYCLING MANAGEMENT METHOD TO REVALUE TOMATO-FRUIT WASTES FROM GREENHOUSE CROPS

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M. (2010). Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops. Waste Management 30, 2461-2468.

RESUMEN

Las inmensas cantidades de frutos desechados (destríos) que generan los cultivos en invernadero representan un problema ambiental a nivel mundial. El objetivo de este trabajo fue valorar la eficiencia del vermicompostaje como una opción de manejo y reciclaje para biotransformar destríos de tomate procedentes de invernaderos en un producto orgánico rico en nutrientes disponible para usos agrícolas. Se construyó un vermirreactor piloto que fue preparado con una capa de estiércol, en la que se inoculó una población inicial de *Eisenia fetida* que fue continuamente alimentada con una alta tasa de carga orgánica (13.6 kg COT m^{-3} $semana^{-1}$) durante 150 días. Diferentes parámetros químicos y enzimáticos del vermicompost así como la estructura de su comunidad bacteriana y fúngica se determinaron durante 210 días (vemicompostaje más un periodo de maduración). La biomasa de lombrices aumentó después de 90 días, y posteriormente descendió debido al incremento de pH, conductividad eléctrica y concentración de amonio. Los patrones temporales de deshidrogenasa, β-glucosidasa, proteasa y ureasa se relacionaron con el crecimiento de las lombrices y con la estabilización de la materia orgánica. Los perfiles bacterianos de DGGE fueron distintos entre el periodo de degradación de los sustratos lábiles y su posterior maduración. Las comunidades fúngicas se diferenciaron más en la fase de máxima producción de biomasa de lombrices, sugiriendo un efecto del tránsito intestinal. El producto final fue químicamente estable y enriquecido en nutrientes, demostrando que los destríos de tomate pueden ser vermicompostados con éxito en una enmienda útil para los suelos. Se sugiere el vermicompostaje de alimentación continua como una opción ambientalmente razonable para la gestión de residuos de invernadero.

Palabras clave: vermicompostaje, *Eisenia fetida*, DGGE, actividad enzimática, residuos de invernadero.

ABSTRACT

Huge quantities of discarded fruits generated from greenhouse crops represent a worldwide environmental problem. The aim of this work was to assess the efficiency of vermicomposting as a recycling management option for biotransforming tomato-fruit wastes from greenhouses into an organic nutrient-rich product available for agricultural purposes. A pilot vermireactor was constructed. It was provided with a manure layer, where an initial population of *Eisenia fetida* was introduced and fed continuously at a high organic loading rate (13.6 kg TOC m^{-3} wk^{-1}) for 150 days. Vermicompost chemical and enzymatic parameters as well as the bacterial and fungal community structure were determined for 210 days (vermicomposting plus a maturation period). Earthworm biomass increased after 90 days, and then declined due to increasing pH, electrical conductivity and ammonium concentration. The temporal patterns of dehydrogenase, β-glucosidase, protease and urease were related to earthworm growth and the stabilization of organic matter. Bacterial DGGE profiles differed between the period of degradation of labile substrates and the maturation step. Fungal communities at the stage of maximum earthworm biomass differed most, suggesting a gut passage effect. The end product was chemically stable and enriched in nutrients, demonstrating that tomato-fruit wastes can be successfully vermicomposted into a valuable soil amendment. We suggest continuous-feeding vermicomposting as an environmentally sound management option for greenhouse wastes.

Keywords: vermicomposting, *Eisenia fetida*, DGGE, enzyme activity, greenhouse wastes.

2.1. INTRODUCTION

In Spain, 66,000 ha are dedicated to greenhouse crops, 65% of which are located in Andalusia (Spanish Ministry of Agriculture, Fisheries and Food, 2007). These profitable systems have a negative impact on the environment where they are located because of the enormous quantities of wastes produced, which have become one of most important problems associated to this agricultural practice. These waste were traditionally eliminated by abandon them in dry ravines or empty areas originating uncontrolled burning, blocking of riverbeds, poisoning of cattle and sheep and a negative visual impact on the landscape. In addition, landfills containing these wastes are a potential breeding ground for diseases and pests and a source of greenhouse gas (methane) (Parra et al., 2008). Therefore, the greenhouse wastes should be recycled and reused in order to achieve environmentally suitable agricultural management. A particular kind of vegetable waste are the fruit wastes, which consist of fruits unsuitable for marketing due to bruising, inappropriate size, rotting, or insect attack, as well as marketable fruits that are discarded so as to raise their market prices. Only in the region of Motril (Granada, Southern Spain), where this study was conducted, 15,000 Tm of tomato-fruit wastes were discarded in 2007, representing 24% of all greenhouse wastes. Tomatoes comprised 80% of all fruit wastes from greenhouses and were generated continuously over the year.

This huge quantity of wastes could be converted into a nutrient-enriched bio-fertilizer and used for agricultural purposes or land restoration. Vermicomposting, a process involving the biostabilization of organic wastes by the joint action of earthworms and microorganisms, has proven to be a low-cost and rapid technique for the efficient management of vegetable wastes (Bansal and Kapoor, 2000; Suthar, 2008), using a variety of epigeic earthworms, e.g. *Eisenia fetida*, *Perionyx excavatus*, and *Eudrilus eugeniae*. Among these species, *E. fetida* might be the best choice for developing a vermicomposting process in temperate climates due to its tolerance of a broad range of environmental conditions, i.e. pH, moisture, and temperature (Nogales et al., 2008). Recent studies have shown the efficiency of *E. fetida* to vermicompost supermarket vegetable and fruit wastes using traditional (non-continuous) vermicomposting processes (Gunadi and Edwards, 2003; Suthar, 2009). To date, the vermicomposting of fruit wastes using a continuous-feeding system has not been studied.

Enzyme activities have been used as indicators of the time course of organic matter in vermicomposting systems from a small to large scale (Benítez et al., 1999, 2002; Nogales et al., 2005). Overall decreases in dehydrogenase and total hydrolytic activities have been

related to the decline in microbial activity and available substrates during the decomposition of organic matter. Despite that these enzymes have been widely used in composting and non-continuous vermicomposting, they have still not been evaluated as tools for monitoring a continuous vermicomposting system. On the other hand, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of genes coding for SSU rRNA has recently been used to determine the genetic structure of bacterial communities during vermicomposting at the laboratory scale with bin systems (Sen and Chandra, 2009). However, temporal changes in enzyme activities and microbial community structure have not been researched jointly throughout a continuously fed vermicomposting process.

In view the above, the objective of this study was to test the efficiency of *E. fetida* to vermicompost tomato-fruit wastes from greenhouse crops using a pilot-scale continuous-feeding vermicomposting system. A rectangular vermireactor was designed and tomato-fruit wastes were added over a 150-day vermicomposting process. The time course of the chemical parameters, enzyme activities, and genetic structure of the fungal and bacterial communities proliferating in the vermibed (i.e. the vermireactor substrate) were tracked in order to evaluate the role of earthworms and monitor the stabilization of organic wastes during this biotransformation process. Finally, the nutrient content was analysed in the end product in order to assess the quality of this vermicompost as an agricultural resource.

2.2. MATERIALS AND METHODS

2.2.1. Experimental set-up

A rectangular metal pilot-scale (0.6 m × 0.9 m × 0.2 m) vermireactor was designed. A 0.1 cm mesh was placed at the bottom of the vermireactor. A 5 cm layer containing 15 kg dry weight of mature sheep manure (36% moisture, pH 8.6, 1.8 dS m^{-1} electrical conductivity (EC), 138 g kg^{-1} total organic carbon (TOC), 9.6 g kg^{-1} total Kjeldahl nitrogen (TKN), and C:N ratio 14; see also Table 2.3) was placed on the mesh to provide an initial habitat for the earthworms. Sheep manure was selected since it is optimal for worm growth and it is intensively produced close to the greenhouses in Southern Spain. A total of 500 g of clitellated and non-clitellated earthworms (*E. fetida*) were inoculated in the manure layer. These were obtained from the earthworm culture bank at the "Estación Experimental del Zaidín" (CSIC) in the city of Granada (Southern Spain). Fifteen days after the earthworms were added, the vermireactor was fed with 10 kg of the liquid-paste of tomato-fruit wastes

(92% moisture, pH 3.9, 1.4 dS m^{-1} EC, 459 g kg^{-1} TOC, 23 g kg^{-1} TKN and C/N ratio 20), which were taken from the organic-waste-treatment plant in Motril (Granada province, Spain). This operation was repeated weekly for 5 months, during which the organic loading rate was kept at 13.6 kg TOC m^{-3} wk^{-1}. After this period, the earthworms were removed by hand, and the vermicompost was matured in a pile for 2 months. The vermicomposting process was conducted under a controlled temperature (25ºC) with no water was applied beyond that contained in the liquid-paste of tomato-fruit wastes.

Throughout the process, five 70 cm^2 cylindrical cores were used to take regularly distributed samples from the vermireactor. The number and weight of earthworms in each core were recorded every 15 days, and the worms were replaced in the vermireactor. The content of each core was collected monthly during vermicomposting and at the end of the maturation period. Each sample was divided into two subsamples, of which one was stored at -20°C for enzyme activity and molecular biological analyses, while the other was air dried and finely ground for chemical analyses.

2.2.2. Chemical analyses

The pH and electrical conductivity (EC) of the vermibed were measured with a glass electrode using a 1:10 sample:water (w:v) ratio. Total organic C (TOC) and total Kjeldahl N (TKN) were determined using the dichromate oxidation and Kjeldahl method, respectively (M.A.P.A., 1986). Water-soluble carbon (WSC) was extracted at 60ºC for 1 h with distilled water (1:10 sample:water w/v) and then determined with potassium dichromate and sulphuric acid at 160ºC for 30 min. Subsequently, the amount of Cr^{3+} produced by the reduction of Cr^{6+} was quantified spectrophotometrically at 590 nm (Sims and Haby, 1971). The ammonium (NH_4^+) concentration was determined after extraction with 2 M KCl using a modified salicylate-nitroprusside colorimetric method (Kandeler and Gerber, 1988). Total phosphorus was measured using the ammonium vanadomolybdate method; total K and Na were measured by flame-photometry; and total Ca and Mg and micronutrients (Fe, Mn, Cu, Zn) were determined by atomic absorption spectrometry after digesting the samples with HNO_3:$HClO_4$ mixture (Williams, 1984).

2.2.3. Enzyme activities

Total enzyme activities were determined in triplicate, each reaction tube containing 0.2 g sample. Dehydrogenase activity was measured after the extraction of iodonitrotetrazolium formazan (INTF), produced by the reduction of 2-p-iodophenyl-3 p-nitrophenyl-5 tetrazolium chloride, with a mixture of 1.5:1 acetone:tetrachloroethylene. INTF was measured in a spectrophotometer at 490 nm (García et al., 1997). The enzyme activities β-glucosidase and acid phosphatase were quantified by estimating the amount of p-nitrophenol (PNP) produced from 4-nitrophenyl-β-D glucanopyranoside (PNG) and 4-nitrophenyl phosphate (PNPP) following Tabatabai (1982), and Tabatabai and Bremner (1969), respectively. Urease and protease activities were determined using urea and N-a-benzoyl-L-argininamide, respectively, as substrates (Nannipieri et al., 1980; Bonmatí et al., 1998). The NH_4^+ released was measured using an ammonium-selective electrode (ORION Research Inc., Beverly, MA, USA, mod. 95-12).

2.2.4. DNA extraction and PCR-DGGE analyses

Total DNA was extracted in triplicate from 0.25 g of samples taken on days 0 (M0), 30 (M1), 90 (M3), and 150 (M5) of vermicomposting and from the matured vermicompost (V) by means of the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. Briefly, this DNA extraction kit involves mechanical and chemical lysis of the cells, precipitation of non-DNA substances and capture of total DNA on a silica membrane for its washing and subsequent elution. DNA solutions were checked for quality by electrophoresis in 1% agarose gels stained with ethidium bromide.

The PCR was performed with 1-2 µl DNA in a total volume of 25 µl containing 0.2 µM of each primer (Table 2.1), 1X reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20] (GeneCraft, Münster, Germany), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin, 0.025 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany) and sterile water. Non-template controls, containing all the components except DNA templates, were included in parallel. Amplification was performed in a PCR Express cycler (ThermoHybaid) using an initial denaturation at 94°C for 8 min, followed by 30 amplification cycles, each consisting of 1 min at 94°C, 1min annealing at the specific temperature in Table 2.1, and 1 min at 72°C, followed by a final extension step for

Table 2.1. PCR primer sets, PCR, and DGGE conditions used in this study.

Primer set	Annealing positions	Secuence 5'-3'	Specificity	Annealing (ºC)	Amplicon length (bp)[c]	Denaturing gradient (%)	References
F984[a]	968-984[b]	AACGCGAAGAACCTTAC	Bacteria	61	433	40-65	Heuer et al. (1997)
–R1378	1378-1401[b]	CGGTGTGTACAAGGCCCGGGAACG					
R1[a]	18 rDNA	AICCATTCAATCGGTAIT (I= Inosin)	Fungi	50	390	30-60	Vainio and Hantula, (2000)
–F390	18 rDNA	CGATAACGAACGAGACCT					

[a] Primers with a GC clamp at the 5' end according references.
[b] Numbering according to the *rrs* gene of *Escherichia coli.*
[c] Calculated from the *rrs* gene of *E. coli* or from the product length described in Vainio and Hantula (2000).

10 min at 72°C. Proper sizes of amplification products were verified by electrophoresis in 1.5% agarose gels stained with ethidium bromide and inspected under a UV-transilluminator. The PCR product concentration was determined with the PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA, USA). Fluorescence was measured with an Anthos Zenyth 3100 multimode detector (Anthos Labtec, Austria) and the Software for Anthos Multimode Detectors (Version 2.0.0.13).

The denaturing gradient gel electrophoresis (DGGE) was conducted by loading 100 ng of PCR products into 8% (w/v) polyacrylamide gel in 1x TAE (20 mM Tris-Cl, 10 mM acetate, 0.5 mM Na_2EDTA) containing a gradient of denaturants (100% denaturants consisting of 40% [v/v] formamide and 7 M urea) as indicated in Table 2.1. A 100 bp DNA ladder (Genecraft®, Germany) served as marker. Gels were run in an INGENYphorU System (Ingeny International BV, The Netherlands) at 60°C for 16 h at 100 V. Gels were stained with silver nitrate (Sanguinetti et al., 1994) using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Germany) and photographed for subsequent image analysis.

2.2.5. Data analysis

The time course of all parameters determined during vermicomposting was statistically tested using a repeated-measures analysis of variance (ANOVA). The Least Significant Difference test (LSD) was used for *post hoc* mean separation. A paired-sample *t*-test was performed to assess the differences of chemical parameters between initial sheep manure and the final mature vermicomposts. All statistical analyses were conducted using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

A comparison of DGGE patterns was made with the GelCompar II software (Applied Maths, Kortrijk, Belgium). After conversion of the scanned gels, the bands were normalized and a reference position defined to align the patterns for proper comparison after associating the bands with the standard. Similarity values among banding patterns were calculated based on the comparison of the corresponding densitometric curves using the Pearson correlation similarity coefficient. Dendrograms were calculated with the unweighted-pair group method using arithmetic averages (UPGMA) clustering algorithm. A position tolerance of 1% was set for band matching.

2.3. RESULTS AND DISCUSSION

2.3.1. Earthworm development and chemical changes during vermicomposting

Weekly applications of tomato-fruit wastes did not increase the total earthworm biomass during the first 75 days of vermicomposting (Fig.2.1a). Nevertheless, a pronounced increase was observed from day 75 to day 90, peaking at 18.6 g earthworm per core (ca. 1400 g earthworms in the whole vermireactor), which was twofold the initial value. This is consistent with the life-cycle of *E. fetida*, which population doubles between 54 and 91 days after cocoon deposition under optimal feeding conditions (Nogales et al., 2008). Importantly, the moisture content in the liquid-paste of tomato-fruit wastes was high enough so as to allow normal earthworm development without further watering. Edwards (1988) reported that *E. fetida* can survive at moisture contents between 50% and 90%, in agreement with our results.

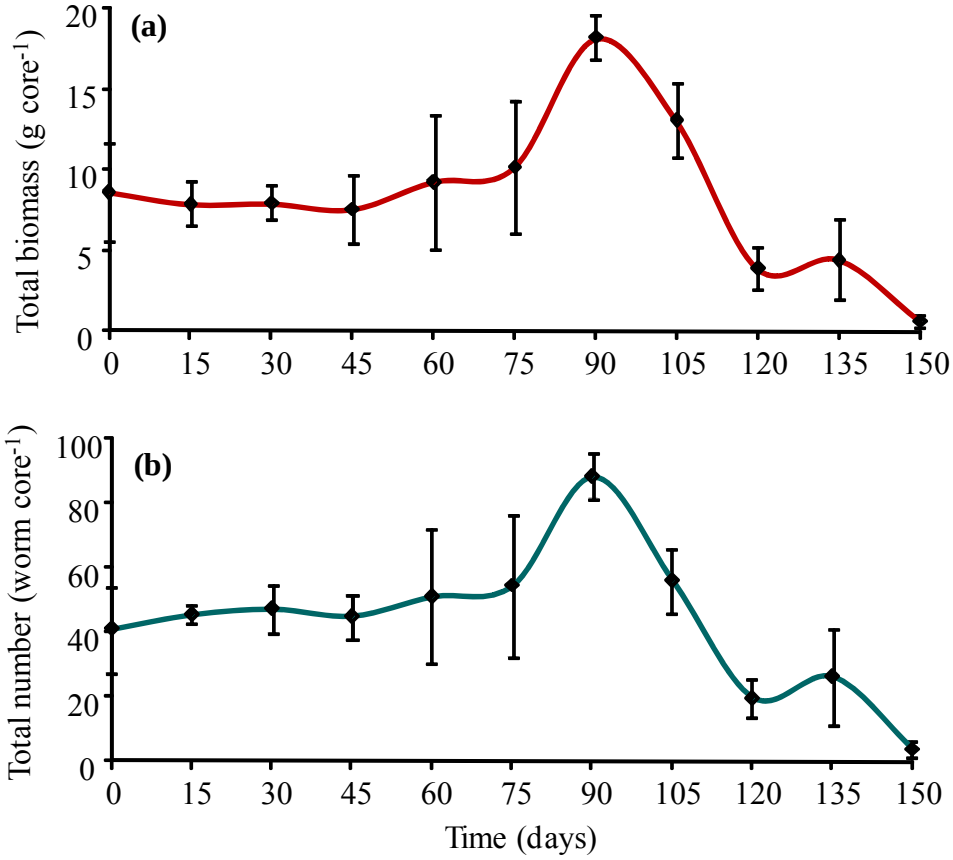


Figure 2.1. Evolution of biomass (a) and total number of earthworms (b) during the continuous-feeding vermicomposting process. Each symbol indicates the mean ±standard error (n = 5).

From day 90 to 120**,** although more tomato-fruit was added, the total earthworm biomass decreased abruptly and continued to decline slightly until the end of vermicomposting (day 150). This trend of earthworm biomass mirrored the reduction in the number of earthworms (Fig. 2.1b), and not the individual worm weights, which remained basically constant throughout the vermicomposting (data not shown). The increased earthworm mortality during this period could be explained by the rise in the pH value, electrical conductivity (EC) and NH_4^+ levels in the vermibed, which have been previously shown to be lethal to earthworms (Edwards, 1988). The vermibed pH increased from 8.3 to 9.5 during the 150 days period of earthworm activity and up to 10.4 after maturation (Table 2.2). This can be attributed to the increase in NH_4^+ concentration, exceeding 3000 mg kg^{-1} on day 90 and remaining high until the end of the process. This could be the result of the high mineralization of proteins from the tomato-fruit waste and sheep manure. In addition, it must be considered that under a basic pH, ammonium (NH_4^+) turns into the more toxic ammonia (NH_3). EC values also increased continuously up to a maximum of 4.5 dS m^{-1} after 150 days (Table 2.2) indicating the release of salts during the decomposition of organic substances. Gunadi and Edwards (2003) reported that *E. fetida* died in vegetable waste (e.g. lettuce, pea, celery, etc.) with a high EC value (12.8 ± 1.7 dS m^{-1}) and NH_4^+ concentrations (1878.1 ± 67.8 mg kg^{-1}), and these worms survived only 3 weeks in supermarket fruit wastes (e.g. cucumber, pear, apple, etc.) at pH 4.1, EC 4.5 dS m^{-1} and 3.5 mg NH_4^+ kg^{-1} recorded in that vermibed. Likewise, Mitchell (1997) found *E. fetida* unable to survive in cattle manure at pH 9.5 and 5.0 dS m^{-1} EC.

During the 150 days vermicomposting, the values of TOC, TKN and WSC significantly increased by 100%, 96% and 35%, respectively, compared to their initial values (Table 2.2). This was not surprising, since fresh organic matter was continuously incorporated by the addition of tomato-fruit waste at a high loading rate. These results contrast with the general decline in TOC value observed in non-continuous-feeding vermicomposting systems (Benítez et al., 1999, 2002; Nogales et al., 2005). Hence, a maturing period was considered necessary to complete the mineralization of the carbonaceous and organic-N compounds contained in the vermibed. Indeed, TOC, TKN, and WSC all decreased after the maturation period, down to levels similar to the initial values. Despite the changes during the vermicomposting process, the C:N ratio (TOC:TKN) remained constant at values from 11 to 12. The C:N ratio has been traditionally used as index for estimating compost maturity and stability. Senesi (1989) reported that although a decline from the initial C:N value, which should be not >30 in the substrate, to a final value <20, generally indicates an advanced

degree of organic matter stabilization in the product and is considered desirable for a mature composts, for particular substrates this index is not considered a reliable indicator, since raw materials used may have a highly variable lignin:cellulose ratio, or they may contain other recalcitrant component, varying their biodegradability. In spite of this fact, the Spanish legislation for fertilizers (Government of Spain, 2005) states that the C:N value of a compost to be used as an organic amendment in agriculture should always be <20.

Table 2.2. Temporal changes in chemical parameters during the continuous-feeding vermicompostig process and after maturation period.

Time (days)	pH	EC (dS m^{-1})	Moisture (%)	TOC (g kg^{-1})	TKN (g kg^{-1})	C/N	WSC (g kg^{-1})	NH_4^+ (mg kg^{-1})
Continuous-feeding vermicomposting process								
0	8.3^{a}	1.68^{a}	56^{b}	139^{a}	12.1^{a}	11.4	17.8	348^{a}
30	9.1^{b}	2.09^{ab}	58^{b}	170^{a}	14.4^{ab}	11.9	21.3	362^{a}
60	9.5^{b}	2.55b	57^{b}	188^{ab}	16.6^{bc}	11.3	24.2	1380^{bc}
90	9.1^{b}	3.01^{c}	69^{a}	208^{b}	17.1^{c}	12.1	23.3	3303^{e}
120	8.6^{a}	3.85^{d}	71^{a}	267^{c}	23.0^{d}	11.6	26.7	1968^{bc}
150	9.5^{b}	4.48^{f}	57^{b}	279^{c}	23.9^{d}	11.7	25.2	2561^{de}
Maturation period								
210	10.4^{c}	4.40^{f}	29^{c}	148^{a}	12.9^{a}	11.4	16.9	2264^{cd}

All values are means of five replicates.
EC: Electrical Conductivity; TOC: Total Organic Carbon; WSC: Water Soluble Carbon.
Data with the same letter are not significantly different ($p < 0.05$).

2.3.2. Enzyme activity during vermicomposting and vermicompost maturation

Dehydrogenases (DH-ase) are intracellular enzymes involved in the oxidative phosphorylation process, so that the activity of these enzymes has been widely used to assess the overall microbial activity in soils as well as in other biotransformation processes (García et al., 1997; Castaldi et al., 2008). The low DH-ase recorded at the start of the experiment

(Fig. 2.2a) indicated reduced microbial activity in the mature manure used as the starter material. This enzyme activity sharply increased at day 30, as a result of microbial stimulation by the initial application of tomato-fruit waste and the supply of microbes thriving in the residue. Parthasarathi and Ranganathan (1999) demonstrated that fresh earthworm castings released into the vermicompost substrate have higher microbial activity and viable cell numbers. Still, a more appreciable increase was recorded between days 90 and 120. This could be a consequence of the significant increase in TOC and TKN content due to an excessive waste-loading rate, which also raised the moisture level reducing the oxygenation conditions in the vermibed between days 90 and 120 (Table 2.2). On the other

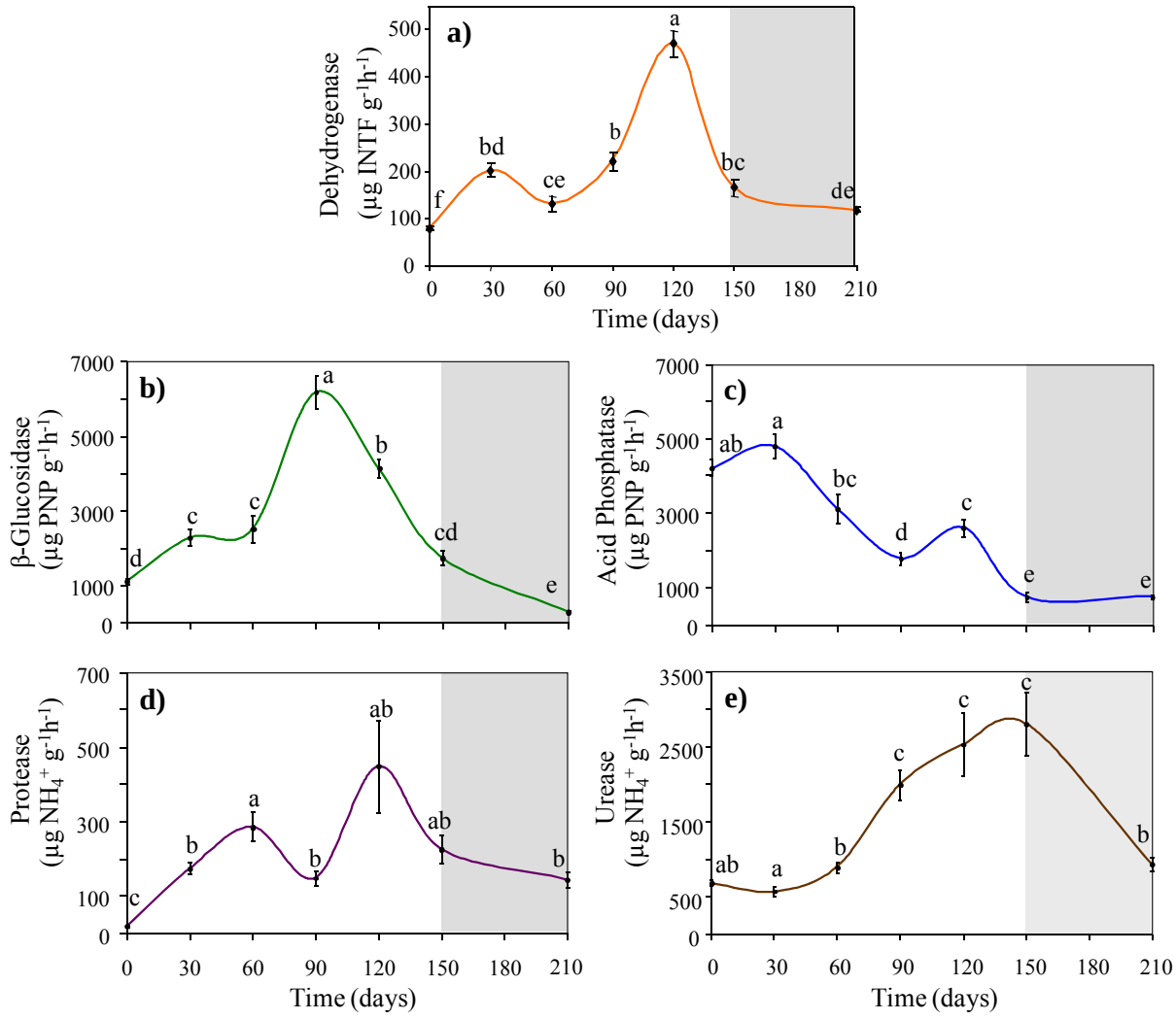


Figure 2.2. Enzyme activities during the continuous-feeding vermicomposting process (□) and after maturation period (■). Data with the same letter are not significantly different ($p < 0.05$). Each symbol indicates the mean ±standard error (n = 5).

hand, the mentioned increase coincided with the sudden decrease in total earthworm biomass, suggesting that the burst in microbial activity could be related to the decomposition of the worm bodies. Immediately after day 120, DH-ase activity fell to values similar to those recorded at the start of the experiment and remained stable during the maturation phase. According to Benítez et al. (1999), during a non-continuously fed vermicomposting systems, a peak in the DH-ase activity pattern would allow the end of the hydrolytic phase to be distinguished, corresponding with the maximum earthworm biomass, from the start of the maturation phase. Our results show that DH-ase is useful for monitoring continuously fed vermicomposting processes, its rise pointing to imminent system instability and thus indicating the need of reducing the organic loading rate.

The β-glucosidase activity, which catalyses the hydrolysis of cellobiose and other disaccharides, resembled dehydrogenase in behaviour, although its maximum was detected on day 90, a month before the peak registered by dehydrogenase (Fig. 2.2b). The application of tomato-fruit waste, which has a high carbohydrate and soluble sugar content (averaging 4% of the total dry weight), activated these enzymes. However, β-glucosidase was presumably also fostered by the high earthworm biomass. This conjecture is supported by the positive correlation between the two parameters (Pearson's correlation coefficient = 0.456, $p < 0.05$). This result agrees with those reported by Parthasarathi and Ranganathan (2000) who detected higher carbohydrase activities in castings from earthworms grown in organic wastes. Thus, the strong decline of this enzyme between days 90 and 120 could be related to the sharp reduction in the earthworm population. Therefore, β-glucosidase activity, which is easy and inexpensive to analyse, appears to be useful to monitor the earthworm population while avoiding the laborious earthworm biomass determination.

Acid phosphatases are enzymes of agricultural value because they catalyse the hydrolysis of organic phosphomonoester to an inorganic phosphate form, which can be taken up by plants. The initial activity was high (Fig. 2.2c) and decreased progressively until the end of the experiment, presumably due to the rise in pH to over 9.0 (Table 2.2), which is considerably higher than the optimum for this enzyme (between 4 and 6.5) (Speir and Ross, 1978). Therefore, acid phosphatases do not seem to be good process indicators in this continuous-feeding vermicomposting system.

Protease activity increased from days 0 to 60 (Fig. 2.2d), indicating the depolymerization of N-containing compounds into dissolved organic nitrogen. The subsequent decline of protease activity at day 90 could be due to the transformation of available N-containing compounds in worm tissues at that moment of maximum earthworm

biomass. A further increase of protease activity was recorded from day 90 to 120, coinciding with increasing microbial activity and decreasing earthworm biomass. The death of earthworms could have released peptides which stimulated protease synthesis by the microbiota. Thus, it should be noted that protease-activity patterns would potentially reflect earthworm mortality.

Urease activity remained more or less stable during the first 60 days, then increased until day 90, and remained high until day 150 (Fig. 2.2e). This enzyme acts on carbon nitrogen (C–N) bonds other than the peptide linkage and is involved in the hydrolysis of urea to carbon dioxide and ammonium. In this sense, the dynamics of urease activity could be used as an indicator of high ammonium production in the vermibed, as it suggests the positive correlation between the two parameters (Pearson's correlation coefficient = 0.600, $p < 0.01$). Eventually, this enzyme activity decreased after maturation as a consequence of the mineralization of urea-type substrates.

2.3.3. Changes in elemental composition in the mature vermicompost

Table 2.3. Elemental composition of initial manure layer and mature vermicompost

Parameters	Initial sheep manure	Mature vermicompost	t-Test[a]
TKN (g kg^{-1})	9.6	12.9	0.001*
P (g kg^{-1})	2.60	2.04	0.020^{ns}
K (g kg^{-1})	8.9	22.7	0.006*
Ca (g kg^{-1})	24.6	18.6	0.020^{ns}
Mg (g kg^{-1})	9.54	7.20	0.033^{ns}
Na (g kg^{-1})	2469	1385	0.001*
Fe (mg kg^{-1})	15166	8588	0.001*
Mn (mg kg^{-1})	315	256	0.222^{ns}
Cu (mg kg^{-1})	13.1	27.4	0.001*
Zn (mg kg^{-1})	68.0	99.3	0.001*

All values represent mean of three replicates.
[a] Paired sample t-test: * significant at $p<0.01$; ^{ns} non-significant difference

Vermicomposting with *E. fetida* converted the sheep manure together with tomato-fruit wastes into a nutrient-rich product which can be used for agricultural purposes. As compared to the initial manure, the mature vermicompost had significantly higher TKN content, as well as other plant nutrients, i.e. K, Cu, and Zn (Table 2.3). This increase may respond to the high mineralization of nitrogen from decaying earthworm tissues jointly to the release of other nutrients by the oxidation of organic matter (Lee, 1985). In agreement with these results, Tripathi and Bhardwaj (2004) reported increases in N and K, 150 days after *E. fetida* inoculation in a non-continuous vermicomposting system. Conversely, total Fe and Na measured in the vermicompost showed lower concentrations than that in the sheep manure, whereas P, Ca, Mg, and Mn did not show any significant change. Except in the case of Na, these results contrast with those commonly observed in non-continuous vermicomposting processes, in which these elements tend to increase as a result of organic matter mineralization (Benítez et al., 2002; Nogales et al., 2005).

2.3.4. Microbial community structure during vermicomposting and vermicompost maturation

The bacterial and fungal DGGE profiles were generated from a total of 15 samples (Fig. 2.3), i.e. three replicate vermicompost samples taken on days 0 (M0), 30 (M1), 90 (M3), 150 (M5), and the mature vermicompost (V). DGGE profiles of triplicate samples were found to be highly reproducible since, with one exception, the replicates from the same period of vermicomposting were more similar to each other than to other samples (2.4). A complex banding pattern with more than 40 detectable bands appeared in all samples, indicating a high bacterial diversity in these complex microbial systems.

An UPGMA analysis of these DGGE profiles showed that the bacterial community profiles clustered into two main groups (Fig. 2.4a): samples from day 0 (M0) were discriminated from another cluster grouping all others (M1, M3, M5 and V). This implies that the bacterial community present in the initial samples was strongly affected by earthworm digestion. Therefore, the earthworm's activity could modify the microbial diversity of the vermibed by selectively feeding on and/or stimulating specific taxa. Recently, Monroy et al. (2009) demonstrated that the passage of the pig slurry through the

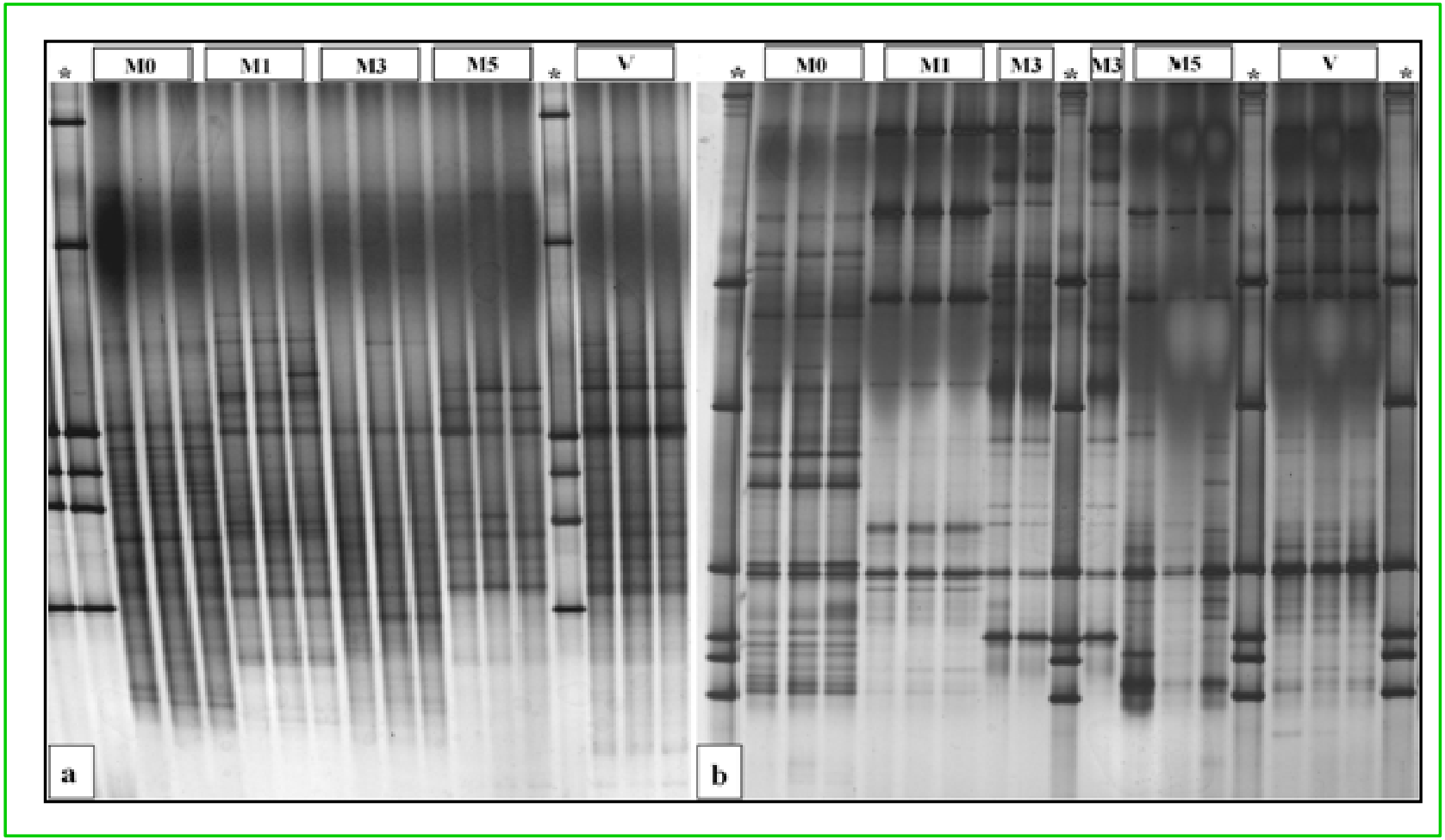


Figure 2.3. Photographed of DGGE gels from triplicate samples taken during the continuous-feeding vermicomposting on days 0 (M0), 30 (M1), 90 (M3), 150 (M5) and mature vermicompost (V). **a)** DGGE from bacterial 16S rDNA gene fragments PCR products. **b)** DGGE from fungal 18S rRNA gene fragments. Ladder lanes are labelled with *.

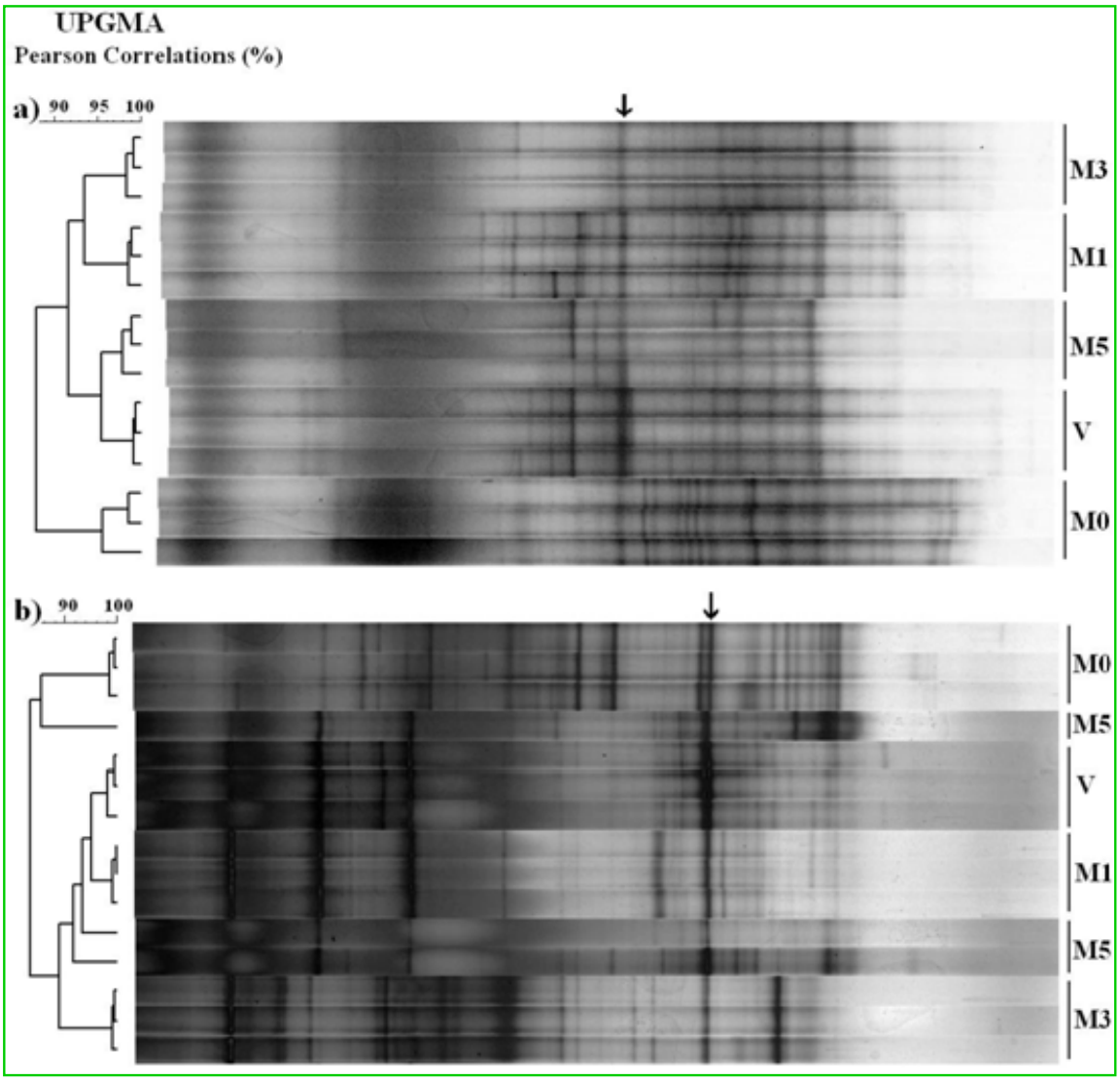


Figure 2.4. Dendrogram depicting the relatedness of bacterial (**a**) and fungal communities (**b**) from triplicate samples taken during the continuous-feeding vermicomposting on days 0 (M0), 30 (M1), 90 (M3), 150 (M5) and mature vermicompost (V).

gut of *E. fetida* reduced the total coliform numbers by 98%, but did not reduce the total abundance of bacteria providing a sound evidence of this selective effect. Within the main cluster including M1, M3, M5, and V, a temporal variation was also evident as M1 and M3 clustered separately from M5 and V. This suggests a change in the bacterial community concurrent with the two steps detected in the dynamics of vermicomposting. The first step was probably characterized by a bacterial community associated with the degradation of labile organic substrates. In the second step, marked by a decrease in microbial activity, the bacterial DGGE profiles presumably reflected a community involved in the stabilization of organic matter.

As for bacteria, UPGMA analysis clustered fungal profiles into two groups (Fig.2.4b): one consisted of samples M0, and the other M1, M3, V and two out of three M5 samples. Within this cluster, a subcluster composed of samples taken on day 90 (M3) was evident. This points out a difference in the fungal community structure as a consequence of the high earthworm biomass recorded in this period (Fig. 1a), indicating that gut passage selected certain fungal species. According to Schönholzer et al. (1999) fungi represent a considerable food source for earthworms and are digested during gut passage. Likewise, Tiunov and Scheu (2000) found distinct effects on the abundance of certain phyla by gut passage through earthworms (*Lumbricus terrestris*).

All DGGE profiles from both bacterial and fungal communities shared a dominant band in the position indicated by an arrow in Fig. 2.4. The intensity of this band increased in the final vermicompost (V). This might represent microbial taxa associated with this type of vermicomposting. Toyota and Kimura (2000) demonstrated that *E. fetida* presents an indigenous gut-associated microflora which could contribute to the microbial community in mature vermicomposts. On the other hand, the gut microbiota of *Lumbricus rubellus* was found to be substantially affected by its diet (Knapp et al., 2009). Lores et al. (2006) reported that the specific microbial community fingerprint of a vermicompost is dependent on the animal manure and earthworm species used in the process.

2.4. CONCLUSIONS

The continuous-feeding system assayed was an effective method to revalue the liquid-paste of tomato-fruit waste under high organic loading rate conditions (13.6 kg TOC m^{-3} wk^{-1}). Thanks to the high moisture content of this waste, an extra water addition was not required to maintain optimal moisture for earthworm development, which is fundamental for the economy of the process under semi-arid conditions. Among all parameters studied, the enzyme activities were useful indicators of the process and should be used in subsequent trials to further optimise the waste-loading rate in order to allow even longer earthworm persistence and shorten the vermicomposting process (e.g. by reducing the amount of tomato-fruit wastes added and/or the frequency of feeding; by reducing the time of the vermicomposting process from 150 to 90 days and/or by starting the maturation step before). The study of the bacterial and fungal communities showed strong and specific changes in the vermibed's microbiota as a consequence of the degradation and stabilization of organic matter. The mature vermicompost constituted a value-added product which may be used as

an organic amendment for agricultural soils. Therefore, the continuous-feeding vermicomposting has proven an adequate option for recycling tomato-fruit waste from greenhouses in an environmentally acceptable way.

ACKNOWLEDGEMENTS

This study was founded by the Junta de Andalucía (P05-AGR-00408). Manuel J. Fernández Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant and his temporal stay in the University of Innsbruck (AP2006-03452). M. Goberna thanks support by the Marie Curie Actions (MEIF-CT-2006-041034). We thank the organic waste treatment plant in Motril (Granada, Southern Spain) for providing the tomato-fruit wastes. The authors also thank Celia Cifuentes for her technical support and David Nesbitt for assisting in the translation of the manuscript into English.

REFERENCES

Bansal, S., Kapoor, K.K., 2000. Vermicomposting of crop residues and cattle dung with *Eisenia foetida*. Bioresour. Technol. 73, 95-98.

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol. 67, 297-303.

Benítez, E., Sainz, H., Melgar, R., Nogales, R., 2002. Vermicomposting of a lignocellulosic waste from olive oil industry: A pilot scale study. Waste Manage. Res. 20, 134-142.

Bonmatí, M., Ceccanti, B., Nannipieri, P., 1998. Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol. Biochem. 30, 2113-2125.

Castaldi, P., Garau, G., Melis, P., 2008. Maturity assessment of compost from municipal solid waste through the study of enzyme activities and water-soluble fractions. Waste Manag. 28, 534-540.

Edwards, C.A., 1988. Breakdown of animal, vegetable and industrial organic wastes by earthworms, in: Edwards, C.A., Neuhauser, E.F. (Eds.), Earthworms in waste and environmental management. SPB Academic Publishing BV, The Hague, pp. 21-31.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan. 28, 123-134.

Government of Spain, 2005. Real Decreto 824/2005 sobre productos fertilizantes, in: BOE. Government of Spain, Madrid, pp. 25602-25669.

Gunadi, B., Edwards, C.A., 2003. The effects of multiple applications of different organic wastes on the growth, fecundity and survival of *Eisenia fetida* (Savigny) (Lumbricidae). Pedobiologia 47, 321-329.

Heuer, H., Krsek, M., Baker, P., Smalla, K., Wellington, E.M., 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63, 3233-3241.

Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertl. Soils 6, 68-72.

Knapp, B.A., Podmirseg, S.M., Seeber, J., Meyer, E., Insam, H., 2009. Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. Soil Biol. Biochem. 41, 2299-2307.

Lee, K.E., 1985. Earthworms. Their ecology and relationships with soils and land use, First ed. Academic Press, Sydney.

Lores, M., Gómez-Brandón, M., Pérez-Díaz, D., Domínguez, J., 2006. Using FAME profiles for the characterization of animal wastes and vermicomposts. Soil Biol. Biochem. 38, 2993-2996.

M.A.P.A., 1986. Official methods of analysis. Volume III. Plants, organic products, fertilizers, soils, waters and organic fertilizers. Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación, Madrid.

Mitchell, A., 1997. Production of *Eisenia fetida* and vermicompost from feed-lot cattle manure. Soil Biol. Biochem. 29, 763-766.

Monroy, F., Aira, M., Domínguez, J., 2009. Reduction of total coliform numbers during vermicomposting is caused by short-term direct effects of earthworms on microorganisms and depends on the dose of application of pig slurry. Sci. Total Environ. 407, 5411-5416.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011-1016.

Nogales, R., Cifuentes, C., Benítez, E., 2005. Vermicomposting of winery wastes: A laboratory study. J. Environ. Sci. Health. 40, 659-673.

Nogales, R., Domínguez, J., Mato, S., 2008. Vermicompostaje, in: Moreno, J., Moral, R. (Eds.), Compostaje. Ediciones Mundi Prensa, Madrid, pp. 187-208.

Parra, S., Aguilar, F.J., Calatrava, J., 2008. Decision modelling for environmental protection: The contingent valuation method applied to greenhouse waste management. Biosystems Engineering 99, 469-477.

Parthasarathi, K., Ranganathan, L.S., 1999. Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. Eur. J. Soil Biol. 35, 107-113.

Parthasarathi, K., Ranganathan, L.S., 2000. Aging effect on enzyme activities in pressmud vermicasts of *Lampito mauritii* (Kinberg) and *Eudrilus eugeniae* (Kinberg). Biol. Fertl. Soils 30, 347-350.

Sanguinetti, C.J., Neto, E.D., Simpson, A.J.G., 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17, 914-921.

Schönholzer, F., Hahn, D., Zeyer, J., 1999. Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. FEMS Microbiol. Ecol. 28, 235-248.

Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresour. Technol. 100, 804-811.

Senesi, N., 1989. Composted materials as organic fertilizers. Sci. Total Environ. 81-82, 521-542.

Sims, J.R., Haby, V.A., 1971. Simplified colorimetric determination of soil organic matter. Soil Sci. 112, 137-141.

Spanish Ministry of Agriculture, Fisheries and Food, 2007. Encuesta sobre Superficies y Rendimientos de Cultivos. Resultados. Madrid.

Speir, T.W.,.Ross, D.J., 1978. Soil phosphatase and sulphatase, in: Burns, R.G.(Ed.), Soil Enzymes. Academic Press, London, pp. 197-250.

Suthar, S., 2008. Bioconversion of post harvest crop residues and cattle shed manure into value-added products using earthworm *Eudrilus eugeniae* Kinberg. Ecol. Eng. 32, 206-214.

Suthar, S., 2009. Vermicomposting of vegetable-market solid waste using *Eisenia fetida*: Impact of bulking material on earthworm growth and decomposition rate. Ecol. Eng. 35, 914-920.

Tabatabai, M.A., 1982. Soil enzymes, in: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. ASA and SSSA, Madison, pp. 907-943.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301-307.

Tiunov, A.V., Scheu, S., 2000. Microfungal communities in soil, litter and casts of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. Appl. Soil Ecol. 14, 17-26.

Toyota, K., Kimura, M., 2000. Microbial community indigenous to the earthworm *Eisenia foetida*. Biol. Fertl. Soils 31, 187-190.

Tripathi, G., Bhardwaj, P., 2004. Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg). Bioresour. Technol. 92, 275-283.

Vainio, E., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104, 927-936.

Williams, S., 1984. Official methods of analysis of the Association of Official Analytical Chemists, fourteenth ed. Association of Official Analytical Chemists, Arlington.

CAPÍTULO 3
CHAPTER 3

ACTIVIDADES HIDROLASAS DE SUSTANCIAS HÚMICAS EXTRAÍDAS DURANTE EL VERMICOMPOSTAJE DE DESTRIOS DE TOMATE UTILIZANDO UN SISTEMA DE ALIMENTACIÓN CONTINUA

HYDROLASES ACTIVITIES OF EXTRACTED HUMIC SUBSTANCES DURING VERMICOMPOSTING OF DAMAGED TOMATOES WASTES USING A CONTINUOUS-SUPPLYING SYSTEM

Fernández-Gómez, M.J., Romero, E., Cifuentes, C., Nogales, R. (2011). Hydrolases activities of extracted humic substances during vermicomposting of damaged tomatoes wastes using a continuous-supplying system. In: Soil Enzymology in the Recycling of Organic Wastes and Environmental Restoration. Environmental Science and Engineering Series. Trasar, C.; Hernández, T.; García, C.; Rad, C.; González-Carcedo, S. (Eds.). Chapter 22, 11 pp. DOI: 10.1007/978-3-642-21162-1-22. Springer-Verlag, Berlin Heidelberg, ISBN: 978-3-642-21161-4.

RESUMEN

El comportamiento de las actividades de enzimas hidrolíticas extracelulares en un sistema de vermicompostaje de alimentación continua no ha sido estudiado hasta ahora. El objetivo de este estudio fue valorar en ese sistema las actividades glucosidasa, ureasa, fosfatasa ácida y proteasa extracelulares que se extrajeron a pH 7.1 siguiendo el método de extracción con pirofosfato. El vermicompostaje se llevó a cabo usando un vermirreactor rectangular de tamaño medio alimentado continuamente sobre una capa de estiércol ovino con destríos de tomate (10 kg semana^{-1}) durante un periodo de 5 meses. Las lombrices fueron entonces retiradas y el material vermicompostado se dejó madurar durante 2 meses. La biomasa total de lombrices alcanzó su mayor valor después de tres meses, coincidiendo con los niveles más altos del contenido en carbono extraíble con pirofosfato y de las actividades extracelulares ureasa, fosfatasa ácida y proteasa. La actividad extracelular β-glucosidasa alcanzó su nivel más alto en el 4º mes, cuando la actividad microbiana estuvo también en su mayor nivel. Al final del periodo de vermicompostaje, las actividades fosfatasa y proteasa disminuyeron hasta niveles similares a los que se registraron al principio del proceso de vermicompostaje. Por el contrario, los valores de las actividades β-glucosidasa y ureasa fueron mayores que los registrados al comienzo del vermicompostaje. Los complejos humus-enzimas generados durante el periodo de vermicompostaje no pudieron resistir la desnaturalización, la desactivación y la degradación causada por la desecación al aire durante la fase de maduración. Aunque el vermicompost maduro obtenido mostró mayor contenido en complejos humus-enzima que el inicial estiércol maduro de oveja, el sistema de vermicompostaje de alimentación continua ensayado no fue eficaz para intensificar la formación de complejos estables de humus y enzimas.

Palabras clave: Destríos de tomates, vermicompostaje, actividades de enzimas hidrolíticas, complejos humus-enzimas.

ABSTRACT

The behaviour of extracelullar hydrolytic enzyme activities in a continuous-supply vermicomposting system has never been studied previously. The aim of this study was to assess under such system the extracellular enzyme activities glucosidase, urease, acid phosphatase, and protease, which were extracted following the pyrophosphate-extraction method at pH 7.1. Vermicomposting was carried out using a medium-sized rectangular vermireactor continually supplied with damaged tomatoes (10 kg $week^{-1}$) during a 5-month period on a layer of mature sheep manure. The earthworms were then removed, and the vermicomposted organic material was matured over a 2-month period. Total earthworm biomass reached its greatest value after 3 months, coinciding with peak levels for pyrophosphate-extractable carbon content and extracellular urease, acid phosphatase and protease activities. Extracellular β-glucosidase activity peaked in the 4^{th} month, when microbial activity was also at its greatest level. At the end of the vermicomposting period, phosphatase and protease activity decreased to levels similar to those recorded at the beginning of the vermicomposting process. By contrast, β-glucosidase and urease activity values were grater that those recorded at the start of the vermicomposting. Humic-enzyme complexes generated during the vermicomposting period were unable to resist denaturation, inactivation, and degradation caused by the air-drying during the maturation phase. Although the mature vermicompost obtained showed higher content in humus-enzyme complexes than the initial mature sheep manure, the assayed continuous-supply vermicomposting system was inefficient for enhancing the formation of stabilized humus-enzymes complexes.

Keywords: damaged-tomatoes wastes, vermicomposting**,** hydrolytic enzyme activities, humic-enzyme complexes.

3.1. INTRODUCTION

In Spain, approximately 66,100 ha are dedicated to greenhouse horticultural crops, most of which is located in Andalusia (Southern Spain). This intensive agricultural system generates large amounts of different types of waste every year, including vegetable wastes, which have become the greatest problem associated with this type of agriculture by exacerbating environmental problems associated with this crop. Damaged fruit waste consists of unmarketable fruits due to bruising, inappropriate size, rotting, and insect attack as well as marketable fruits that are discarded to raise market prices. In the Motril region alone (Granada, Southern Spain), about 15000 Tm of fruit waste were rejected in 2007, representing 24% of all greenhouse waste generation. Particularly, tomato fruit accounts for 80% of all damaged greenhouse fruit waste generates in this region.

Vermicomposting is an effective low-cost method of transforming organic waste into a stable end-product called vermicompost, which is characterized by relatively high levels of humus-like substances, active microorganisms and enzymes. This biological process can be carried out on a small, medium, or large scale by using a windrow, bed, or bin system and flow-through reactors. The type of system adopted – non-continuous batch-supply mode or continuous-flow supply mode – depends on the nature of the waste input recycled (Edwards, 1995). Previous works have shown that non-continuous vermicomposting systems are capable of producing organic soil amendments and biofertilizers from agricultural, agroindustrial, and urban waste (Elvira et al., 1998; Nogales et al., 2005; Melgar et al., 2009). However, to date, a continuous-supply system has never been used to transform fruit wastes into stabilized organic soil amendment.

Formation of complexes between humic substances and extracellular enzymes is a mechanism to stabilize and protect enzymes in soil, avoiding its denaturation, inactivation and degradation (Burns, 1982; Nannipieri et al., 1996). In non-continuous vermicomposting processes, the activity of some extracellular hydrolytic enzymes extracted with pyrophosphate increased or remained constant, thus suggesting that the humus enzyme complexes resisted microbial and earthworm attack (Benítez et al., 2000, 2005). This is particularly relevant from an ecological point of view, as stabilized and active humus-enzyme complexes in soil environments can reactivate soil C, N, and P-cycles (Pascual et al., 2002). In addition, the active humus-enzyme complexes in the vermicomposts have been found to be particularly useful for soil biochemical remediation when they are used as soil amendments (Benítez et al., 2004, Romero el al., 2005).

As the behavior of extracellular hydrolytic enzyme activity in continuous-supply vermicomposting systems has never been studied, the objective of this study was to analyze the evolution of extracellular enzyme activities during a continuous vermicomposting in order to monitor the process. The relationship between extracellular enzyme activities and humic-like substances was also studied as a possible tool for characterizing the degree of stability of the vermicompost obtained as well as its potential biochemical contributions to soil quality and fertility. To do this, β-glucosidase, acid phosphatase, protease, and urease extracellular enzyme activities were determined in the pyrophosphate extract during a continuous-supply vermicomposting process using damaged tomato-fruit.

3.2. MATERIALS AND METHODS

A medium-sized rectangular metallic vermireactor (0.6 x 0.9 x 0.2 m) was designed, and 0.1 cm mesh was placed at the bottom of the vermireactor. A 5 cm layer containing 15 kg dry weight of sheep manure, whose chemical characteristics are described in Table 3.1, was placed on the mesh to provide an appropriate habitat for earthworms. A total of 500 g of clitellated and non-clitellated earthworms (*Eisenia fetida*) were inoculated in this layer. The worms came from a culture stock in the Estación Experimental del Zaidín (CSIC) in Granada (Spain). Fifteen days after earthworm inoculation, the vermireactor was filled with liquid-paste from damaged tomatoes (moisture: 92%, pH: 3.9, EC: 1.8 dS m^{-1}, TOC: 459 g kg^{-1}, TKN: 23 g kg^{-1}, and C/N: 20) at a loading rate of 10 kg $week^{-1}$. The damaged tomatoes were obtained from greenhouse crops in the Motril region (Granada, Southern Spain).

Vermicomposting was carried out under controlled temperature conditions (25ºC), and no water was added other than that already present in the semiliquid tomato paste. After 5 months, the earthworms were removed by hand, and the vermicomposted organic substrate was left in a pile for a 2-month maturation period without water addition. During the vermicomposting process, the organic substrate was sampled monthly by means of five cylindrical cores (600 cm^3), which were evenly placed in the vermireactor. In each core, earthworm biomass was recorded, and the worms were replaced in the vermireactor. A fraction of the organic substrate (~100 g) contained in each core was taken, homogenized and stored in plastic vials at -20ºC for analysis. In addition, five cores were also taken from the initial layer of sheep manure and the vermicompost obtained after the maturation period for analysis. Chemical and enzyme activities analysis of organic substrate contained in each core was carried out in triplicate.

The pH, electrical conductivity (EC), total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) were determined according to validated methods (M.A.P.A., 1986). The ammonium-N concentration (NH_4^+-N) was determined after extraction with 2 M KCl using a modified salicylate-nitroprusside colorimetric method (Kandeler and Gerber, 1988). Water-soluble carbon (WSC) was extracted with distilled water (1:10 w/v) by mechanical shaking at 60ºC for 1 h. WSC was analyzed in the supernatant after centrifugation 8000 x*g*.

The pyrophosphate extractable carbon (PEC) was extracted with $Na_2P_4O_7$ (0.1 M, pH 7.1) in a 1:10 (w:v) ratio by mechanical shaking at 37°C for 24 h. The suspension was centrifuged at 8000 x*g*; the supernatant was filtered through a 0.45 μm Millipore membrane, which was then dialysed for 7 days against distilled water, which was changed daily, in order to generate the pyrophosphate extractable carbon (PEC) solution. Humic acid-like compounds (HAL) were extracted from 10 ml of a PEC solution by means of acidification with 97% H_2SO_4 (pH 1.0) and then centrifuged. The precipitate was dissolved in 10 ml of 0.5 M NaOH to obtain the HAL solution. To determine the C content of the WSC, PEC, and HAL solutions, 1 ml of each solution was digested with 1 ml of 1N $K_2Cr_2O_7$ and 2 ml of 97% H_2SO_4 at 160 °C for 30 min. Spectrophotometry (λ = 590 nm) was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} (Sims and Haby, 1971).

Dehydrogenase activity was determined incubating 0.5 of solid organic sample at 25ºC for 20 h with 0.2 mL of 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium chloride (INT). Controls without INT were also incubated. The iodonitrotetrazolium formazan (INTF) produced by the reduction of INT was extracted with acetone:tetrachloroethene (1.5:1) and measured in a spectrophotometer at 490 nm (García et al., 1997). To determine the absolute extracellular β-glucosidase and the absolute extracellular acid phosphatase activity, 0.5 ml of PEC solution was incubated at 37°C for 2 h with 0.5 ml of each enzyme-substrate solution and 2 ml of 0.1 M maleate buffer at pH 6.5. An enzyme-substrate solution with 0.05 M 4-nitrophenyl-β-D-glucanopyranoside (*p*-NG) or 0.115 M *p*-nitrophenyl phosphate (*p*-NPP) was used as enzyme-substrate for β-glucosidase or acid phosphatase activity, respectively. Controls were run as an enzyme test, but the enzyme-substrate was added at the end of incubation, before the determination of enzyme-product. The *p*-nitrophenol (*p*-NP) produced in the enzyme reaction was extracted and determined spectrophotometrically at 398 nm as described by Tabatabai and Bremner (1969). Absolute extracellular urease and protease activities were determined using 0.5 ml of PEC solution, 2 ml of 6.4% urea or 0.5 ml N-α-benzoyl-L-argininamide respectively as substrates, and 2 ml of 0.1 M phosphate buffer at pH 7.0. Controls were run as an enzyme test, but distilled waster was added instead of the

enzyme-substrate. Enzyme tests and controls were incubated at 37°C for 2 h and straight afterwards the reaction was stopped cooling down at 2°C for 15 min as described by Nannipieri et al. (1980). The NH_4^+ released into the solution from the hydrolytic reaction was measured using an ammonium-selective electrode (ORION, mod. 95–12). The specific extracellular enzyme activity was calculated from the absolute extracellular enzyme activity values divided by the C content of the PEC solution.

The evolution of all parameters determined during the whole process was statistically tested using repeated analysis of variance (ANOVA) measures. The least significant difference test (LSD) was used for post-hoc mean separation. A paired-sample *t*-test was performed to assess the differences in chemical parameters between initial sheep manure and mature vermicompost. All statistical analyses were conducted using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

3.3. RESULTS AND DISCUSSION

The weekly application of damaged tomatoes did not significantly increase total earthworm biomass during the first two months of vermicomposting (Figure 3.1). Nevertheless, a pronounced increase was observed in the 3rd month, when the percentage of total earthworm biomass was 3.3-fold higher than that at the beginning of the process. However, earthworm biomass decreased sharply in the 4th month, in coincidence with the peak in the microbial activity, as indicated by dehidrogenase activity (Figure 3.1), which is used to assess overall microbial activity during vermicomposting (Benítez et al., 1999). From the 4th month until the end of the vermicomposting process, total earthworm biomass decreased slightly, though not significantly. Conversely, a significant fall in dehydrogenase activity could suggest a microbial turnover in the vermireactor microbiota. The reduction in earthworm biomass could be due to the following factors (shown in Table 3.1): i) an increase in ammonium concentration in the substrate as a consequence of the mineralization of proteins from the tomato-fruit waste and manure layer, ii) an increase in pH value due to the decomposition of abundant organic anions in tomatoes, as reported for other plant residues (Xu et al., 2006), and iii) an increase in electrical conductivity (EC) recorded in the vermireacot substrate due to the weekly salt intake by the tomatoes waste added to the vermireactor. These increases in the organic substrates are well known to negatively affect normal earthworm development during the vermicomposting process (Nogales et al., 2008). The increases in ammonium, pH, and EC could be explained by: i) excessive fresh organic matter in the vermireactor at the 3rd

month as consequence of continuous additions of tomato-fruit waste and ii) the high level of earthworm activity in the 3rd month which greatly stimulated microbial activity. These two factors triggered microbial growth and activity in the vermireactor substrate, leading to imminent system instability. To optimize the benefits of this continuous-supply system in subsequent trials, the organic loading rate should therefore be reduced, the earthworm population halved in the 3rd month, and another vermireactor constructed with an additional manure layer.

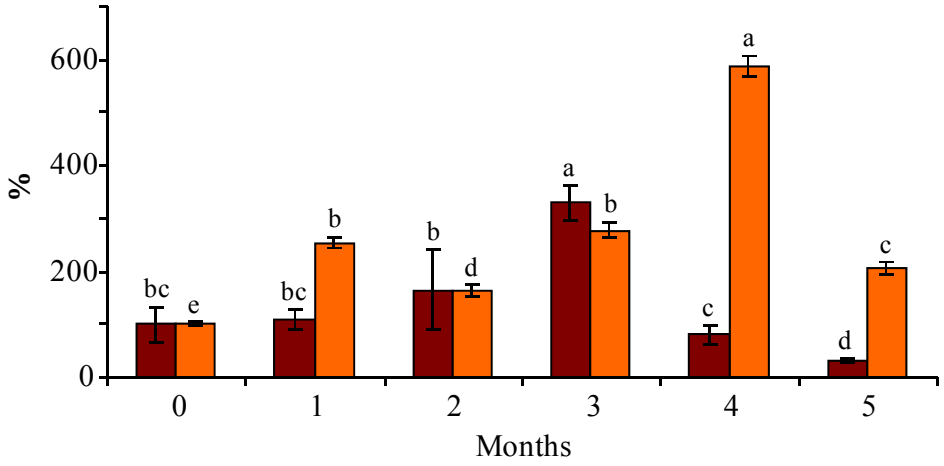


Figure 3.1. Percentage changes in total earthworm biomass (■) and dehydrogenase activity (■) during the vermicomposting period. Bars represent standards errors. In each parameter, columns with different letters indicate significant differences ($P< 0.05$).

Table 3.1. Chemical analyses in the sheep manure (S), at initial (V0) and after the vermicomposting period (V5), and in mature vermicompost (MV).

	pH	EC (dS m^{-1})	NH_4^+-N (mg kg^{-1})	TOC (g kg^{-1})	TKN (g kg^{-1})	WSC (g kg^{-1})	C/N	Moisture (%)
S	8.6±0.3^{c}	1.8±0.1^{b}	438±32^{c}	138±6^{b}	9.6±0.2^{c}	12±0.5^{c}	14±0.1^{a}	7±0.2^{c}
V0	8.3±0.5^{c}	1.7±0.1^{b}	271±42^{b}	140±3^{b}	12±1.2^{b}	18±1.5^{b}	11±0.6^{b}	55±1^{a}
V5	9.5±0.5^{b}	4.5±0.1^{a}	1992±285^{a}	280±12^{a}	24±0.9^{a}	25±3^{a}	12±0.5^{b}	57±3^{a}
MV	10.4±0.3^{a}	4.4±0.03^{a}	1761±71^{a}	148±6^{b}	13±0.4^{b}	17±0.2^{b}	11±0.1^{b}	25±0.8^{b}

In each parameter, different letters indicate significant differences ($P< 0.05$).

The pyrophosphate extractable carbon (PEC) and the humic-acid like substances (HAL) contents (Figure 3.2) peaked in the 3rd month as a result of large amounts of organic matter from additions of tomato-fruit waste. The increase in both PEC and HAL may indicate that all the fresh substrate added was efficiently transformed by earthworms. However, from the 4th month until the end of the vermicomposting process, while more tomato-fruit waste was added, PEC and HAL content fell sharply. This could be attributed to a fall in earthworm biomass together with an increase in microbial activity. The PEC solution contains both humic-like and more recalcitrant compounds (Benítez et al., 2000) which were possibly not degraded by earthworms. Both types of compound could have subsequently been degraded by the proliferation of microorganisms in the 4th month. From the 4th month onwards, PEC and HAL content remained more or less stable.

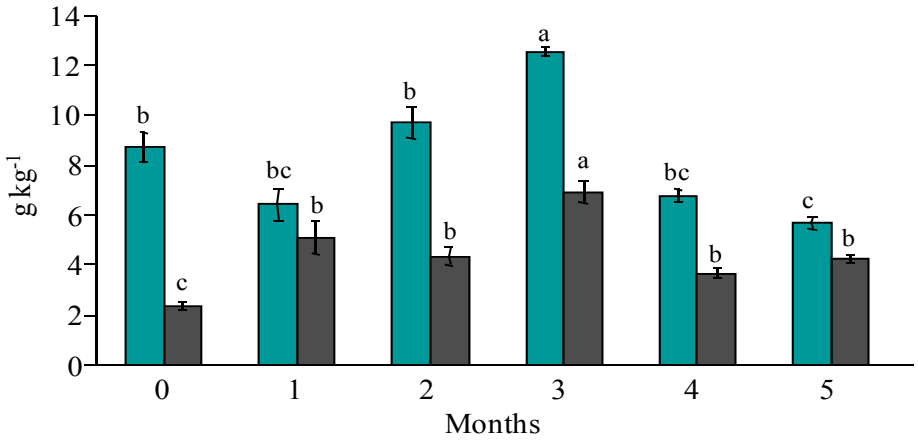


Figure 3.2. PEC (■) and HAL (■) during the vermicomposting process. Bars represent standards errors. In each parameter, columns with different letters indicate significant differences ($P< 0.05$).

Absolute extracellular β-glucosidase activity significantly increased and reached peak levels in the 4th month (3.3a). Although this extracellular enzyme activity later decreased in the 5th month, it remained above the level recorded at the beginning of the vermicomposting process. The maximum level recorded by extracellular β-glucosidase activity coincided with the peak in microbial activity (Figure 3.1). This suggests that extracellular β-glucosidase activity greatly depends on microbes which are responsible for enzyme synthesis, whereas that the reduction in earthworm biomass, PEC, and HAL observed from the 4th month onwards did not affect this extracellular enzyme.

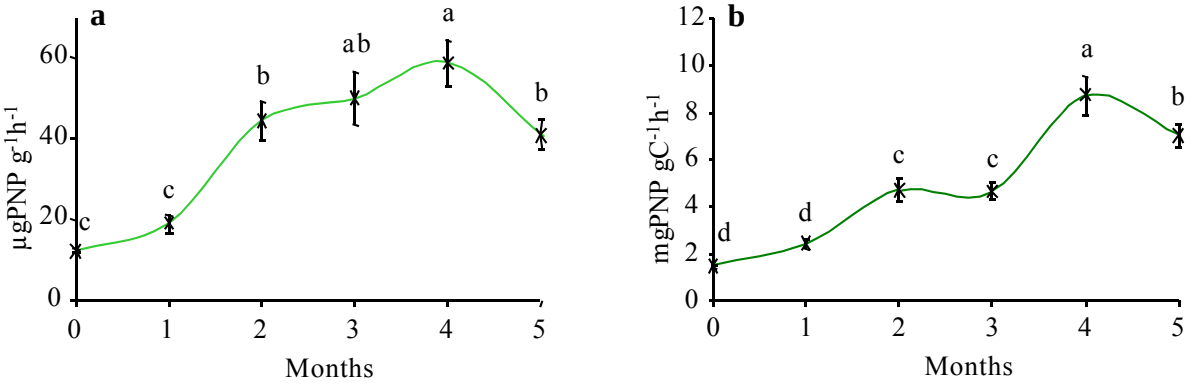


Figure 3.3. Absolute (a) and specific (b) extracellular β-glucosidase activity during the vermicomposting process. Values are means of fifteen replicates. Bars represent standards errors. Different letters indicate significant differences ($P< 0.05$).

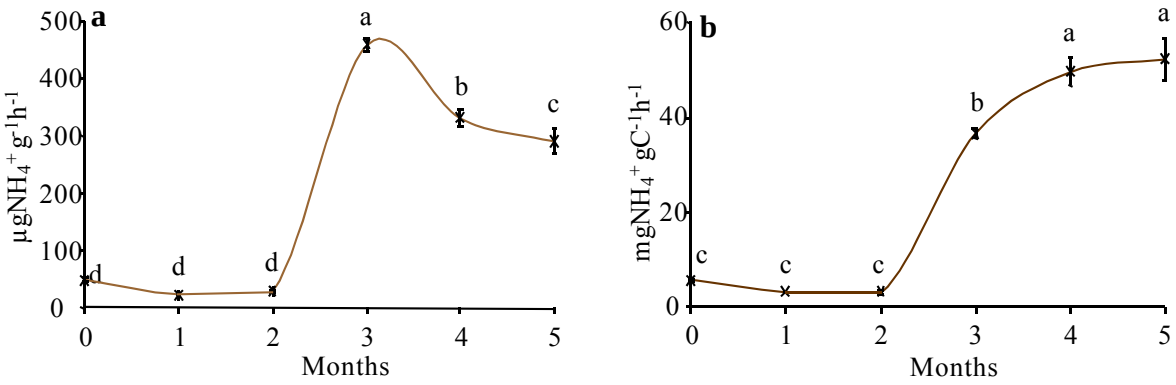


Figure 3.4. Absolute (a) and specific (b) extracellular urease activity during the vermicomposting process. Values are means of fifteen replicates. Bars represent standards errors. Different letters indicate significant differences ($P< 0.05$).

In contrast with absolute extracellular β-glucosidase activity, absolute extracellular urease activity (Figure 3.4a) behaved differently, recording a sharper increase in the 3rd month, coinciding with growth in earthworm biomass (Figure 3.1), and then fell significantly at the end of the vermicomposting process. This pattern of enzyme growth suggests that urease activity in organic extracts was strongly influenced by the earthworm population. On the other hand, despite the reduction in PEC and HAL recorded in the 5th month, the level of absolute extracellular urease activity was higher than that recorded at the beginning of the process. This suggests that a fraction of this extracellular enzyme may bind to humic matter during the vermicomposting process, thus able to remain active in the 5th month despite the high NH_4^+-N concentrations recorded in the substrate (Table 3.1), which inhibit extracellular enzyme activity (McCarty et al., 1992).

The absolute extracellular activity of acid phosphatase and protease showed a similar trend during the vermicomposting process (Figure 3.5a and 3.6a). In both cases, enzyme activity increased significantly and reached a peak in the 3rd month, coinciding with maximum earthworm biomass and PEC content levels, and then decreased sharply to below the values recorded at the start of the process. As with the pattern for absolute extracellular urease activity, the highest level of acid phospatase and protease activity was recorded in the 3rd month, suggesting that the large earthworm population transformed the biological composition of the fresh organic matter through enrichment with microorganisms and extracellular enzymes. In line with these findings, Parthasarathi and Ranganathan (1999, 2000) reported that, compared to initial wastes, the casts freshly produced by earthworms have higher microbial, phosphatase and protease activity as well as larger microbial populations. The decrease in both types of absolute extracellular enzyme activities from the

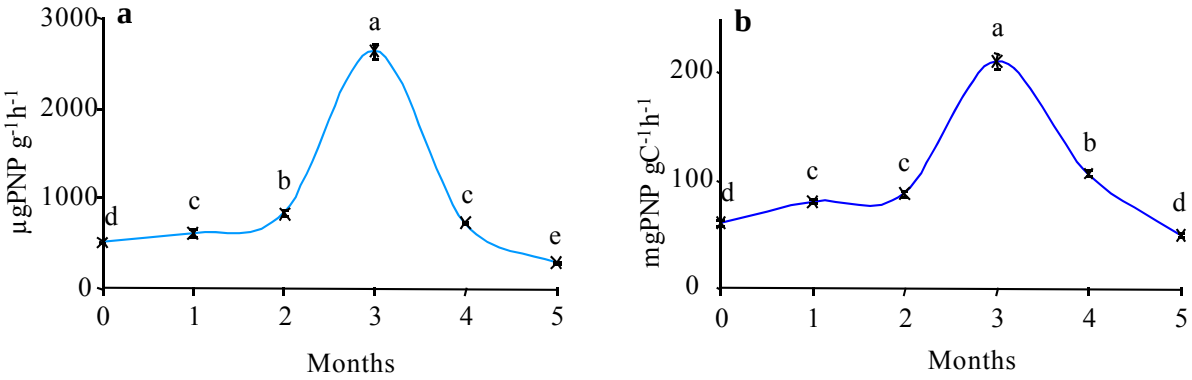


Figure 3.5. Absolute (a) and specific (b) extracellular acid phosphatase activity during the vermicomposting process. Bars represent standards errors. Different letters indicate significant differences ($P< 0.05$).

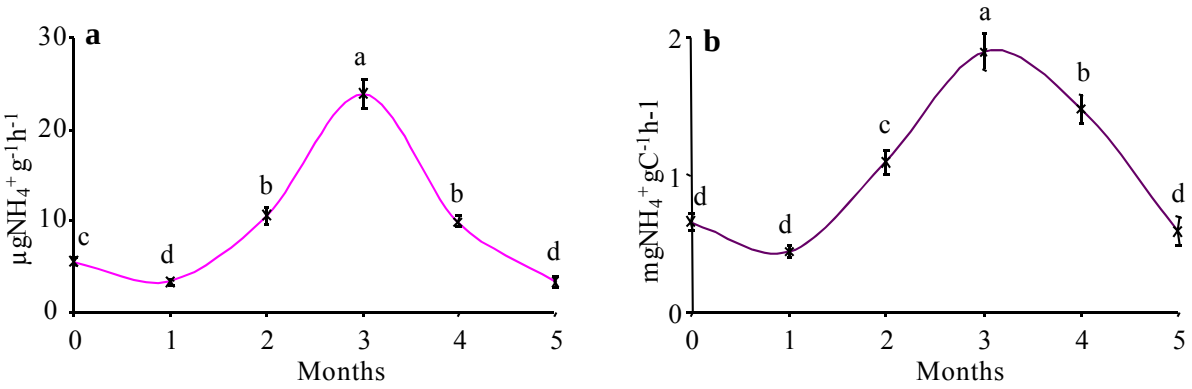


Figure 3.6. Absolute (a) and specific (b) extracellular protease activity during the vermicomposting process. Bars represent standards errors. Different letters indicate significant differences ($P< 0.05$).

3rd month onward could be explained by the reduction in earthworm biomass and the environmental constraints of adverse physico-chemical conditions (increased pH) and product inhibition (NH_4^+-N) observed in the vermireactor (Figure 3.1 and Table 3.1).

The specific extracellular activity (activity per unit of extracted carbon) of theses enzymes showed a similar pattern to absolute extracellular activity (Figure 3.3b, 3.5b and 3.6b), except in the case of specific extracellular urease activity (Figure 3.4b), which unlike its absolute extracellular activity (Figure 3.4a), continued to increase from the 3rd month up to the end of vermicomposting period. Benítez et al. (2000) concluded that the fact that both absolute and specific extracellular enzyme activity increase during vermicomposting gave rise to several assumptions: 1) the association of enzyme with humic substances did not affect activity enzyme sites, 2) the humic-enzyme complex is capable of resisting the microbial or earthworm attack as well as extracellular proteases and others inhibitory and degrading effects, and that 3) extracellular enzymes in vermicompost are more dependent on the type of humic compound than the quantity of C in the extract (García et al., 1993).

Table 3.2. PEC, HAL, absolute and specific extracellular hidrolases in the sheep manure (S), after the vermicomposting period (V5), and in the mature vermicompost (MV).

		S	V5	MV
PEC (g kg^{-1})		2.1±0.1^c	5.7±0.3^b	6.3±0.1^a
HAL (g kg^{-1})		0.6±1^b	4.3±0.2^a	4.4±0.03^a
β-glucosidase	Absolute (μg PNP g^{-1} h^{-1})	1.7±0.2^c	41±4^a	11±0.4^b
	Specific (mg PNPg C^{-1} h^{-1})	0.8±0.1^c	7±0.5^a	1.8±0.1^b
Acid phosphatase	Absolute (μg PNP g^{-1} h^{-1})	7.1±1.5^c	287±14^a	211±4^b
	Specific (mg PNP gC^{-1} h^{-1})	2.9±0.7^c	50±1.3^a	34±0.7^b
Protease	Absolute (μg NH_4^+ g^{-1} h^{-1})	14± 0.9^a	3.2±0.5^b	0.7±0.1^c
	Specific (mg NH_4^+ g C^{-1} h^{-1})	7.2±0.4^a	0.6±0.1^b	0.11±0.01^c
Urease	Absolute (μg NH_4^+ g^{-1} h^{-1})	4.1±0.9^c	291±23^a	29±1.6^b
	Specific (mg NH_4^+ g C^{-1} h^{-1})	2.0±0.4^c	52±4^a	4.6±0.2^b

In each parameter, different letters indicate significant differences ($P< 0.05$).

Slight increases in PEC and HAL was observed in mature vermicompost (MV) as compared with those recorded in fresh vermicompost (V5) (Table 3.2). In comparative terms, PEC and HAL content in the mature vermicompost (MV) was 3- and 7.7-fold higher, respectively, than the levels recorded in sheep manure (S). However, the absolute and specific extracellular enzyme activities were significantly lower in MV than those recorded

in V5 (Table 3.2). It is important to note that most of the humic-enzyme complexes generated by the vermicomposting process were unable to resist denaturation, inactivation, and degradation caused by the air-drying of the vermicompost during the maturation phase (Table 3.1). Burns (1982) reported that the formation of humic complexes stabilizes enzymes and ensures their persistence, which would otherwise be impossible under adverse extracellular environmental conditions. On the other hand, it is well known that air-drying leads to inactivation of extracellular enzymes (Dick, 1994). Since extracellular enzyme activity depends mainly on free enzymes in the vermicompost solution and a smaller fraction linked to humic substances, the drying of the vermicompost caused inactivation of free extracellular enzymes in the organic extract, while stabilized enzymes were protected against the adverse effects of low water content. Absolute and specific extracellular enzyme activities measured in air-dried mature vermicompost thus resembles the activity of enzymes closely linked to humus colloids. We found that the levels of absolute and specific extracellular β-glucosidase, urease, and acid phosphatase activity were significantly higher in the mature vermicompost than in the initial sheep manure used (Table 3.2). However, extracellular protease enzyme complexes produced during the vermicomposting process were not sufficiently stabilized and were thus strongly degraded by drying during the maturation phase, as suggested by the lower extracellular enzyme activity recorded in the mature vermicompost as compared to the initial sheep manure.

3.4. CONCLUSIONS

Extracellular hydrolytic enzyme activities measured in the pyrophosphate extract during the continuous-supplying vermicomposting process showed a pattern that has not previously been reported. Extracellular β-glucosidase activity was related to the microbial activity, while extracellular urease, acid phosphatase, and protease activities were influenced by the earthworm biomass observed during the vermicomposting period. After the maturation period, all extracellular enzyme activity measured in the pyrophosphate extract eventually decreased due to air-drying as the free extracellular enzymes and enzymes weakly linked to humus colloids were denatured or inactivated. Therofore, the assayed continuous-supply vermicomposting system was inefficient for enhancing the formation of stabilised humus-enzymes complexes. Despite this fact, the mature vermicompost obtained showed greater extracelular enzyme activities (β-glucosidae, acid-phosphatase and urease) as compared with

those recorded in the sheep mature used as an initial layer for tomato-fruit waste bioconversion.

ACKNOWLEDGEMENTS

This study supported by Junta de Andalucía (Project P05-AGR-00408). M. Fernández-Gómez thanks the Science and Innovation Ministry for his FPU doctoral Grant. Finally, we would also like to thank Michael O'Shea for proofreading.

REFERENCES

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzymes activities as indicators of the stabilization of sewage sludges composting by *Eisenia andrei*. Bioresource Technology 67, 297-303.

Benítez, E., Nogales, R., Masciandaro, G., Ceccanti, B., 2000. Isolation by IEF of humic–urease complexes from earthworms processed sewage sludges. Biology & Fertility of Soils 31, 489-493.

Benítez, E., Melgar, R., Nogales, R ., 2004 Estimating soil resilience to toxic waste by measuring enzyme activities. Soil Biology & Biochemistry 36, 1615-162.

Benítez, E., Sainz, H., Nogales, R., 2005. Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresource Technology 96, 785–790.

Burns, R.G., 1982. Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biology & Biochemistry 14, 423-427.

Dick, R.P., 1994. Soil Enzyme Activities as Indicators of Soil Quality. In: J.W. Doran, D.C. Coleman, D.F. Bezdicek, B.A. Stewart (Eds), Defining Soil Quality for a Sustainable Environment. American Soil Association-Soil Science Society of America Special Publication No. 35, Madison, WI, USA, pp. 107–124.

Edwards, C.A., 1995. Historical overview of vermicomposting. BioCycle 36, 56-58.

Elvira, C., Sampedro, L., Benítez, E., Nogales, R., 1998. Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: a pilot scale study. Bioresource Technology 6, 205-211.

García, C., Henández, T., Costa, F., Ceccanti, C., Ganni, A., 1993. Hydrolases in the organic matter fractions of sewage sludge: changes with composting. Bioresource Technology 45, 47-52.

García, C., Hernández, T., Costa, F., 1997. Potencial use of dehydrogenase activity as an index of microbial activity in degraded soils. Communications in Soil Science and Plant Analysis 28,123-134.

Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biology & Fertility of Soils 6, 68-72.

MAPA, 1986. Métodos Oficiales de Análisis. Vol. III. Plantas, productos orgánicos fertilizantes, suelos, agua, productos fitosanitarios y fertilizantes inorgánicos. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.

McCarty, G.W., Shogren, D.R., Bremner, J.M., 1992. Regulation of urease production in soil by microbial assimilation of nitrogen. Biology & Fertility of Soils 12, 261-264.

Melgar, R., Benítez, E., Nogales R., 2009. Bioconversion of wastes from olive oil industries using the epigeic earthworm *Eisenia andrei*. Journal of Environmental Science and Health-Part B 44, 488-495.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Science Society of America Journal 44, 1011-1016.

Nannipieri, P., Sequi, P., Fusi, P., 1996. Humus and Enzyme Activity. In: A. Piccolo (Ed.) Humic Substances in Terrestrial Ecosystems. Elsevier Science, Amsterdam, pp. 293–328

Nogales, R., Cifuentes, C., Benítez, E., 2005. Vermicomposting of winery wastes: A laboratory study. Journal of Environmental Science and Health-Part B, 49: 659-673

Nogales, R., Domínguez, J., Mato, S., 2008. Vermicompostaje. In: J. Moreno, R. Moral (Eds.). Compostaje. Editorial Mundi-Prensa, Madrid, Spain, pp. 187-2008.

Parthasarathi, K., Ranganathan, L.S., 1999. Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. European Journal of Soil Biology 35, 107-113.

Parthasarathi, K., Ranganathan, L.S., 2000. Aging effect on enzyme activities in pressmud vermicasts of Lampito mauritii (Kinberg) and Eudrilus eugeniae (Kinberg). Biology & Fertility of Soils 30, 347-350.

Pascual, J.A., Moreno, J.L., Hernández, T., García, C., 2002. Persistence of immobilised and total urease and phosphatase activities in a soil amended with organic wastes. Bioresource Technology 82, 73-78.

Romero, E., Benítez, E., Nogales, R., 2005. Suitability of wastes from olive-oil industry for initial reclamation of a Pb/Zn mine tailing. Water Air and Soil Pollution 165, 153-165.

Sims, J.R., Haby, V.A., 1971. Simplified colorimetric determination of soil organic matter. Soil Science 112, 137-141.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenylphosphate for assay of soil phosphatase activity. Soil Biology & Biochemistry 1, 301-307.

Xu, J.M., Tang, C., Chen, Z.L., 2006. The role of plant residues in pH change of acid soils differing in initial pH. Soil Biology & Biochemistry 38, 709-719.

CAPÍTULO 4
CHAPTER 4

RECICLAJE DE RESIDUOS VEGETALES AMBIENTALMENTE PROBLEMÁTICOS GENERADOS POR CULTIVOS DE TOMATE EN INVERNADERO MEDIANTE VERMICOMPOSTAJE.

RECYCLING OF ENVIRONMENTALLY PROBLEMATIC PLANT WASTES GENERATED FROM GREENHOUSE TOMATO CROPS THROUGH VERMICOMPOSTING

Fernández-Gómez, M.J., Díaz-Raviña, M., Romero, E., Nogales, R (2011). Recycling of environmentally problematic plant wastes generated from greenhouse tomato crops through vermicomposting. International Journal of Environmental Science and Technology. Enviado / *Submitted*. (3417-IJEST).

RESUMEN

La gran cantidad de residuos vegetales producidos por cultivos de tomate en invernadero es un problema ambiental que debería ser solucionado reciclando este residuo en productos orgánicos de valor mediante tecnologías de bajo coste tales como el vermicompostaje. En el presente estudio, se investigó la viabilidad de vermicompostar residuos de invernadero de plantas de tomate (P) utilizando como residuo acondicionante lodos residuales de industrias papeleras (S). El desarrollo de lombrices que tuvo lugar en P, S y en dos mezclas de ambos residuos se monitorizó durante 24 semanas y se comparó con el sucedido en estiércol de vaca (D), un residuo orgánico óptimo para ser vermicompostado. La eficacia del vermicompostaje para bioestabilizar esos residuos se valoró analizando la composición en ácidos grasos, las características químicas, el contenido en nutrientes vegetales, la concentración de metales, las actividades enzimáticas y el índice de germinación. Un vermicompost comercial fue también analizado y considerado como referente de un vermicompost de calidad. Las lombrices no sobrevivieron en P, pero mezclando P con S en una relación 2:1 o 1:1 fue posible obtener un desarrollo de las lombrices similar al observado en el estiércol (D). El análisis PLFA reveló que la actividad de las lombrices transformó intensamente la microbiota inicial residente en los residuos, dando lugar a comunidades microbianas en los vermicomposts que fueron similares a las de un vermicompost comercial. Ambas mezclas de P y S fueron biostabilizadas correctamente mediante vermicompostaje según dejó patente, tras el proceso, las disminuciones de sus relaciones C:N y sus actividades enzimáticas junto con los aumentos de sus grados de madurez (GI~100%). Este estudio demostró que el vermicompostaje de ressiduos de planta de tomate junto con lodos de papelera permite el reciclaje de ambos residuos, mejorando la sostenibilidad ambiental de los cultivos de invernadero.

Palabras clave: Bioestabilización, lombrices *Eisenia fetida*, actividad enzimática, ácidos grasos fosfolipídicos microbianos, lodo de la fabricación de papel.

ABSTRACT

The enormous quantity of plant waste produced from greenhouse tomato crops is an environmental problem that should be solved by recycling of that waste into valuable organic-products through low-cost technologies such as vermicomposting. Feasibility of vermicomposting greenhouse tomato-plant waste (P) by using paper-mill sludge (S) as complementary waste was investigated by this study. Earthworm development occurred in P, S, and two mixtures of both wastes was monitored over 24 weeks and compared with that in cow dung (D), an optimum organic-waste to be vermicomposted. The effectiveness of vermicomposting to biostabilize those wastes was assessed by analysing phospholipid fatty acids composition, chemical features, plant nutrient content, metal concentration, enzyme activities, and germination index. A commercial vermicompost was also analysed and taken as a reference mark of vermicompost quality. Earthworms did not survive in P alone, but mixing P with S in a ratio of 2:1 or 1:1 was possible to obtain an earthworm development similar to that observed in D. PLFA analysis revealed that earthworm activity strongly transformed initial microbiota inhabiting the wastes, giving rise to vermicompost microbial communities which were similar to that of a commercial vermicompost. Both mixtures of P and S were properly biostabilized through vermicomposting as indicated by decreases in their C:N ratio and enzyme activities together with raises in their degree of maturity (GI~100%) after the process. This study demonstrated that the vermicomposting of tomato-plant waste together with paper-mill sludge allows recycling of both wastes, improving the environmental sustainability of greenhouse crops.

Keywords*:* Biostabilization, *Eisenia fetida* earthworms, enzyme activity, microbial phospholipid fatty acids, paper-mill sludge.

4.1. INTRODUCTION

Greenhouse crops are rapidly expanding in many countries because these agricultural systems are able to product high-value vegetables in great amounts during all year. Although current statistical data on greenhouse crops at world level are quite difficult to find, previous surveys reported that there are more than 2 million hectares of greenhouses in the world (Pardossi et al., 2004). Among all different plant grown in greenhouse systems tomato is the predominant crop (Alkoaik and Ghaly, 2006). Despite growing tomato in greenhouses is a profitable agricultural practice, such intensive crop causes an environmental impact due to the enormous quantity of tomato-plant discarded after greenhouse crop harvest. Indeed, greenhouse tomato crops produce the greatest amount of plant waste, around 49 tonnes per greenhouse hectare and year, as compared with other greenhouse crops which produce a average amount of 28.5 t ha $^{-1}$ year $^{-1}$ (Manzano-Agugliaro, 2007). Greenhouse plant wastes are often eliminated by abandon them in dry ravines or empty areas originating uncontrolled burning, blocking of riverbeds, poisoning of cattle and sheep, and a negative visual impact on the landscape (Parra et al., 2008). In addition, the disposal of greenhouse plant waste into landfills is an environmental problem as that waste is a potential source of methane, a gas which negatively contributes to global warming (Bicheldey and Latushkina, 2010). Therefore, the annual production of plant waste from greenhouse crops arise a global environmental problem which requires urgent actions.

The large quantity of plant waste produced from greenhouse crops should be considered an opportunity to generate energy or to produce valuable organic-products. Given that the vegetable production in greenhouses needs abundant amounts of organic matter to renew the organic layer placed within greenhouse soil, the transformation of greenhouse plant waste into a valuable organic-material through low-cost technologies such as composting or vermicomposting can be the preferred recycling method (Parra et al., 2008). Furthermore, the production of organic soil amendments through composting or vermicomposting of greenhouse plant waste should be a priority taking into consideration that most of southern European soils are degraded owing to a lack of organic matter (Zdruli et al., 2004). Despite those facts, greenhouse plant waste is hardly recycled into a well stabilized organic material due to this type of waste has features that hinder a proper composting or vermicomposting, producing therefore unusable organic materials for agricultural practices or soil restoration. Greenhouse plant wastes from diverse crops has features in common such as a high moisture, low C/N, and high portion of lignin fibres, which protect this waste type from

microbial attack, hindering composing processes. In the particular case of greenhouse tomato-plant waste, previous studies have concluded that waste has a bioavailable carbon content that is insufficient to develop an satisfactory thermophilic phase, which is necessary for its proper composting (Alkoaik and Ghaly, 2005, 2006). The vermicomposting of greenhouse tomato-plant waste have been also reported unfeasibly as that waste has lethal concentrations of salts and ammonia to earthworms (Fernández-Gómez et al., 2010). Consequently, to date, there not studies which have successfully transformed greenhouse plant wastes into organic-materials with agricultural value by using composting or vermicomposting.

Several previous studies have been reported that paper-mill sludge is also inadequate waste for earthworm development, although that waste can be successfully vermicomposted by preparing mixtures with another waste rich in nitrogen (Elvira et al., 1996, 1997; Kaur et al., 2010). Taking into consideration the physical structure and chemical composition of greenhouse tomato-plant waste and paper-mill sludge, both organic wastes could complement each other. Thus, mixing both wastes can produce an organic substrate which may have a nutritive value for earthworms greater than that of each single waste. Moreover, tomato-plant waste could act as a bulking agent for the paper-mill sludge, improving the microclimatic condition of that waste and thus accelerating the simultaneously decomposition of both waste. In short, mixing both wastes types in a suitable waste ratio may result in an organic substrate which could be more suitable to be stabilized through vermicomposting than each waste alone. To test this hypothesis, the feasibility of vermicomposting greenhouse tomato-plant waste mixed with paper-mill sludge in two different ratios was investigated by this study. Those wastes were produced in 2010 at the area of Motril (Granada, Southern Spain), where an intensive production of tomato is carried out by using greenhouse systems and a paper-mill company is located. The earthworm species *Eisenia fetida* was used for vermicomposting due to this earthworm species is commonly used in temperate climates and has been recently demonstrated to be able of vermicomposting of paper-mill sludge (Kaur et al., 2010). Consequently, an optimum waste to be biostabilized by using *E. fetida* (i.e. cow dung) was assayed as a control vermicomposting as described by previous studies (Gupta and Garg, 2009). The effectiveness of vermicomposting to biostabilize the different organic wastes was assessed by analysing their chemical features, enzyme activities, germination index, and phospholipid fatty acids before and after vermicomposting. In addition, a commercial vermicompost

produced from cattle manure by using *E. fetida* was analysed and taken as a reference mark of vermicompost quality for assessing the vermicomposts produced in this study.

4.2. MATERIALS AND METHODS

4.2.1. Earthworms and organic-waste collection

Non-clitellated earthworms (*E. fetida*) were selected from a culture bank at the Estación Experimental del Zaidín (CSIC), Granada, Spain. Tomato-plant waste (P) consisted of leaves, stems and roots from whole tomato-plants discarded by greenhouse crops after harvest. P was collected from an organic-waste-treatment installation for greenhouse wastes located in Motril (Granada, Southern Spain) where they were being accumulated and air dried. Paper-mill sludge (S) was a secondary sludge (paper pulp) which was collected from the wastewater-treatment plant of Torraspapel S.A, a paper-mill company also located in Motril (Granada, Southern Spain). Cow dung (D), which was a mixture of faeces and urine without any bedding material, was collected from a farmyard of dairy cows located in Granada (Spain). A stable and mature commercial vermicompost (CV) was purchased from Humus-Fertil Company (La Roda, Albacete, Spain). CV was produced from cattle manure which was processed in large-scale windrow system by using *E. fetida*.

4.2.2. Experimental set-up

P was chopped (<1 cm) and mixed with S in two ratios (dw:dw): a ratio of two to one (PS 2:1) and another ratio of one to one (PS 1:1). Three mixtures of 200 g (dw) each one were separately made by mixing P and S at 2:1 ratio. Similarly, three mixtures were made by mixing P and S at 1:1 ratio. Each single mixture was placed on a rectangular glass container (15 cm × 15 cm × 5 cm) and its moisture content was adjusted to 80% with water. After a 2-week period in which the mixtures were air-dried (20ºC) in order to eliminate the volatile substances potentially toxic to earthworms, each organic substrate was remoisturized and placed inside a plastic circular pot (13 cm diameter x 12 cm high), which had a perforated bottom for allowing aeration and drainage. Thus, three replicated vermibeds constituted of PS 2:1 and three of PS 1:1 were constructed. Another two types of vermibeds were constructed in triplicate as similar way as described above. One vermibed type made of P as the unique material, and the other type constituted of S alone. In addition, three control

vermibeds constituted of 200 g (dw) of cow dung (D) each one were similarly constructed. A layer of material partially vermicomposted (1 cm), which was collected from the earthworm culture bank, was placed on the top surface of each vermibed and 25 non-clitellated earthworms, weighing 0.20 g each one were inoculated within. All vermibeds were kept in darkness at 20ºC for 24 weeks, maintaining their moisture percent around 80% by periodical watering. The earthworms were counted and weighed every week during the vermicomposting period. At the end of the process, the earthworms were removed by hand. Vermibed substrates were sampled immediately before earthworm inoculation and also at the end of vermicomposting. Samples were homogenized and divided into two subsamples, one of which was dried and finely ground for chemical analyses and maturity test, and the other was stored at –20°C for enzyme activities and PLFA analyses.

4.2.3. Chemical analyses

The pH and electrical conductivity (EC) were measured in a 1:10 sample:water (dw:v) ratio by using a pH meter and a conductivity meter, respectively. Total organic carbon (TOC) and total nitrogen (N) were determined with a CN analyzer. Water-soluble carbon (WSC) was extracted by mechanical shaking at 60ºC for 1 h with distilled water (1:10 sample:water; dw:v). Afterwards, 1 ml of the extract was digested with 1 ml of 1 N $K_2Cr_2O_7$ and 2 ml of 97% H_2SO_4 at 160°C for 30 min. Total P was measured using the ammonium vanadomolybdate method. Total K and Na were measured by flame-photometry and total Ca, Mg, Fe, Mn, Cu, Zn were determined by atomic absorption spectrometry after a digestion of the samples with HNO_3:$HClO_4$ (Williams, 1984).

4.2.4. Enzyme activity analyses

Total enzyme activities were determined in each sample (0.2 g) per triplicate. Dehydrogenase activity was determined according to the method described by García et al. (1997). The β-glucosidase and acid phosphatase activities were determined by measuring the amount of p-nitrophenol (PNP) produced from 4-nitrophenyl-β-D glucanopyranoside (PNG) and 4-nitrophenyl phosphate (PNPP) according to the methods reported by Tabatabai (1982) and Tabatabai and Bremner (1969), respectively. Urea or N-α-benzoyl-L-argininamide was used as the substrate for determination of urease or protease activities as described by Kandeler and Gerber (1988) and Bonmatí et al. (1998), respectively. The NH_4^+ produced by

both types of enzymes was measured after extraction with 2 M KCl through a salicylate-nitroprusside colorimetric method (Kandeler and Gerber, 1988).

4.2.5. Germination index

Germination index (GI) was determined in each sample per triplicate according to the method reported by Zucconi et al. (1981). Briefly, GI was estimated by using 10 cress seeds (*Lepidium sativum*) inside a Petri plate containing a 1 ml of 1:10 sample:water (dw:v) solution. After incubation in darkness at 25ºC for 24 h, seed germination percentage and radicle growth was determined.

4.2.6. Phospholipid fatty acid (PLFA) analysis

PLFA analysis is a technique based on the precept that some PLFAs are considered to be absolute signature substances (biomarkers) for specific groups of organisms, which provides qualitative and quantitative information on viable microorganisms inhabiting complex sample since PLFAs are quickly degraded upon microbial death (Marschner, 2007). The concentration of phospholipid fatty acids (PLFA) in each sample was determined as described by Fostergård et al. (1993). Briefly, the lipids were extracted from 0.5 g of each sample with a chloroform:methanol:citrate buffer mixture 1:2:0.8 (v:v:v) and separated into neutral lipids, glycolipids, and phospholipids by using a pre-packed silicic acid column. Subsequently, the phospholipids were subjected to a mild alkaline methanolysis and the resulting fatty acid methyl esters were identified by gas chromatography (flame-ionization detector) according their relative retention times of the fatty acids, using methyl nondecanoate (19:0) as an internal standard. The PLFAs were designated in terms of total number of carbon atoms and position of the double bonds (ω). Prefixes ‘a’, ‘i’, ‘cy’ and ‘Me’ refer to anteiso, iso, cyclopropyl and methyl branching. As described by previous studies (Marschner, 2007; Moore-Kucera and Dick, 2008) the sum of i15:0, a15:0, i16:0, i17:0, and a17:0 was used to estimate the biomass of Gram-positive bacterial in each sample; the sum of 16:1ω7c, cy17:0, 18:1ω7, and cy19:0 to estimate the biomass of Gram-negative bacteria; the sum of 10Me16:0, 10Me17:0, and 10Me18:0 to measure the biomass of actinomycetes; and the sum of a16:1ω5, 18:1ω9, and 18:2ω6 to calculate the biomass of fungi. The sum of PLFA biomarkers for Gram-positive bacteria, Gram-negative bacteria, and actinomycetes,

plus the fatty acids 14:0, 15:0, and 17:0 were taken as total biomass of all bacteria contained in a sample. Total viable microbial biomass was estimated as the sum of all the extracted PLFAs per gram of material (nmol PLFA g^{-1}).

4.2.7. Data analysis

Repeated-measures ANOVA analyses were performed to evaluate the earthworm development over time in each vermicomposting process. Paired-sample *t*-Student test was performed to assess differences in the parameters measured in each organic substrate before and after vermicomposting. In addition, one-way ANOVA with mean separation based on Tukey test was performed to asses the difference in the analysed parameters among the organic wastes or among the resulting vermicompost. Concerning PLFA analysing, statistical analyses were performed using a normally distributed data set resulting of transform PLFA concentrations (log (x_i+1)). Only a principal component analysis (PCA) was performed by using the untransformed PLFA date set. The statistical analyses were conducted at >95% confidence level (p <0.05) using the software SPSS® 13.0 (Chicago, Illinois, USA).

4.3. RESULTS AND DISCUSSION

4.3.1. Earthworm development in the different vermibeds

All the earthworms placed in the vermibeds constituted of P alone died after 24 h. This could be due to the excessive salts concentration in that waste (Table 4.2). Salts concentration in organic waste over 8 dS m^{-1} has been considered to be lethal for *E. fetida* (Edwards, 1988). In line with this result, Fernández-Gómez et al. (2010) found that *Eisenia andrei*, an earthworm species closely related to *E. fetida*, died after 24 h when was placed in a vermibed constituted of greenhouse heterogeneous-plant wastes due to its great salinity (17 dS m^{-1}). Therefore, P cannot be vermicomposted as single waste by using *E. fetida*. The vermibeds made of P alone were therefore discarded since an earthworm activity is compulsory for the successful stabilization of a wastes through vermicomposting (Domínguez et al., 2010). Previous studies have demonstrated that the degradation of organic wastes under mesophilic condition without earthworms results in materials which are insufficiently degraded and

mineralized as compared with vermicomposted materials (Atiyeh et al., 2000; Elvira et al., 1996; Kaur et al., 2010).

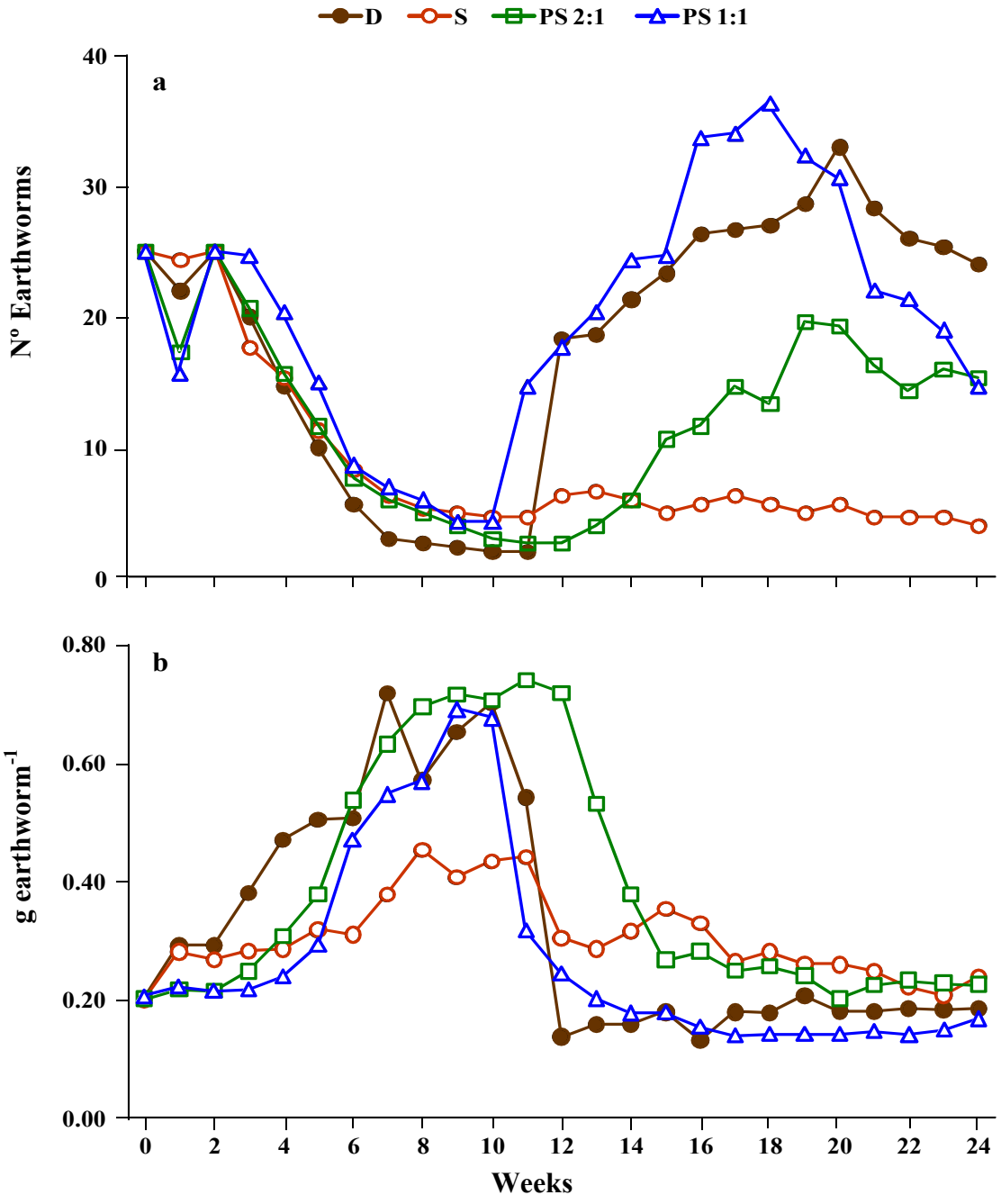


Figure 4.1. Number (a) and mean earthworm weigh (b) during the vermicomposting of cow dung (D, control), paper-mill sludge (S), and plant waste mixed with paper-mill sludge in 2:1 ratio (PS 2:1) or 1:1 ratio (PS 1:1). Each symbol indicates the mean of three replicates.

All earthworms were alive in the vermibeds constituted of PS 2:1, PS 1:1, and S after 24 h, indicating that those organic substrates were not lethal for *E. fetida*. Nevertheless, 39%,

43%, and 20% earthworm mortality was observed after two week in the vermicomposting of PS 2:1, PS 1:1, and S, respectively (Figure 4.1a). However, those percents of earthworm mortality were not significant different from that registered in control vermibeds after two weeks (31%) (F=1.53, p<0.28). To offset the possible earthworm death because of the stress caused by the initial earthworm handling, new juvenile earthworms (0.20 g) were carefully inoculated again at the 2nd week, reaching the same earthworms population (25) of as at the beginning of the experiment. However, from the 2nd until the 10th week, a significant declines in earthworm population (F= 281.78, p <0.05) were still observed in the vermicomposting of PS 2:1, PS 1:1, and S, in which cases were similar to that occurred in the vermicomposting of D (F= 1.78, p= 0.23) (Figure 4.1a). As the earthworm population decreased over time all the surviving earthworms significantly gained weigh (F= 28.02, p <0.05) (Figure 4.1b). This suggests that the reduction in earthworm population observed in all the vermibeds was due to a high competition among earthworms inoculated during the earthworm acclimation phase. At the 10th week of vermicomposting, the earthworms fed on PS 2:1 or PS 1:1 had mean weighs that were 3.3- or 3.1-fold initial value, while mean weight of earthworm fed on D was 2.4-fold initial. Earthworm fed on S alone had a mean weight which was 1.6-fold initial individual weight after teen weeks. These results suggest that mixing S with P produces organic substrates which are more nutritious for *E. fetida* than S alone. From the 10th week to the end, significant differences were observed in the earthworm populations developed over time in different organic substrates (earthworm population: F= 2.90, p <0.05). During that period, the earthworm population rose in vermibeds constituted of PS 2:1 or PS 1:1, showing growth curves which were similar to that occurred in control vermibeds (D). By contrast, the earthworm population observed in vermibeds constituted of S did not increase its number over time. This was due to a low reproductive rate of the earthworms fed on S, which produced a small number of new hatched earthworms as compared with that in the other vermibed types. Previous studies found a lower earthworm reproduction during the vermicomposting of paper-mill sludge as compared with that in the vermicomposting of this waste mixed with sewage sludge (Elvira et al., 1997) or with cattle dung (Kaur et al., 2010), concluding that paper-mill sludge must be supplemented with other organic materials in order to achieve an adequate biostabilization by vermicomposting. Finally, the absence of a new increase in the mean earthworm weigh suggests that the organic substrates have been completely degraded and more fresh material had to be added to maintain earthworm growth (Figure 4.1b).

4.3.2. Assessment of waste transformation through PLFA analysis

Microbial biomass initially contained in all substrates, estimated as the sum of all the extracted PLFA per gram of material, decreased after the vermicomposting process (Table 4.1). More pronounced decreases were found in the substrates D and PS 1:1 whereas S and PS 2:1 showed less accentuated decreases which were non-significant. This may be related to the comparatively better earthworm development observed in D and PS 1:1 since individual can reduce microbial biomass by selectively feeding on bacteria and fungi or indirectly by depleting resources for these microorganisms (Domínguez et al., 2010). Aira et al. (2006) reported that the sharp decrease in the microbial biomass after vermicomposting of pig slurry is a result of mineralization and stabilization of that waste. Interestingly, vermicomposting caused significant decreases in fungal PLFA in all substrate types. This could be explained because fungi cells are considered to be one of the main components of the earthworms' diet (Schönholzer et al., 1999). Thus, the inadequate earthworm development observed in earthworms fed on S could be partially attributed to its lower fungal biomass compared to that in the other organic substrates. In this sense, PLFA analysis could be useful for exploring organic wastes before vermicomposting in order to assess whether the wastes could be microbiologically adequate for earthworm growth.

The PCA was performed with the whole PLFA data set was useful to compare initial waste substrates and resulting vermicomposts according to their microbial community structure (Figure 4.2). The distribution of samples on the plane defined by PC1 and PC2 (Figure 4.2a), which explained 42% of the total variance, clearly separated the plant waste (P) from the rest of initial substrates (lower values along PC2) and from the resulting vermicomposts (higher values along PC2). Likewise, a separation of the initial substrates from the resulting vermicomposts was shown by the plane defined by PC3 and PC4 (Figure 4.2b), which explained 27% of the total variance. This PCA analysis indicated that: 1) microorganisms of greenhouse plant wastes differed greatly from those of the other samples, perhaps due to the lower pH, higher TOC content, and EC recorded in P as compared with the other initial substrates and end vermicomposts; 2) microbial communities of different vermicomposts were grouped together, indicating that vermicomposting played a key role in shaping the microbial community structure in different substrates. In agree with these results, Gómez-Brandón et al. (2010) reported that PLFA analysis is useful to discriminate waste stabilized by earthworms from unprocessed wastes.

Table 4.1. Phospholipid fatty acid contents (nmol PLFA g^{-1}) analysed before (B) and after (A) vermicomposting.

	Total microorganism		Fungi		Bacteria		Gram-negative bacteria		Gram-positive bacteria		Actinomycetes	
Substrate	B	A	B	A	B	A	B	A	B	A	B	A
P	41c	-	6d	-	11d	-	0d	-	8.5d	-	2b	-
S	293b	71b*	25c	8b*	171b	43b	106b	19b	50bc	18b	6b	4b
PS 2:1	301b	198a*	66b	33a*	84c	106a	44c	56a	32c	34a	2b	11a*
PS 1:1	465b	89b*	125a	15b*	196b	45b*	112b	24b*	67b	13b*	7b	7b
D	1134a	76b*	181a	8b*	663a	37b*	319a	17b*	212a	12b*	99a	6b*
CV	-	173a	-	20ab	-	107a*	-	40a	-	45a	-	17a

D: cow dung; P: tomato-plant waste; S: paper-mill sludge; CV: commercial vermicompost. Values in the same column followed by same letters are not significantly different from each other (ANOVA, p <0.05). The asterisk (*) denotes a significant difference between the initial organic substrate and the resulting vermicomposts (Paired-sample t-Student test, p <0.05).

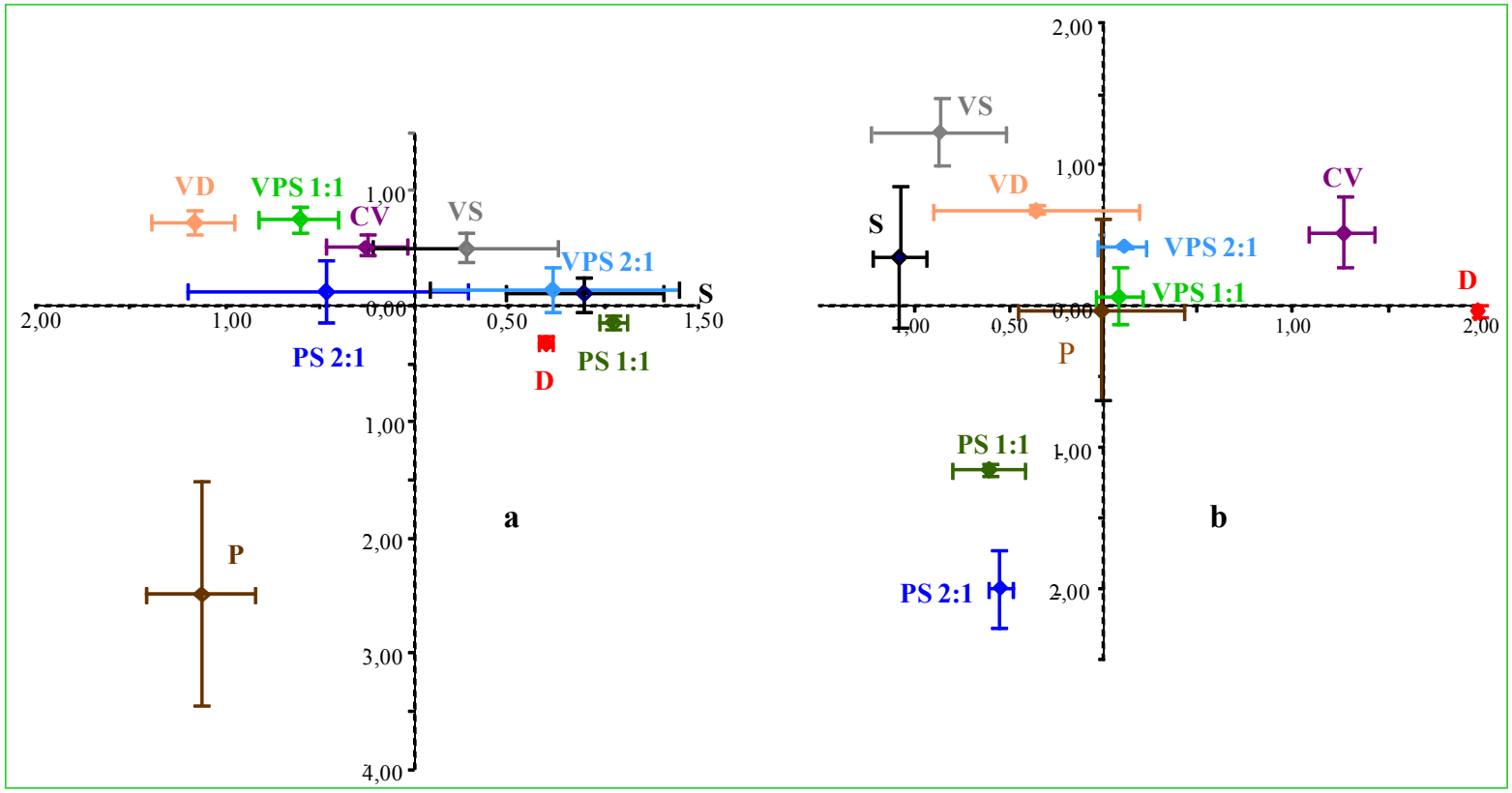


Figure 4.2. Principal component analysis of PLFA content in initial substrates (i.e. P, D, S, PS 2:1, and PS 1:1), resulting vermicomposts (i.e. VD, VS, VPS 2:1, and VPS 1:1), and a commercial vermicompost (i.e. CV). Error bars represent the mean standard error (n=3). D: cow dung; P: plant wastes; S: paper-mill sludge

The relative abundance of biomarker PLFAs for different microbial groups recorded in the vermicomposts produced in this study was compared with that of a commercial vermicompost (CV), which is considered stable, mature, and suitable for agricultural practices (Figure 4.3). For all vermicomposts produced (VD, VS, VPS 2:1, VPS 1:1), PLFA percentages for Gram-positive bacteria, Gram-negative bacteria, actinomycetes and fungi relative to the total PLFA amount were in the range of 23-28%, 15-25%, 5-8%, and 10-17%, respectively. These values were quite close to the relative abundance of these microbial groups registered in CV by PLFA analysis. However, a smaller percentage of Gram-negative bacteria and a greater percentage of fungi were observed in the vermicomposts produced from both mixtures of P and S (PS 2:1 and PS 1:1) compared to those in CV. This suggests that P could boost the fungal growth in these vermibeds. These results are supported by Fernández-Gómez et al. (2010), who reported that earthworms greatly transformed different initial substrates into vermicomposts which were characterized by quite similar fingerprints to each other. These authors concluded that the dissimilarities found among those fingerprints can be attributed to the different chemical composition of each initial substrate vermicomposted.

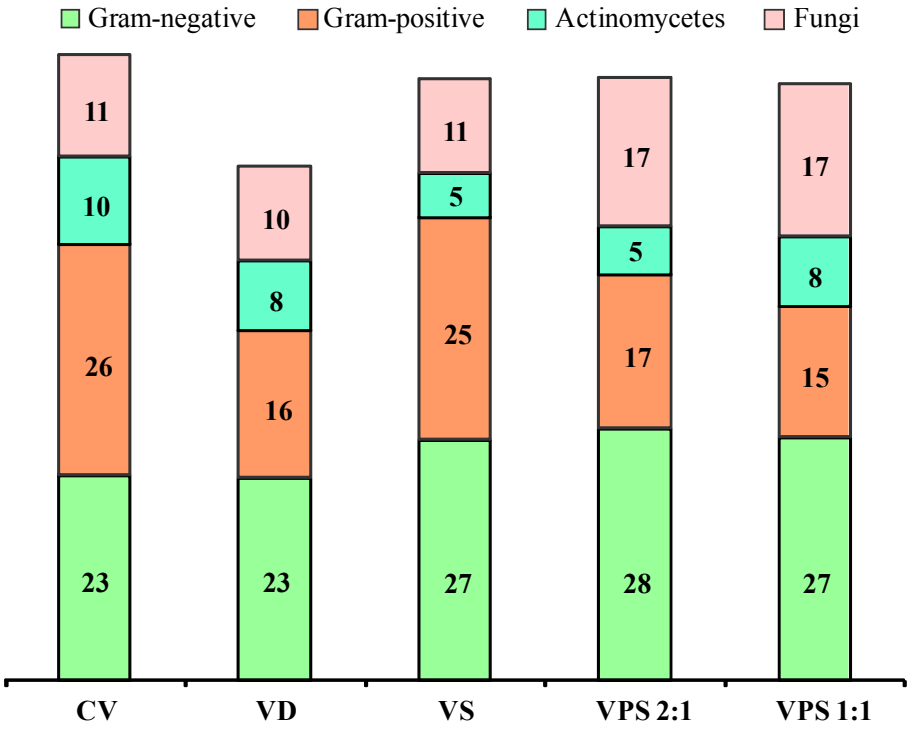


Figure 4.3. Relative concentrations (%) of PLFA used as biomarkers for specific microbial groups recorded in vermicomposts produced in this study (i.e. VD, VS, VPS 2:1, and VPS 1:1), and in a commercial vermicompost used as reference (i.e. CV). D: cow dung; P: plant wastes; S: paper-mill sludge.

4.3.3. Assessment of waste biostabilization through chemical analyses

After 24 weeks initial chemical properties of the different vermicomposting substrates varied depending on the wastes which comprised the vermibeds (Table 4.2). The value of pH increased after vermicomposting only in the case of organic substrate which contained P. This may be a consequence of degrading organic acids contained in P during the vermicomposting of PS 2:1 and PS 1:1. Degradation of P mixed with S was shown by significant decreases in the initial TOC of PS 2:1 (39%) and PS 1:1 (34%), whereas a non-significant reduction in TOC was observed after vermicomposting of S; maybe because the TOC content in S was already little prior to vermicomposting. This indicates that a large fraction of organic matter content in P was mineralized into CO_2 or earthworm biomass during vermicomposting. In the case of vermicomposting control, 29% decrease in the initial content in TOC of D was recorded. Degradation of the waste was also evidenced by decreases in WSC, which has been used as a indicator of the easily metabolised organic-matter fraction in organic wastes during vermicomposting (Benítez et al., 1999), occurred after all the vermicomposting processes. Earthworms can directly break down organic waste and thus assimilate carbon from the more labile fractions of organic wastes (Edwards, 1988). In addition, earthworms indirectly accelerate the mineralization of organic wastes by stimulating the development of degrading microorganisms during vermicomposting (Domínguez et al., 2010).

The consumption of organic compound by the earthworm and the microbiota involved in vermicomposting caused a significant decrease in the C:N ratio of all the organic substrates after vermicomposting. Percents of reduction in C:N ratio observed after the vermicomposting of PS 2:1, PS 1:1, or S were similar to that occurred in vermicompsting control (D). That ratio is currently used as an indicator of biostabilization of organic waste during biological processes, since a significant reduction in the initial C:N ratio of a organic has been commonly reported from early studies (Senesi, 1989). On the other hand, the Spanish legislation for organic fertilizers oblige vermicomposts to register a C:N ratio <20 since organic fertilizers which have a higher C:N ratio may contain a great proportion of inorganic nutrients in unavailable forms to be uptake by plants because of adsorption of nutrients to organic C-compounds (Gobierno de España, 2005). As the commercial vermicompost (CV), all the vermicomposts produced by this study met this requirement. On the other hand, EC has been considered to be an important to assess the quality of vermicompost. Although there are not mandatory limit values for EC in vermicomposts,

Table 4.2. Chemical properties analysed before (B) and after (A) vermicomposting.

	pH		EC (dS m^{-1})		TOC (g kg^{-1})		WSC (g kg^{-1})		C/N	
Substrates	B	A	B	A	B	A	B	A	B	A
P	7.2b	-	10a	-	386a	-	15.2a	-	22a	-
S	8.2ab	8.2ab	0.6e	0.9b*	175c	166c	2.1c	0.4c*	24a	19a*
PS 2:1	7.6b	9.3a*	7.2b	0.9b*	286b	190b*	13.3a	1.3b*	19b	15b*
PS 1:1	7.7b	8.8a*	5.3c	0.8b*	276b	196b*	13.8a	1.6ab*	21ab	17ab*
D	9.0a	9.0a	2.8d	0.8b*	237b	145c*	8.9b	2.4a*	15c	12c*
CV	-	8.9a	-	3.3a	-	228a	-	1.9ab	-	12c

D: cow dung; P: tomato-plant waste; S: paper-mill sludge; CV: commercial vermicompost; EC: electrical conductivity; TOC: total organic carbon; WSC: water soluble carbon. Values in the same column followed by same letters are not significantly different from each other (ANOVA, $p < 0.05$). The asterisk (*) denotes a significant difference between the initial organic substrate and the resulting vermicomposts (Paired-sample t-Student test, $p < 0.05$).

Lasaridi et al. (2006) suggested EC value <1.5 dS m^{-1}, for stabilized materials used as growing media, and <4 dS m^{-1}, for organic soil amendments, as those values are tolerable by plants of medium sensitivity for salinity. Initial salt content in the wastes was reduced by vermicomposting due to soluble elements were pulled out by the excess of applied water that leached. Thus, all the vermicomposts can be used as growing media or organic soil amendment according their EC values.

Concerning concentrations of elements considered to be essential for plant nutrition (i.e. N, P, and K), the vermicomposts produced from PS 2:1 or PS 1:1 had concentrations of NPK very similar to those of CV (Table 4.3), whereas the vermicompost from S had the lowest concentrations of those elements. This could be related to the inadequate earthworm development occurred during the vermicomposting of S (Figure 4.1). Previous studies have reported that earthworm can enrich the N-content of waste by adding nitrogenous compound in the form of mucus and excretory substances or even as a consequence of decaying tissues of earthworms which die during the vermicomposting acclimation phase (Suthar and Singh, 2008). Furthermore, N-fixation performed by microorganisms which are stimulated by the earthworms have been reported during vermicomposting (Bhattacharya and Chattopadhyay, 2004). Other element such as Ca, Mg, Fe, Mn recorded concentration which were different from those of CV (Table 4.3). However, those elements are plant micronutrients so that the vermicomposts produced in that study contains sufficient amount of Ca, Mg, Fe, and Mn for plant nutrition. As CV, all vermicompost produced in this study had total concentrations of heavy metals (i.e. Cu, Zn, Ni, Cd, and Pb) below the limit of heavy metals for organic fertilizers and soil amendments authorized by the Spanish legislation (Gobierno de España, 2005).

4.3.4. Assessment of waste biostabilization through enzyme activities analyses

A significant reduction in the dehydrogenase activity values recorded initially in the organic substrates was observed after their vermicomposting (Figure 4.4a). During a vermicomposting process, a significant decrease in the dehydrogenase activity of a waste is due to the mineralization of its organic matter that reduces available C substrates for microorganisms (Benítez et al., 1999). Dehydrogenases are intracellular enzymes involved in the respiratory chains of all active microorganisms, and thus determination of dehydrogenase activity has commonly been used for estimating overall microbial activity in biotransformation processes, providing a clear indication of the dynamics of organic-matter

degradation (García et al., 1997). Among all the vermicompost produced, higher dehydrogenase activities were recorded in vermicomposts from PS 2:1 and PS 1:1. This suggests that the plant waste could maintain populations of degrading microorganisms of lignin fibres after vermicomposting process.

Table 4.3. Plant nutrients and heavy metals concentrations in vermicomposts produced from paper-mill sludge (S), mixtures of plant waste and paper-mill sludge in 2:1 ratio (PS 2:1) or 1:1 ratio (PS 1:1), cow dung (D, control), and a commercial vermicompost (CV).

	S	PS 2:1	PS 1:1	D	CV
N (g kg $^{-1}$)	8.8b	12.4a	11.6a	11.9a	19a
P (g kg $^{-1}$)	1.7c	4.4a	4.3a	2.9bc	4.4a
K (g kg $^{-1}$)	1.8c	6.3b	5.3b	5.6b	14.8a
Ca (g kg $^{-1}$)	131ab	113b	146a	52d	85c
Mg (g kg $^{-1}$)	5c	7b	8b	10a	8b
Na (mg kg $^{-1}$)	330d	480c	430c	719b	1868a
Fe (mg kg $^{-1}$)	1866d	2114c	2100c	6015a	3228b
Mn (mg kg $^{-1}$)	57d	127c	122c	327a	192b
Cu (g kg $^{-1}$)	23b	54a	50a	26b	43a
Zn (mg kg $^{-1}$)	63c	105b	108b	110b	153a
Ni (mg kg $^{-1}$)	9b	9b	7b	43a	8b
Cd (mg kg $^{-1}$)	<0.2a	<0.2a	<0.2a	<0.2a	<0.2a
Pb (mg kg $^{-1}$)	<0.2a	<0.2a	<0.2a	<0.2a	<0.2a

Values in the same row followed by same letters are not significantly different from each other (ANOVA, $p < 0.05$).

As with dehydrogenase activity, β-glucosidases, which catalyse the hydrolysis of cellobiose and other disaccharides, recorded lower activity in vermicomposted material than in initial substrates as a consequence of the mineralization of carbohydrates occurred during

vermicomposting (Figure 4.4b). The great reduction in this enzyme activity recorded in PS 2:1 as well as PS 1:1 after vermicomposting suggests that the large labile organic C fraction initially contained in plant wastes was degraded by the joint action of earthworms and microorganisms involved in vermicomposting.

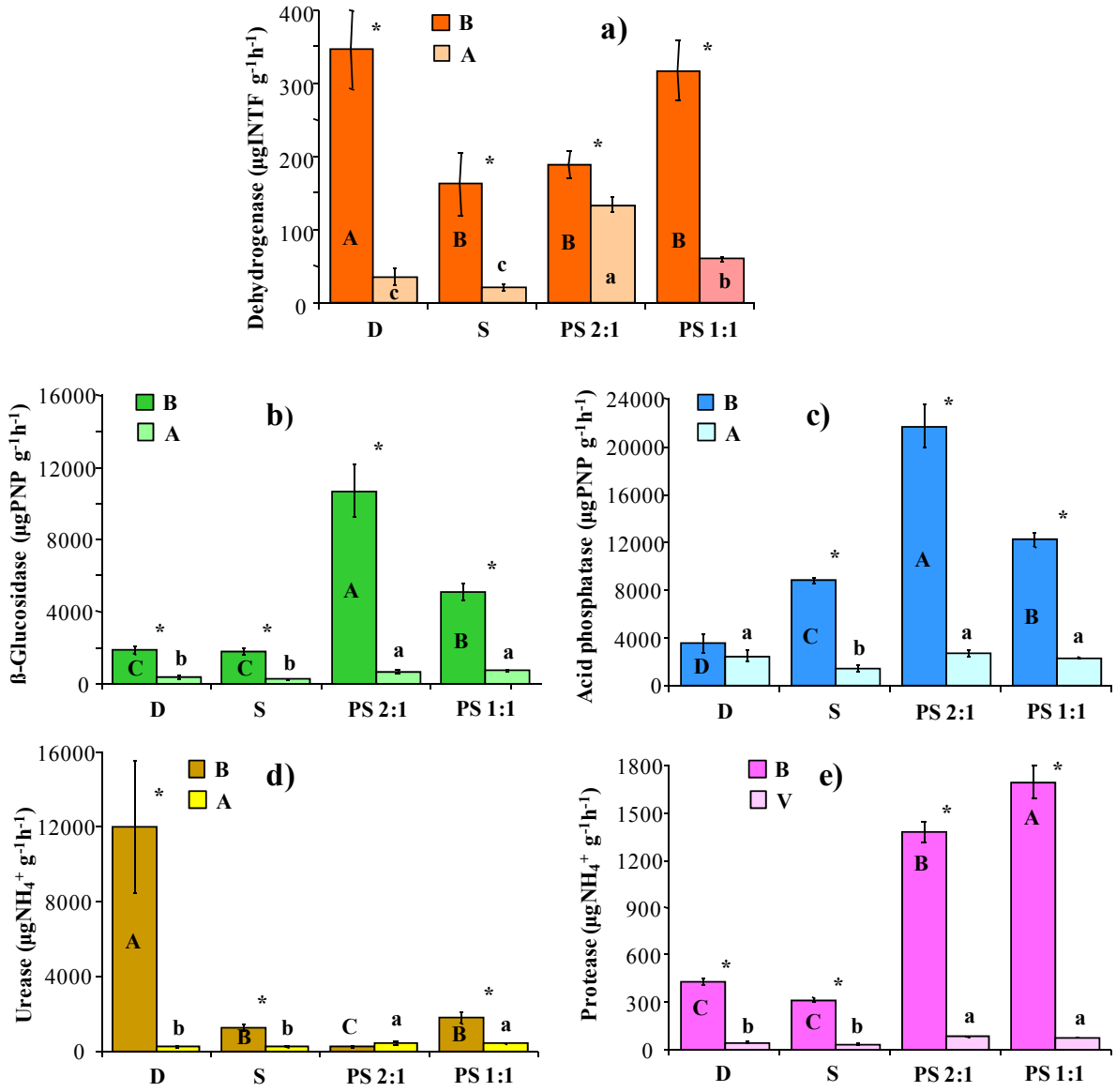


Figure 4.4. Enzyme activities in organic substrates before (B) and after vermicomposting (A). Error bars represent the mean standard error (n=3). The asterisk (*) denotes enzyme activity values significantly different between each initial organic substrate and its resulting vermicompost (Paired-sample t-Student test, p <0.05). Different capital letter and different lowercase indicate differences among initial substrates and vermicomposted substrates, respectively (ANOVA, p <0.05). D: cow dung; P: plant wastes; S: paper-mill sludge**.**

Acid phosphatases, which catalyse the hydrolysis of organic phosphomonoester to an inorganic phosphate form, behaved in a way similar to that of β-glucosidases, although in the case of cow dung (D) the initial value of acid phosphatase did not significantly decline after vermicomposting (Figure 4.4c). This could be because D had a initial pH value higher than that considered to be optimum for this type of enzymes (between 4.0 and 6.5 according to Speir and Ross (1978) (Table 4.2).

Regarding the enzymes involved into the N mineralization, the activity of urease enzymes recorded in D before vermicomposting was greater than that in the others (Figure 4.4d). This indicates a great amount of ureic compounds in D, which acted as enzyme-synthesis-inducing substrate. However, as a consequence of the hydrolysis of those compounds during vermicomposting, this activity felt significantly after the vermicomposting of D. Protease activity also diminished in all the organic substrates after their vermicomposting, recording strong reductions after the vermicomposting of PS 2:1 and PS 1:1 (Figure 4.4e). This sharp fall in protease suggests that earthworms and microorganisms together could be able first to depolymerise and afterwards to degrade the available peptides from the tomato-plant waste. Urease and protease activity values were significantly higher in vermicomposts PS 2:1 and PS 1:1 as compared with the other vermicomposts, suggesting that the level of these enzymes depends on the greater microbial activity in those vermicomposts as indicated by their higher dehydrogenase activity values (Figure 4.4a). Benítez et al. (1999) found that both enzyme activities were closely correlated with dehydrogenase activity during the vermicomposting of sewage sludge.

In short, a significant fall in dehydrogenase activity along with decreases in hydrolytic activities has been reported to be indicative of a significant decomposition and stabilization of organic wastes due to the joint action of earthworms and microorganisms during vermicomposting (Aira et al., 2006; Benítez et al., 1999).

4.3.5. Assessment of maturity degree in initial substrate and resulting vermicomposts through germination index

Proper stabilization of wastes should allow their transformation in mature products, defined as a substrate which has no adverse effects on plants. Germination index (GI) is one of the most used indices for determining the maturity degree in organic fertilizers. GI values close to zero were initially recorded in P, PS 3:1, PS 2:1 and PS 1:1 (Figure 4.5). This strong suppression of seed germination suggests that plant waste produced from greenhouse tomato

crops is inadequate to be used as soil organic amendments because of its potential adverse effect on plants. According to Zucconi et al. (1981), only the organic substrate which registers a GI value higher than 60% can be considered to be safe and suitable for agricultural application. Low GI values initially found in organic substrates which contained P may be due to the high EC values of this waste (Table 4.2). Supporting this idea, previous studies conduced to assess the features of a variety of composts and vermicomposts from different wastes found a large negative correlation between GI and EC (Campitelli and Ceppi, 2008; Lasaridi et al., 2006). After vermicomposting, GI values of the different organic substrate increased significantly up to values around 100%, or even greater in the case of cow dung. This denotes that all vermicomposted substrates had a great maturity, registering GI values which were similar to that of a commercial vermicompost, which is recommended as organic fertilizer for agriculture. These results point out that vermicomposting was capable of eliminating the adverse plant effects of unprocessed tomato-plant waste when this waste was processed together with paper-mill sludge.

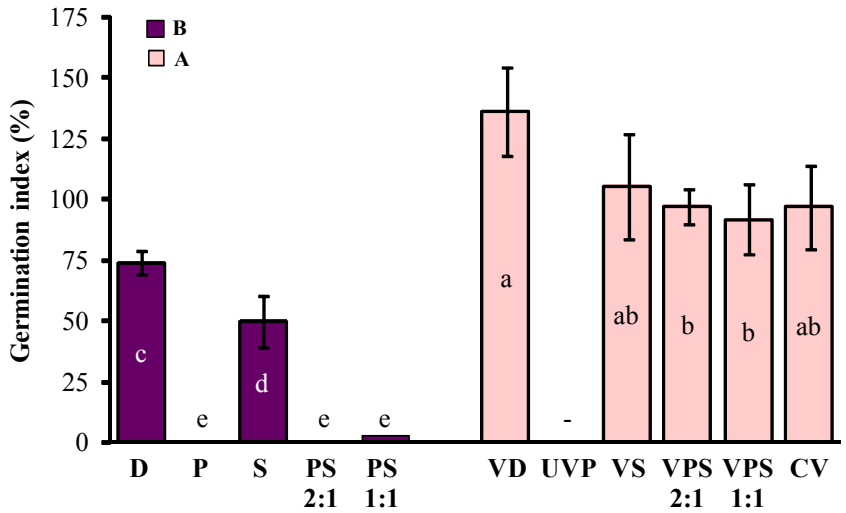


Figure 4.5. Germination index of organic substrates before (B) and after vermicomposting (V). Error bars represent the mean standard error of three replicates. Materials with different lowercase letters are significantly different (ANOVA, p <0.05). Substrates successfully vermicomposted were named by the preceding letter "V" whereas preceding letters "UV" denote substrate unviable for vermicomposting. D: cow dung; P: plant wastes; S: paper-mill sludge**;** CV: commercial vermicompost.

4.4. CONCLUSIONS

By mixing tomato-plant waste with paper-mill sludge in a ratio of 2:1 or 1:1 was possible to improve the earthworm development occurred during the vermicomposting of paper-mill sludge alone, allowing also to vermicompost tomato-plant waste which is harmful for *E. fetida*. Analysis of PLFA composition, chemical features, enzymatic activities, and germination index indicated that the vermicomposting of mixtures of plant waste and paper-mill sludge improves the nutrient content, stability and maturity of these waste mixtures. Our results also suggest that PLFA analysis provides complementary information that could be useful for selecting wastes for vermicomposting, assessing the process efficiency, and comparing different vermicomposts according to their microbial community structure.

ACKNOWLEDGEMENTS

This study was financed by "Junta de Andalucía" project P05-AGR-00408. M. J. Fernández-Gómez thanks the Science and Innovation Ministry for their FPU doctoral grant (AP2006-03452). The authors also thank C. Cifuentes, A. Martín and J. Benitez for technical support and D. Nesbitt for assisting in the translation of the manuscript into English.

REFERENCES

Aira, M.; Monroy, F.; Domínguez, J., (2006). Changes in microbial biomass and microbial activity of pig slurry after the transit through the gut of the earthworm *Eudrilus eugeniae* (Kinberg, 1867). Biol. Fertil. Soils, 42 (4), 371-376.

Alkoaik, F.; Ghaly, A. E., (2005). Effect of inoculum size on the composting of greenhouse tomato plant trimmings. Compost Sci. Util., 13, 262-273.

Alkoaik, F.; Ghaly, A. E., (2006). Influence of dairy manure addition on the biological and thermal kinetics of composting of greenhouse tomato plant residues. Waste Manage., 26 (8), 902-913.

Atiyeh, R. M.; Domínguez, J.; Subler, S.; Edwards, C. A., (2000). Changes in biochemical properties of cow manure during processing by earthworms (*Eisenia andrei*, Bouche) and the effects on seedling growth. Pedobiologia, 44 (6), 709-724.

Benítez, E.; Nogales, R.; Elvira, C.; Masciandaro, G.; Ceccanti, B., (1999). Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol., 67 (3), 297-303.

Bhattacharya, S. S.; Chattopadhyay, G. N., (2004). Transformation of nitrogen during vermicomposting of fly ash. Waste Manage. Res., 22 (6), 488-491.

Bicheldey, T. K.; Latushkina, E. N., (2010). Biogass emission prognosis at the landfills. Int. J. Environ. Sci. Technol., 7 (4), 623-628.

Bonmatí, M.; Ceccanti, B.; Nannipieri, P., (1998). Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol. Biochem., 30 (14), 2113-2125.

Campitelli, P.; Ceppi, S., (2008). Chemical, physical and biological compost and vermicompost characterization: A chemometric study. Chemometr. Intell. Lab., 90 (1), 64-71.

Domínguez, J.; Aira, M.; Gómez-Brandón, M., (2010). Vermicomposting: earthworms enhance the work of microbes, in: H. Insam, I. Franke-Whittle, M. Goberna, (Eds.), Microbes at Work. Springer, Berlin.

Edwards, C. A., (1988). Breakdown of animal, vegetable and industrial organic wastes by earthworms, in: C. A. Edwards, E. F. Neuhauser, (Eds.), Earthworms in waste and environmental management. SPB Academic Publishing BV, The Hague.

Elvira, C.; Goicoechea, M.; Sampedro, L.; Mato, S.; Nogales, R., (1996). Bioconversion of solid paper-pulp mill sludge by earthworms. Bioresour. Technol., 57 (2), 173-177.

Elvira, C.; Sampedro, L.; Domínguez, J.; Mato, S., (1997). Vermicomposting of wastewater sludge from paper-pulp industry with nitrogen rich materials. Soil Biol. Biochem., 29 (3-4), 759-762.

Fernández-Gómez, M. J.; Romero, E.; Nogales, R., (2010). Feasibility of vermicomposting for vegetable greenhouse waste recycling. Bioresour. Technol., 101 (24), 9654-9660.

Fostergård, A.; Bååth, E.; Tunlio, A., (1993). Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol. Biochem., 25 (6), 723-730.

García, C.; Hernández, T.; Costa, F., (1997). Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan., 28 (1-2), 123-134.

Gobierno de España, (2005). Real Decreto 824/2005 sobre productos fertilizantes, BOE. Gobierno de España, Madrid.

Gómez-Brandón, M.; Lazcano, C.; Lores, M.; Domínguez, J., (2010). Detritivorous earthworms modify microbial community structure and accelerate plant residue decomposition. Appl. Soil Ecol., 44 (3), 237-244.

Gupta, R.; Garg, V. K., (2009). Vermiremediation and nutrient recovery of non-recyclable paper waste employing *Eisenia fetida*. J. Hazard. Mater., 162 (1), 430-439.

Kandeler, E.; Gerber, H., (1988). Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils, 6 (1), 68-72.

Kaur, A.; Singh, J.; Vig, A. P.; Dhaliwal, S. S.; Rup, P. J., (2010). Cocomposting with and without *Eisenia fetida* for conversion of toxic paper mill sludge to a soil conditioner. Bioresour. Technol., 101 (21), 8192-8198.

Lasaridi, K.; Protopapa, I.; Kotsou, M.; Pilidis, G.; Manios, T.; Kyriacou, A., (2006). Quality assessment of composts in the Greek market: The need for standards and quality assurance. J. Environ. Manage., 80 (1), 58-65.

Manzano-Agugliaro, F., (2007). Gasificación de residuos de invernadero para la obtención de energía eléctrica en el sur de España: Ubicación mediante SIG. Interciencia, 32 (2), 131-136.

Marschner, P., (2007). Soil microbial community structure and function assessed by FAME, PLFA and DGGE-Advantages and Limitations, in: A. Varma, R. Oelmüller, (Eds.), Advanced Techniques in Soil Microbiology. Springer, Berlin.

Moore-Kucera, J.; Dick, R. P., (2008). PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. Microb. Ecol., 55 (3), 500-511.

Pardossi, A.; Tognoni, F.; Incrocci, L., (2004). Mediterranean Greenhouse Technology. Chron. Hort., 44, 28-34.

Parra, S.; Aguilar, F. J.; Calatrava, J., (2008). Decision modelling for environmental protection: The contingent valuation method applied to greenhouse waste management. Biosyst. Eng., 99 (4), 469-477.

Schönholzer, F.; Hahn, D.; Zeyer, J., (1999). Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. FEMS Microbiol. Ecol., 28 (3), 235-248.

Senesi, N., (1989). Composted materials as organic fertilizers. Sci. Total Environ., 81-82 (C), 521-542.

Speir, T. W.; Ross, D. J., (1978). Soil phosphatase and sulphatase, in: R. G. Burns, (Ed.), Soil Enzymes. Academic Press, London.

Suthar, S.; Singh, S., (2008). Vermicomposting of domestic waste by using two epigeic earthworms (*Perionyx excavatus* and *Perionyx sansibaricus*). Int. J. Environ. Sci. Technol., 5 (1), 99-106.

Tabatabai, M. A., (1982). Soil enzymes, in: A. L. Page, E. M. Miller, D. R. Keeney, (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. ASA and SSSA, Madison.

Tabatabai, M. A.; Bremner, J. M., (1969). Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem., 1 (4), 301-307.

Williams, S., (1984). Official methods of analysis of the Association of Official Analytical Chemists., 14th Ed. Association of Official Analytical Chemists.

Zdruli, P.; Jones, R. J. A.; Montanarella, L., (2004). Organic matter in the soils of southern Europe, European Soil Bureau Research Report, EUR 21083 EN. Office for Official Publications of the European Communities, Luxembourg.

Zucconi, F.; Forte, M.; Monaco, A.; De Bertoldi, M., (1981). Biological evaluation of compost maturity. BioCycle, 22 (4), 27-29.

CAPÍTULO 5
CHAPTER 5

IMPACTO DE DE IMIDACLOPRID SOBRE EL DESARROLLO DE *EISENIA FETIDA* DURANTE EL VERMICOMPOSTAJE DE RESIDUOS VEGETALES DE INVERNADERO

IMPACT OF IMIDACLOPRID RESIDUES ON THE DEVELOPMENT OF EISENIA FETIDA DURING VERMICOMPOSTING OF GREENHOUSE PLANT WASTE

Fernández-Gómez, M.J., Romero, E., Nogales, R. (2011). Impact of imidacloprid residues on the development of *Eisenia fetida* during vermicomposting of greenhouse plant waste. Journal of Harzadous Materials 192, 1886-1889.

RESUMEN

El uso de plaguicidas en agricultura ocasiona la presencia de esos xenobioticos, a diferentes concentraciones, en los residuos postcosecha de las plantas cultivadas. Por ello, conocer como las concentraciones de los plaguicidas pueden afectar a las lombrices es fundamental para reciclar residuos vegetales de invernadero mediante vermicompostaje. En el presente estudio, se han evaluado los efectos de residuos de imidacloprid (IMD) sobre las lombrices (*Eisenia fetida*) durante el vermicompostaje de residuos vegetales procedentes de cultivos de invernaderos en España. Previamente, el efecto de diferentes concentraciones de IMD en las lombrices se ensayó utilizando estiércol de vaca como un residuo óptimo para su desarrollo. Los resultados obtenidos con estiércol vacuno indican que las dosis de IMD $\geq$ 5 mg kg^{-1} dificultan el crecimiento de las lombrices e incluso provocan su muerte. Los resultados obtenidos del vermicompostaje de residuos vegetales revelan que el IMD no permite un crecimiento satisfactorio de las lombrices y aumenta su mortalidad. Aunque el 89% de las lombrices llegaron a ser maduras sexualmente en substratos que contuvieron 2 mg IMD kg^{-1}, éstas no produjeron cápsulas. El IMD también afectó a los microorganismos del substrato, como se puso de manifiesto por la reducción de su actividad deshidrogenasa. Esta actividad enzimática se restableció después del vermicompostaje. Este estudio proporciona una base científica sólida para el vermicompostaje de residuos vegetales contaminados con plaguicidas.

Palabras clave: Vermicompostaje, imidacloprid, residuos vegetales, desarrollo de lombrices, actividad deshidrogenasa.

ABSTRACT

Pesticide application in agriculture causes residues in post-harvest plant waste at different concentrations. Knowledge concerning how pesticide concentrations in such waste affect earthworms is essential for recycling greenhouse plant debris through vermicomposting. Here, we have evaluated the effects of imidacloprid (IMD) residues on earthworms (*Eisenia fetida*) during the vermicomposting of plant waste from greenhouse crops in Spain. Before, the effect of different IMD concentrations on earthworms was tested using cattle manure as an optimum waste for worm development. The results after using cattle manure indicate that IMD dose $\geq$ 5 mg kg^{-1} hinders worm growth and even causes death, whereas IMD dose $\leq$ 2 mg IMD kg^{-1} allows worm growth similar to control but impedes reproduction. The results from the vermicomposting of plant waste reveal that IMD inhibits adequate worm growth and increases mortality. Although 89% worms became sexually mature in substrate containing 2 mg IMD kg^{-1}, they did not produce cocoons. IMD also affected microorganisms harboured in the substrates for vermicomposting, as indicated by the reduction in their dehydrogenase activity. This enzyme activity was restored after vermicomposting. This study provides a sound basis for the vermicomposting of pesticide-contaminated plant waste.

Keywords: Vermicomposting, imidacloprid, plant waste, worm development, dehydrogenase activity.

5.1. INTRODUCTION

Greenhouse crop systems are expanding worldwide, occupying more than 1.5 million hectares (Espi et al., 2006), since these systems protect crops from adverse meteorological conditions, allowing the production of high-value vegetables over the entire season. However, these profitable crops cause an environmental impact on the land where the greenhouses are located due to the enormous quantities of plant wastes discarded after the harvest, around 28.5 tonnes per greenhouse hectare and year (Manzano-Agugliaro, 2007). In the region of Motril (Granada, Southern Spain), where this study was conducted, this type of waste consists of leaves, stems, and roots of tomato plants, which is the predominant greenhouse crop in the region (Ministerio de Medio Ambiente y Medio Rural y Marino, 2010). Contrary to plant waste from conventional crop systems, greenhouse plant wastes are hardly recycled through traditional composting because of its high moisture, low C/N, high salinity, and heavy pesticide load. Concerning pesticides, imidacloprid can frequently be contained in greenhouse plant wastes since this neonicotinoid systemic insecticide is marketed in 120 countries for controlling various species of whitefly and aphids in over 140 different agricultural crops (Drobne et al., 2008). In Spain, imidacloprid has been widely applied since 1992, becoming one of the most widely used products in diverse Spanish greenhouse crops (tomatoes, peppers, cucumbers, zucchini, and potatoes, etc.) (Hernández et al., 1999). Since this pesticide has been reported to persist in different concentration in stems and roots of plants, depending on its application method (Juraske et al., 2009), variables amount of imidacloprid are expected in greenhouse plant wastes. Thus, greenhouse plant wastes containing imidacloprid are an important hazard to the environment because this pesticide is categorized as moderately toxic and with a high potential to leach into groundwaters (World Health Organization, 2006). Hence, the recycling of the greenhouse plant wastes is necessary to avoid soil and water pollution as well as to establish sustainable agriculture.

Vermicomposting is a biotechnological process that enables the recycling of organic wastes into fertilizers through the joint action of earthworms and microorganisms. Among the epigeic earthworm species for vermicomposting, *Eisenia fetida* is the worm most commonly used in temperate climates. Although cattle manure is the optimum substrate to culture *E. fetida* (Reinecke and Viljoen, 1990), previous studies have demonstrated that this worm enables the vermicomposting of other organic wastes such as greenhouse debris (Fernández-Gómez et al., 2010a,b). Taking into account that earthworm growth and sexual

development have a key role driving waste stabilization during vermicomposting (Ndegwa et al., 2000), the evaluation of the effect of the imidacloprid on *E. fetida* development and survival is essential to provide full information on the feasibility of vermicomposting greenhouse plant waste containing this insecticide. The impact of pesticides on *E. fetida* growth and reproduction has been reported by Yasmin and D'Souza (2010), who compiled information on how different pesticides affect to this earthworm species. Concerning imidacloprid, previous studies reported that this pesticide is harmful to *E. fetida* (Luo et al., 1999; Zang et al., 2000). However, to date, the literature available provides information only on effects of imidaclopird on *E. fetida* worms placed in aqueous solutions, paper-filters, or soils. Therefore, it remains to be determined how this pesticide affects this worm species during vermicomposting, a biological process in which other factors influence earthworm development.

The aim of the present study was to evaluate how different concentrations of imidacloprid residues affect *E. fetida* growth and reproduction during the vermicomposting of tomato-plant waste generated from greenhouses. The effect of imidacloprid on the microbial activity in this vermicomposting substrate was also studied by analysing dehydrogenase enzyme activity. Previously, a basic experiment was conducted with worms raised on an optimum organic substrate for *E. fetida* (i.e. cattle manure), which was fortified with four increasing concentrations of imidacloprid. This first experiment was made to assess how imidacloprid affects well-nourished worms, determining what minimum concentration of imidacloprid negatively affects *E. fetida* as well as what imidacloprid concentration impedes the worm growth (or survival) on an optimal organic waste. Thus, it was decided that imidacloprid concentrations should be assayed for the vermicomposting of greenhouse tomato-plant waste, besides the effect of this sub-optimum substrate.

5.2. MATERIALS AND METHODS

Imidacloprid (IMD) (1–[(6-cloro-3 piridinyl)methyl]-N-nitro-2-imidazolidinimina) 99% purity from Ehrenstorfer (Augsburg, Germany) was used into milliQ-water solution. Non-clitellated earthworms (*E. fetida*) were selected from a culture bank at the Estación Experimental del Zaidín (Granada, Spain). Cattle manure (75% moisture, 8.5 pH, 9 dS m^{-1} electrical conductivity, 401 g kg^{-1} total organic carbon, 15 g kg^{-1} total nitrogen) constitutes by a mixture of faeces and urine without any bedding material, was collected from a farmyard of dairy cows (Granada, Spain). Plant waste (23% moisture, 7.2 pH, 10 dS m^{-1}

electrical conductivity, 386 g kg^{-1} total organic C, 17 g kg^{-1} total N) was produced by tomato crops without pesticide treatment in greenhouses located in Motril (Granada, Spain). This plant waste was air-dried, chopped to particle sizes of less than 1 cm and mixed with paper-mill sludge at a 2:1 ratio (dw:dw) in order to improve the structure and moisture conditions and thereby optimise the vermicomposting process. Paper-mill sludge (64% moisture, 8.2 pH, 0.6 dS m^{-1} electrical conductivity, 175 g kg^{-1} total organic C, 7.3 g kg^{-1} total N) was collected from a wastewater treatment plant of a paper company also located in Motril.

Two vermicomposting processes were undertaken. Firstly, Petri dishes (10 cm diameter) were filled with 30 g of cattle manure containing IMD at the following concentrations: 0 (control), 1, 2, 5, and 10 mg kg^{-1}. Three Petri dishes per each IMD concentration were inoculated with four juvenile non-clitellated earthworms (*E. fetida*) weighing 250 mg each one. All Petri dishes were kept in darkness at 20ºC, maintaining their moisture by periodical watering at 80%. Earthworm growth, sexual development, and reproduction were successively monitored in each Petri dish over the time (1, 2, 3, 6, 10, 14, 21, 28, 35, 42, and 49 days). The second vermicomposting process consisted of using plastic containers (12 cm diameter x 13 cm high) which had perforated bottoms for aeration and drainage. Containers were filled with 200 g of the mixture of plant waste and paper-mill sludge contaminated with 0 (control), 2, 4, and 8 mg IMD kg^{-1} plant waste. A thin layer of cattle manure (1 cm) was placed on top of each treatment and 25 non-clitellated earthworms (total worm biomass ~5000 mg) were placed within. All containers were kept under the same conditions as described above. Earthworm development was monitored weekly for 15 weeks. Dehydrogenase enzyme activity was analysed in the substrate containing IMD before worm inoculation and also at the end of vermicomposting period, following the method described by García et al. (1997).

Repeated-measures ANOVA analyses were performed to evaluate the effect of time on the worm development during the vermicomposting processes, determining the interactive effect of the time with the assayed IMD concentrations. The Kruskall-Wallis and Mann-Whitney tests were applied to assess the differences in dehydrogenase activity among treatments due to the non-normality of these data. Statistical analyses were conducted at >95% confidence level ($P<0.05$) using SPSS® Windows (Chicago, Illinois, USA).

5.3. RESULTS

The weight of the worms fed on cattle manure significantly varied over time (F=6.99, *P*<0.01), depending on the IMD concentration contained in this substrate (F=2.96, *P*<0.01) (Figure 5.1). Thus, *E. fetida* worms raised on cattle manure containing concentrations of IMD ≥2 mg kg^{-1} were unable to significantly increase their weight over time, while a significant increase in the individual worm biomass was recorded in cattle manure containing 1 mg IMD kg^{-1} (F=2.35, *P*=0.03), which was similar to that in the control without IMD. By contrast, a significant reduction in the individual worm biomass was observed over time in the manure containing 10 mg IMD kg^{-1} (F=5.50, *P*<0.01).

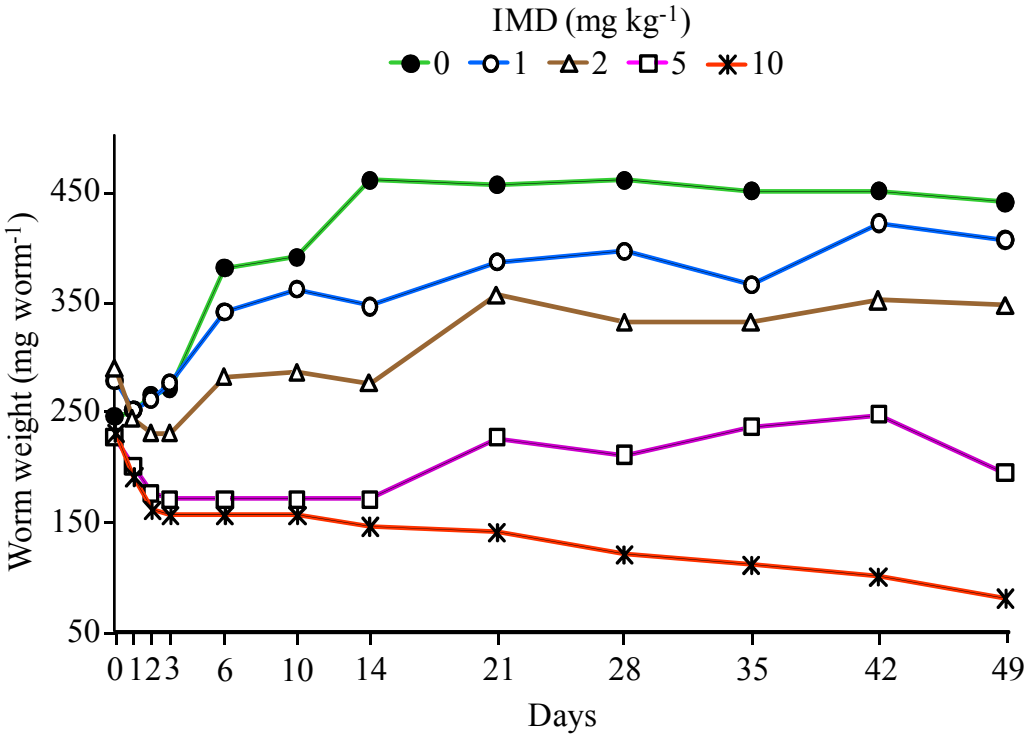
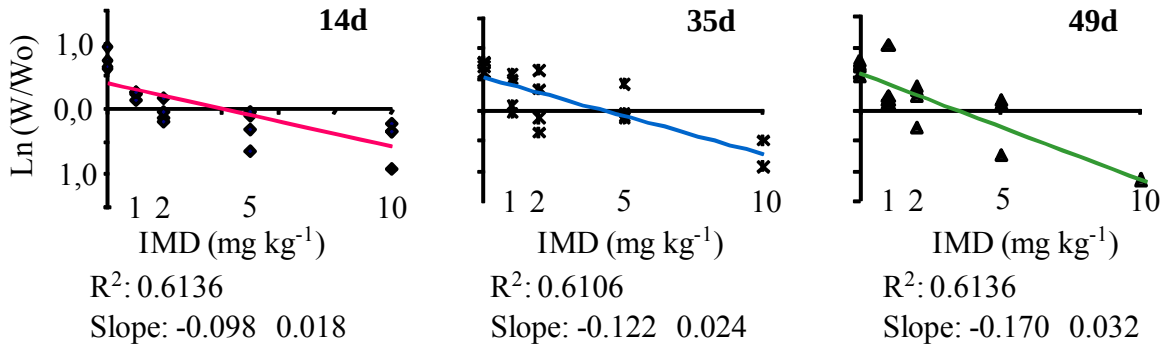


Figure 5.1. Changes in the weight of *Eisenia fetida* individuals over time in cattle manure containing imidacloprid (IMD) at 0, 1, 2, 5 and 10 mg kg^{-1}.

Figure 5.2 offers an overall view of the effect of increasing concentrations of IMD in worm weight, revealing that the logarithmic worm-weight gain (with respect of initial worm weight), at 14, 35, and 49 days, significantly fit a negative linear regression in relation to IMD concentrations at (*P*<0.01). In this figure the regression slope decreases almost two-fold from 14 to 49 days. This indicates that high doses of IMD strongly hindered worm growth as opposed to low doses, which allowed worms to increase their initial weight over

time. With regard to sexual development, in contrast to control, none of the worms growing in manure with IMD developed a clitellum, thus remaining sexually immature. Only the higher IMD concentrations, 5 and 10 mg kg^{-1}, caused worm mortality of 25% and 80%, respectively.



W: Survinving worm weight at 14, 35 and 49 days; Wo: worms weight at initial time

Figure 5.2. Logarithmic worm-weight gain at 14, 35 and 49 days (worm weigh [W]/ initial worm weight [W0]) in cattle manure containing imidacloprid (IMD) at 0, 1, 2, 5 and 10 mg kg^{-1} manure.

When *E. fetida* was used to vermicompost the plant waste mixed with paper-mill sludge, the mixture without IMD did not provide good nourishment, resulting in a sub-optimal worm development as compared with cattle manure. Indeed, a strong reduction of 31% in the number of worms (F=18.89, *P*=0.05) was recorded after one week in the control without IMD (Figure 5.3a). Plant wastes containing IMD made this decline significantly stronger in the initial worm population, for reductions of 89% at 2 mg IMD (F=179.56, *P*<0.01), 81% at 4 mg IMD (F=75.94, *P*=0.01, and 95% at 8 mg IMD kg^{-1} plant waste (F=720.14, *P*<0.01) over a week. To offset the substrate influence on worm development and to continue assessing the effect of IMD, we restocked the dead worms in the 2nd week, reaching the same worm number (25) and biomass (~5g) as at the beginning of the experiment. Then, during the next 10 weeks, an overall significant decline in the worm population was registered in all the vermicomposting processes (F=74.83, *P*<0.01), in which decreasing tends were similar to each other (Figure 5.3a). This reduction in worm number coincided with overall increases in the weights of surviving worms from the 2nd until the 10th week (F=32.12, *P*<0.01; Figure 5.3b). The average weight gain recorded over time was significantly different among the

substrates with different IMD concentrations (F=5.10, *P*=0.03). Thus, individual worm biomass rose by 245% in control after seven weeks, while gains of 120, 116, and 32% were

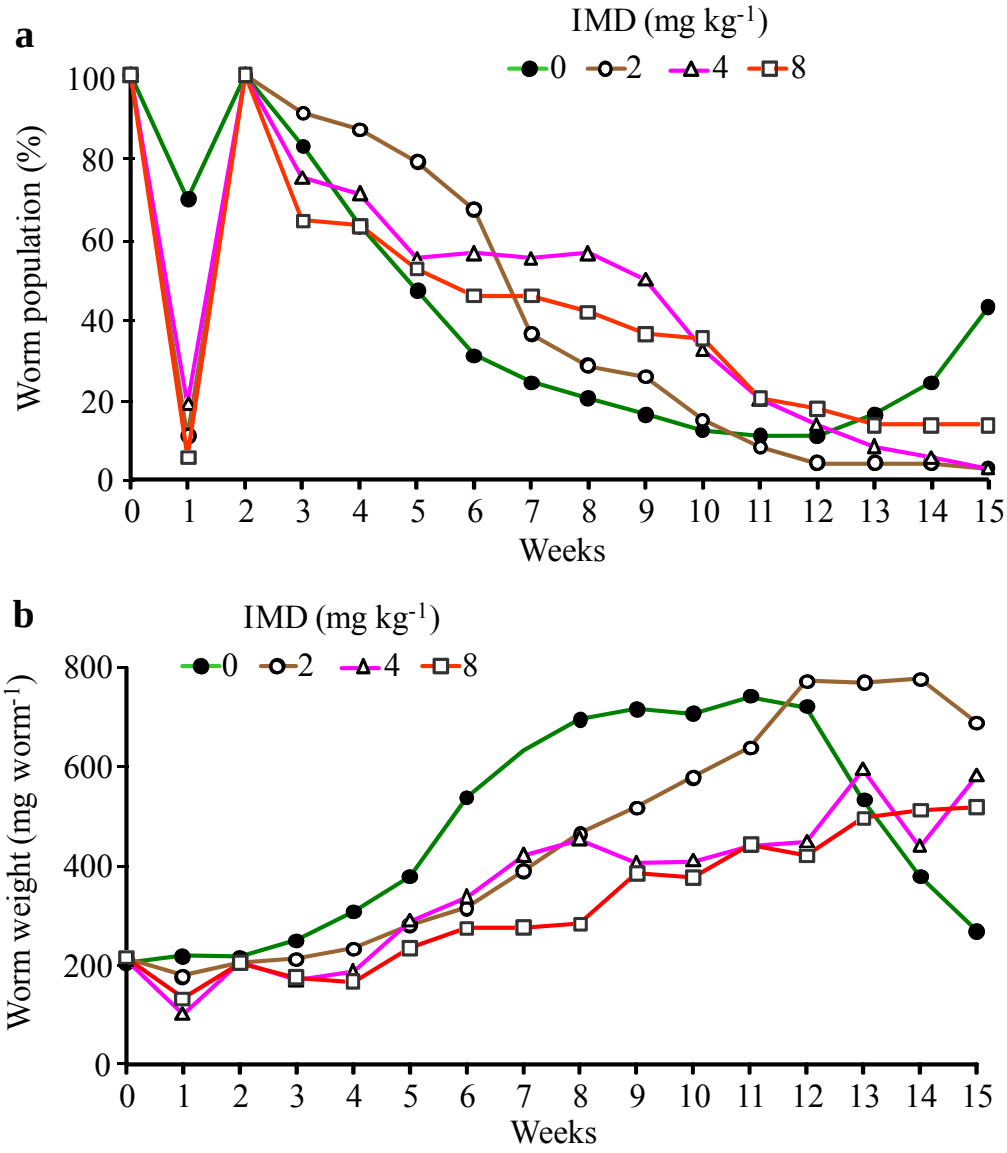


Figure 5.3. Percentage of worm number in relation to initial worm number (a), and weight of surviving-individuals of *Eisenia fetida* (b) during the vermicomposting of greenhouse tomato-plant waste containing imidacloprid (IMD) at 0, 2, 4, 8, and 10 mg kg^{-1}.

found in substrates containing 2, 4, 8 mg IMD kg^{-1} plant waste, respectively. With respect to the sexual development of *E. fetida*, worms became mature in the substrate without IMD by the 3rd week, while this occurred at the 6th week in the case of substrates containing any IMD concentration. At the 7th week, 43, 47, and 13% of the worms displayed a clitellum in the

vermicomposting substrates containing 2, 4, or 8 mg IMD kg^{-1} plant waste, respectively, while 83% of control worms reached maturity. At the 10th week, the percentage of mature worms found in the vermicomposting substrate containing 2 mg IMD kg^{-1} plant waste was similar to that in the control without IMD (89%), but only 58 and 39% maturity was found in the case of the substrates containing 4 and 8 mg IMD kg^{-1} plant waste, respectively. Despite that some worms reached sexual maturity in the substrates containing IMD, they did not produce cocoons. By contrast, great numbers of cocoons were detected in the substrate control, with newly hatched earthworms appearing after the 11th week. Consequently, the worm population recorded in the vermicomposting substrate without IMD at the end of the vermicomposting was significantly higher period compared with that in the substrates with IMD (F=7.59, *P*=0.01) (Figure 5.3b).

Before worm inoculation, the dehydrogenase activity in the vermicomposting substrates containing the assayed IMD concentrations was significantly lower than control without IMD (χ^2=8.13, *P*=0.04) (Figure 5.4). The IMD concentration of 8 mg kg^{-1} plant waste caused the greatest reduction in this activity, which diminished 60% as compared to that of the control. After vermicomposting, dehydrogenase activity values in the vermicomposts resulting from plant wastes containing IMD were similar to those of the vermicompost from the control (χ^2=4.44, *P*=0.22).

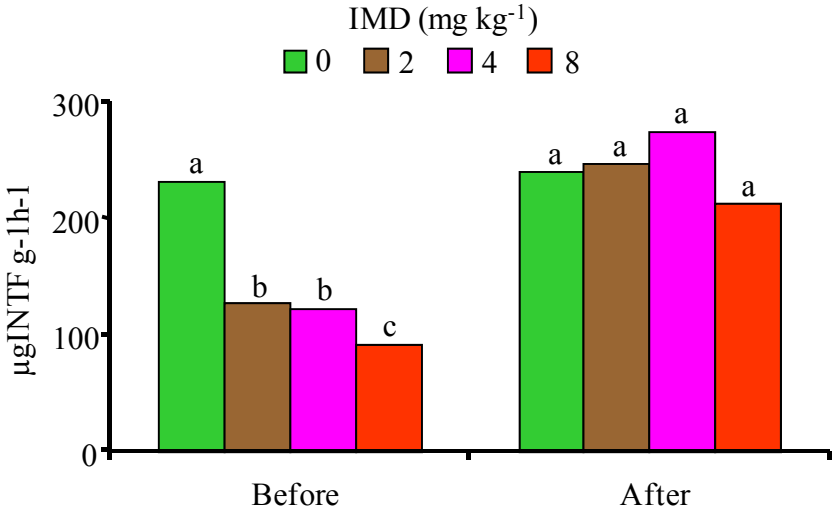


Figure 5.4. Dehydrogenase activities in greenhouse tomato-plant waste containing imidacloprid (IMD) at 0, 2, 4, 8, and 10 mg kg^{-1}, before and after their vermicomposting. Different letters indicate significant differences (*P*<0.05) among the dehydrogenase activities of the initial substrates or among the dehydrogenase activities of the end vermicomposts.

5.4. DISCUSSION

The results for worms optimally fed on cattle manure suggests that IMD concentrations ≥ 2 mg kg^{-1} depresses *E. fetida* growth, whereas at concentrations ≥ 10 mg IMD kg^{-1} the high mortality caused impedes vermicomposting. The 25% mortality noted after 7 weeks in manure containing 5 mg IMD kg^{-1} differs from the results of studies using artificial soil contaminated with 3.5 mg IMD kg^{-1}, in which 50% of mortality in *E. fetida* population was reported after a week (Luo et al., 1999). This suggests that the toxicity of IMD for *E. fetida* could be tempered by vermicomposting process if optimum substrates are used.

In the case the vermicomposting of tomato-plant waste from greenhouse, its mixture with paper-mill sludge was initially inadequate for worm development, as indicated the worm mortality in the substrate without IMD. This may be because this mixture was not sufficiently nutritious to support the initial population added. Thus, after a reduction in the worm population, the surviving worms could properly feed on this substrate, gaining weight and they became sexually mature, giving rise to new worm offspring in the control without IMD. The presence of IMD in the plant waste intensified the reduction in worm population, and also hindered the weight gain of surviving worms, as occurred in the control without IMD. Among the different concentrations of IMD tested, only the lowest (2 mg IMD kg^{-1} plant waste) enabled worms to grow acceptably and become sexually mature. However, the presence of IMD impeded cocoon production by the sexually mature worms. This suggests that IMD prevent the *E. fetida* reproduction. This could be explained by the finding of Luo et al. (1999), who reported that 0.5 mg IMD kg^{-1} in artificial soil caused significant sperm deformity in *E. fetida*.

Dehydrogenases are intracellular enzymes involved in oxidative phosphorylation, the analysis of which provides a measure of the overall activity of microorganisms (Gracia et al., 1997). Furthermore, this activity has previously been used as a reliable biomarker for analysing the negative impact of pesticides on the resident microbiota in vermicomposting (Benítez et al., 1999). Hence, the reduction in dehydrogenase activity initially caused by the presence of IMD in vermicomposting substrate indicates that this pesticide depleted its inhabiting microorganisms. This negative effect may be partially responsible for the lower worm fattening observed in the substrates containing IMD, since it is well known that microorganisms constitute a major part of the diets of earthworms (Edwards and Fletcher, 1988). The restoration of dehydrogenase activity after vermicomposting suggests that this process was able to recover the formerly depleted microbial population.

In conclusion, in practical terms, the vermicomposting of greenhouse plant-waste containing IMD concentrations ≤ 2 mg kg^{-1} is feasible. However, the presence of this insecticide in the vermicomposting substrate impedes the *E. fetida* reproduction. Further research is needed to ascertain whether the vermicomposting of organic wastes contaminated at low concentrations of this pesticide would enable the mineralization of this pesticide.

ACKNOWLEDGEMENTS

This study was financed by "Junta de Andalucía" project P05-AGR-00408. M. J. Fernández-Gómez thanks the Science and Innovation Ministry for their FPU doctoral grant (AP2006-03452). The authors thank D. Nesbitt for assisting in the translation of the manuscript into English.

REFERENCES

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme and earthworm activities during vermicomposting of carbaryl- treated sewage sludge. J. Environ. Qual. 28, 1099-1104.

Drobne, D., Blazic, M., Van Gestel, C.A.M., Leser, V., Zidar, P., Jemec, A., Trebse, P. 2008. Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). Chemosphere 71, 1326-1334.

Edwards, C.A., Fletcher, K.E., 1988. Interactions between earthworms and microorganisms in organic-matter breakdown. Agric. Ecosyst. Environ. 24, 235-247.

Espi, E., Salmeron, A., Fontecha, A., García, Y., Real, A.I., 2006. Plastic films for agricultural applications, J. Plastic Film Sheet. 22, 85-102.

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M., 2010. Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops. Waste Manage. 30, 2461-2468.

Fernández-Gómez, M.J., Romero, E., Nogales, R., 2010. Feasibility of vermicomposting for vegetable greenhouse waste recycling. Bioresour. Technol. 101, 9654-9660.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plant. Anal. 28, 123-134.

Hernández, D., Mansenét, V., Puiggrós Jové, J.M. 1999. Use of Confidor 200 SL in vegetable cultivation in Spain. Pflanzenschutz-Nachrichten Bayer 52, 364-375.

Juraske, R., Castells, F., Vijay, A., Muñoz, P., Antón, A. 2009.Uptake and persistence of pesticides in plants: measurements and model estimates for imidacloprid after foliar and soil application. J. Hazard. Mater. 165, 683-689.

Luo, Y., Zang, Y., Zhong, Y., Kong, Z., 1999. Toxicological study of two novel pesticides on earthworm *Eisenia foetida*. Chemosphere 39, 2347-2356.

Manzano-Agugliaro, F. 2007. Gasificación de residuos de invernadero para la obtención de energía eléctrica en el sur de España: Ubicación mediante SIG. INCI, 32, 131-136.

Ministerio de medio ambiente y medio rural y marino. 2010. Encuesta sobre Superficies y Rendimientos de Cultivos, Gobierno de España, Madrid.

Ndegwa, P.M., Thompson, S.A., Das, K.C., 2000, Effects of stocking density and feeding rate on vermicomposting of biosolids. Bioresour. Technol. 71, 5-12.

Reinecke, A.J., Viljoen, S.A.,1990. The influence of feeding patterns on growth and reproduction of the vermicomposting earthworm *Eisenia fetida* (Oligochaeta), Biol. Fertil. Soils 10 ,184-187.

World Health Organization, 2006. Recommended classification of pesticides by hazard and guidelines to the classification. World Health Organization, Geneva.

Yasmin, S., D'Souza, D., 2010. Effects of pesticides on the growth and reproduction of earthworm: a review. Appl. Environ. Soil Sci. 2010, 1-9.

Zang, Y., Zhong, Y., Luo, Y., Kong, Z.M., 2000. Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. Environ. Pollut. 108, 271-278.

CAPÍTULO 6
CHAPTER 6

UTILIZACIÓN DE DGGE Y COMPOCHIP PARA INVESTIGAR LAS COMUNIDADES BACTERIANAS DE DIVERSOS VERMICOMPOSTS PRODUCIDOS BAJO DISTINTAS CONDICIONES A PARTIR DE RESIDUOS DIFERENTES

USE OF DGGE AND COMPOCHIP FOR INVESTIGATING BACTERIAL COMMUNITIES OF VARIOUS VERMICOMPOSTS PRODUCED FROM DIFFERENT WASTES UNDER DISSIMILAR CONDITIONS

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M. (2011). Use of DGGE and COMPOCHIP for investigating bacterial communities of various vermicomposts produced from different wastes under dissimilar conditions. The Science of Total Environment. Enviado/*Sent.* (STOTEN-S-11-02220)

RESUMEN

A pesar de que los vermicomposts se consideran materiales orgánicos bioactivos que pueden ser utilizados con éxito para resolver o prevenir problemas ambientales como improductividad y degradación de suelos, plagas y enfermedades vegetales, etc., las comunidades bacterianas de los vermicompost producidos a partir de diferentes residuos orgánicos han sido escasamente investigadas. Este estudio describe la utilización de la electroforesis en gel con gradiente desnaturalizante (DGGE) y el COMPOCHIP (un microarray enfocado hacia bacterias típicas de materiales orgánicos estabilizados y bacterias patógenas) para investigar las comunidades bacterianas de cuatro vermicomposts diferentes: un vermicompost comercial producido a partir de estiércol vacuno (CM) y tres vermicompost producidos a escala piloto mediante el reciclaje de destríos de tomate (DT), residuos oleícolas y biosólidos (OB), y residuos vitivinícolas (WW), respectivamente. El DGGE proporcionó inconfundibles huellas indentificativas de cada vermicompost, las cuales se relacionaron estadísticamente con sus características químicas. La comparación de las huellas indentificativas de los vermicomposts dejó patente que éstos albergaban comunidades bacterianas con un coeficiente de similitud medio cercano al 80%. El COMPOCHIP detectó la presencia de *Sphingobacterium*, *Streptomyces*, Alpha-Proteobacteria, Delta-Proteobacteria, y Firmicutes en todos los vermicomposts. También mostró diferencias entre los vermicomposts en la abundancia de los taxones bacterianos examinados, ofreciendo una idea sobre el uso potencial de cada vermicompost para buscar bacterias con capacidades provechosas en biotecnología. El uso conjunto del DGGE y el COMPOCHIP fue una estrategia rápida para comparar las comunidades bacterianas de diversos vermicomposts, valorando también su potencial como materiales orgánicos bioactivos. Esta aproximación también puede proporcionar una valiosa información para nuevos estudios dirigidos al aprovechamiento de las bacterias presentes en los vermicompost.

Palabras clave: Reciclaje, vermicompostaje, material orgánico bioactivo, *Eisenia fetida*, comunidad bacteriana, gen ARN 16S, bacteria patógena.

ABSTRACT

Despite that vermicomposts are considered as bioactive organic materials which can be successfully used for solving or preventing environmental problems such as soil infertility and degradation, environmental pollution, plant pest and diseases, etc., bacterial communities of various vermicomposts produced from different wastes have been scantily investigated. This study describes the use of denaturing gradient gel electrophoresis (DGGE) and COMPOCHIP (i.e. a microarray targeting typical bacteria of stabilized organic materials and pathogenic bacteria) for investigating the bacterial communities of four different vermicomposts: a commercial vermicompost produced from cattle manure (CM) and three vermicomposts produced at pilot-scale by recycling of damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and winery wastes (WW), respectively. DGGE provided distinctive fingerprints of each vermicompost, which were statistically related with their particular chemical features. The comparison of the vermicomposts' fingerprint showed that they housed bacterial communities with an average similarity coefficient near 80%. COMPOCHIP detected the presence of *Sphingobacterium*, *Streptomyces*, Alpha-Proteobacteria, Delta-Proteobacteria, and Firmicutes in all the vermicomposts. COMPOCHIP showed differences in the abundance of inspected bacterial taxa among the vermicomposts, giving an idea about the potential use of each vermicompost for searching bacteria with useful abilities in biotechnology. The joint use of DGGE and COMPOCHIP was a rapid strategy for comparing among bacterial communities of various vermicomposts, assessing also their potential as bioactive organic materials. This approach can also provide a worthwhile guidance for subsequent in-depth studies aimed at profiting from bacteria inhabiting vermicomposts.

Keywords: Recycling, vermicomposting, bioactive organic material, *Eisenia fetida*, bacterial community, 16S RNA gene, pathogenic bacteria.

6.1. INTRODUCTION

Nowadays, the recycling of organic wastes through low-cost technologies, such as composting or vermicomposting, is necessary to overcome environmental problems caused by accumulating of organic wastes in landfills. Particularly, vermicomposting –defined as a process able to stabilize organic matter under aerobic and mesophilic conditions through the joint actuation of earthworms and microorganisms– has become an environmentally appropriate technology for recycling hazardous and worthless organic wastes into safe and valuable products, which are called vermicomposts (Edwards et al., 2010a). Vermicomposts have been early reported as soil organic amendments which house microbial communities relevant to improve soil fertility (Kale et al., 1992). Indeed, vermicomposts are considered as bioactive organic materials with multiple applications. For instance, diverse vermicomposts have been successfully used for increasing crops productivity (Arancon et al., 2005a; Gutiérrez-Miceli et al., 2007), suppressing plant pest and diseases (Arancon et al., 2005b; Szczech, 1999), restoring polluted soil (Delgado-Moreno and Peña, 2009; Fernández-Bayo et al., 2007), or developing mechanism such as biobarriers (Moreno et al., 2009), biocovers (Moon et al., 2010), and biofilers (Fu et al., 2011), which prevent environmental pollutions. Thus, it can be stated that besides the amount of organic matter and nutrients content in a vermicompost, the biological component of a vermicompost determines its usefulness in agriculture, soil restoration, bioremediation, and other applications. In addition, recent studies have isolated various bacteria from diverse vermicomposts, which are interesting for different biotechnological purposes, reporting that vermicomposts are valuable resources of bacteria with interesting applications (Gopalakrishnan et al., 2011; Yasir et al., 2009; Zambare et al., 2011). Therefore, studies focussed on bacterial communities of different vermicomposts may help to predict their usefulness in agriculture, soil restoration, bioremediation, and other applications.

Compared with composts, there is little information on bacteria inhabiting vermicomposts, which is still an obscure topic. To date, there are only a handful of studies which reported relevant information on bacterial composition in vermicomposts. Vaz-Moreira et al. (2008) reported a dominance of strains of the genus *Bacillus* in a vermicompost produced from kitchen refuse through culture-dependent methods. Fracchia et al. (2006) used a culture-independent method (single-strand conformation polymorphism of 16S rRNA gene amplified fragments) to study the bacterial community in three

vermicomposts produced from manure mixed with straw and agricultural crop residues by using independent windrows. Thus, they reported that the replicated vermicompost contained bacterial communities which were so high that they were indistinguishable. Those bacterial communities were dominated by uncultured bacteria belonging to Chloroflexi, Acidobacteria, Bacteroidetes and Gemmatimonadetes. Yasir et al. (2009) also used a culture-independent method (clone libraries of 16S rRNA gene) for investigating the bacterial community in a vermicompost produced from a mixture of paper and dairy sludge. They reported that the majority of clones from that vermicompost belonged to Bacteroidetes (31.2%), Gamma-Proteobacteria (21.3%), and Alpha-Proteobacteria (17.0%). Given that same earthworm species (*Eisenia fetida*) was used in the above mentioned studies, the highlighted findings suggest that dissimilar bacterial communities are present in vermicomposts if they are produced from different wastes. Nevertheless, this statement can not be totally accepted due to those studies investigated bacterial communities in a particular vermicomposts by using incomparable methods. In addition, vermicomposting processes differing in type of processing system and duration may cause differences among bacterial communities of various vermicomposts despite those were produced from similar wastes. To date, no studies have been conducted with the aim of comparing among bacterial communities of various vermicomposts produced from very different organic wastes through dissimilar vermicomposting processes. Hence, it remains to be elucidated in what extent bacterial communities may vary among vermicomposts if they are produced from different wastes by using *E. fetida* (the most frequent worm used in vermicomposting) under dissimilar vermicomposting conditions.

Recent studies have reported that denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments is a fast and reliable technique to obtain genetic fingerprints of vermicomposts' bacterial communities, which can be compared in order to determine what degree of similarity have bacterial communities of various vermicomposts (Fernández-Gómez et al., 2010b). On the other hand, the COMPOCHIP is an oligonucleotide microarray targeting bacteria associated with composting processes, which inhabit well stabilized organic materials (Franke-Whittle et al., 2005). This microarray also includes several oligonucleotide probes for detecting several important human, animal, and plant pathogenic bacteria in an organic material through a single experiment, assessing thus its potential microbiological hazard. Taking into consideration that an integrative approach using a classical fingerprinting technique together with a phylogenetic microarray have been recommended for a rapid characterization of bacterial communities in environmental

samples (Wagner et al., 2007), COMPOCHIP would be a complementary technique to DGGE for investigating bacterial communities of different vermicomposts.

In view of the above observations and with the aim of adding a better understanding on bacterial communities inhabiting vermicompost, this study describes the use of DGGE and COMPOCHIP as a rapid approach for investigating the bacterial communities of four vermicomposts produced from different wastes which were stabilized by using *E. fetida* under dissimilar vermicomposting conditions.

6.2. MATERIALS AND METHODS

6.2.1. Vermicompost production

Four vermicomposts were obtained after recycling of several organic wastes from different origin and nature by using *E. fetida* under various vermicomposting conditions: a vermicompost from cattle manure (CM), one from damaged tomato fruits (DT), one from olive-mill waste mixed with biosolids (OB), and one from winery wastes (WW). CM was commercially produced from cattle manure by Lumbricor S.L. (Córdoba, Spain). This vermicompost was produced from cattle manure through a windrow system operated at large-scale after four months of vermicomposting and one month of maturation without earthworms. DT was experimentally produced from damaged tomato fruits, an abundant waste generated by greenhouse crops. This waste was processed using an indoor continuous-flow vermicomposting reactor as described by Fernández-Gómez et al. (2010a). This reactor was initially filled with a layer of sheep manure (15 kg) where 500 g of earthworms were inoculated. After 15 days the vermireactor was continuously fed with a liquid-paste of damaged tomato fruit for five months. Finally, the earthworms were removed by hand and the pre-processed organic substrate was matured for two months. Whole vermicomposting process was carried out under a controlled temperature (25ºC) with no water was applied beyond that contained in the liquid-paste of tomato fruit. OB was produced from a mixture constituted of wet olive-cake mixed with municipal biosolids at a ratio 8:1 (dw:dw), which was vermicomposted into wood-beds (2 m^2) by adding a total earthworm biomass equivalent to 10% the amount of waste (dw) contained in the bed. Waste moisture was kept at 80-85% by periodical watering during six months of vermicomposting, which was carried out under a controlled temperature (25ºC). Finally, the earthworms were removed by hand and the resulting organic substrate was matured for two months without further water addition. WW

was produced from a mixture of spent grape marc and lees cake at a ratio 1:1 (dw:dw), which are two important organic wastes products of wine production. WW was obtained after a vermicomposting process which was similar to that of OB.

Three samples were taken from each vermicompost. The samples were homogenized, and divided into two subsamples: one was dried and finely ground for chemical analyses and the other stored at -20°C for DNA extraction and molecular analyses.

6.2.2. Chemical analyses

Vermicompost pH and EC were measured with a glass electrode using a 1:10 sample:water (dw:v) ratio. TOC and N were determined with a LECO TruSpec CN analyzer (LECO Corporation, St. Joseph, USA). Total P was measured by the nitrovanadomolybdate method, total K and Na by photometry, and total Ca, Mg, Fe, Mn, Cu, Zn, Ni, Pb, and Cd by atomic-absorption spectrometry after digestion of the samples with HNO_3:$HClO_4$ (Williams, 1984).

6.2.3. DNA extraction

Total DNA was independently extracted using 250g of each one of the three samples collected from the four vermicomposts by means of the PowerSoil^{TM} DNA Isolation Kit (MO BIO Laboratories, Solana Beach, USA). DNA solutions were checked for quality by electrophoresis in 1% agarose gels stained with ethidium bromide and inspected under a UV-transilluminator.

6.2.4. DGGE analysis

The primers 984F (5'-AACGCGAAGAACCTTAC-3') and 1378R (5'-CGGTGTGTACAAGGCCCGGGAACG-3') were used for amplifying fragments of 433 bp (according to the 16S rRNA gene of *Escherichia coli*) including the V6 to V8 bacterial hypervariable regions (Heuer et al., 1997). PCR reactions were performed by adding 2 μl of each DNA extract to a total volume of 25 μl containing 0.2 μM each primer, 1 X reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20], 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin, and 0.025 U BioTherm^{TM} DNA polymerase (GeneCraft, Münster, Germany). A non-template control, containing all PCR components except DNA extract, was included in parallel. Thermal cycling was

performed in a PCR Express cycler (ThermoHybaid, Ulm, Germany) as described by Fernández-Gómez et al. (2010a). Proper sizes of amplification products were verified by electrophoresis in 1.5% agarose gels stained with ethidium bromide. PCR product concentrations were determined with the PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, USA) using an Anthos Zenyth 3100 multimode detector (Anthos labtec, Salzburg, Austria) and the Software for Anthos Multimode Detectors (Version 2.0.0.13).

DGGE was performed by loading 100 ng of PCR products into a polyacrylamide gel (8%, w/v) which contained a denaturing gradient of 40% to 65% (100% denaturants consisting of 40% [v/v] formamide and 7 M urea). A 100 bp DNA ladder (Genecraft®, Germany) served as marker. The gel was run in 1 X TAE (20 mM Tris-Cl, 10 mM acetate, 0.5 mM Na_2EDTA) at 60°C for 16 h at 100 V using an INGENYphorU System (Ingeny International BV, The Netherlands). After electrophoresis, the gel was stained with silver nitrate using a Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Freiburg, Germany) and photographed for subsequent image analysis.

6.2.5. Microarray analysis

The COMPOCHIP microarray, holding 398 different 16S rRNA gene oligonucleotide probes was used for the simultaneous detection of 145 bacterial types at different taxonomic levels, including multiple probes targeting each taxon as described by Franke-Whittle et al. (2005). To perform the microarray analysis, the DNA extracted from two replicates of each vermicompost was subjected to PCR amplification of the nearly complete 16S rRNA genes with the following universal bacterial primers (Lane, 1991): 8F (5'-AGAGTTTGATCMTGG-3'), with a Cy5 label attached at the 5' end, and 1492R (5'-TACCTTGTTACGACTT-3'), with a PO_4^- group conjugated at the 5' end. PCR amplifications were performed by adding 2µl of DNA extract to a total volume of 25 µl, with each reaction mix containing a final concentration of 0.8 µM of the forward primer, 0.2 µM of the reverse primer, 200 µM each dNTP, 1 X reaction KAPA2G GC buffer A, 1X reaction KAPA2G enhancer 2 and 0.5 U KAPA2G Robust DNA Polymerase (KAPABiosystems, Boston, USA) and sterile water. Non-template controls were included in parallel. Amplification was performed in a PCR Express cycler (ThermoHybaid, Ulm, Germany) using an initial denaturing at 94°C for 5 min, followed by 33 amplification cycles, each consisting of 1 min at 94°C, 1 min at 50ºC, and 3 min at 72°C, followed by a final extension step for 10 min at 72°C. Proper sizes of amplification products were verified by

electrophoresis in 1.5% agarose gels stained with ethidium bromide. PCR products were purified with the GenElute PCR Clean-Up Kit (Sigma, Missouri, USA). Fluorescently labelled single-stranded DNA targets were prepared by digesting 1000 ng of purified PCR products using 30 U Lambda exonuclease (Epicentre Technologies, Madison, USA) in 1 X Lambda exonuclease buffer, at 37ºC for 3 h. The subsequent hybridisation was performed in a Hybex hybridisation oven (SciGene, Sunnyvale, USA) as described by Franke-Whittle et al. (2005). Hybridised slides were scanned at 550 nm with a ScanArray Gx microarray scanner (Perkin Elmer, Waltham, USA).

6.2.6. Data analyses

Significant differences among means of the chemical parameters determined in each vermicompost were tested by using a one-way analysis of variance (ANOVA) with *post-hoc* mean separation based on Tukey's test. This statistical analysis was conducted at >95% confidence level ($P < 0.05$) using SPSS® Windows Version 13.0 (IBM, Chicago, USA).
The DGGE patterns were compared using the GelCompar II software (Applied Maths, Kortrijk, Belgium). After the conversion of the scanned gel, band patterns were normalized using the reference positions defined by the molecular weight marker to align the bands for proper comparison. Similarity values among the DGGE band patterns recorded in the vermicompost samples were calculated by comparing their densitometric curves using the Pearson correlation similarity coefficient. The dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) clustering algorithm. A position tolerance of 1% was set for band matching. The binary matrix representing the occurrence of DGGE bands (band presence/absence) was exported to analyse the correlation between the bacterial community structure and the chemical features of the vermicomposts. Statistical correlations between the matrix of occurrence (presence/absence of DGGE bands) and the chemical matrices were evaluated by applying a Mantel test with 999 iterations. This was performed after calculating the binary distances for the occurrence matrix and Euclidean distances for the chemical matrix. Mantel test was performed using the Vegan package for R 2.12.1 (R Foundation for Statistical Computing, Vienna, Austria).

For array analysis, fluorescent images were captured and analysed using the software Scan Array Express (Perkin Elmer, Waltham, USA). Background fluorescence signal of each probe at 550 nm was subtracted from the fluorescence intensity of the same probe. Hybridisation signals were calculated by using the average signal intensity of two spots for

each probe minus the average signal intensity of the non-template spots. Hybridisation signal for each probe was expressed as a percentage of the average signal intensity to the two bacterial positive-control probes (EUB 338 and UNIV 1389) spotted on the same array. A cut-off value of 5% in relation to the control probes was used to define positive (≥5%) and negative signals (<5%). Standardized fluorescence signals were subjected to correspondence analysis (CA) using the PC-ORD program Version 5.0 (MjM Software Design, Gleneden Beach, USA).

6.3. RESULTS AND DISCUSSION

6.3.1. Chemical characterization of the vermicomposts

The vermicomposts showed significant differences in relation to their chemical composition (Table 6.1). The TOC and N contents in the vermicomposts were ranked as follows: WW>OB>DT>CM. All the vermicomposts had a TOC content over the limit of TOC in organic fertilizers (200 g kg^{-1}) that establishes the European Union to award the Eco-label to soil improvers and growing media materials European Union, 2001. On the other hand, in view of the vermicomposts had C:N ratios < 20, they can be legally marketed as organic fertilizers according to the Spanish legislation for fertilizer products (Gobierno de España, 2005). In addition, the total concentration of N along with that of others plant nutrients, such as P and K, can reflect the agronomic value of vermicompost as fertilizer (Edwards et al., 2010b). In this sense, DT would be the most powerful fertilizer as it contained the highest concentrations of these plant macronutrients and also the highest concentrations of the micronutrients Mg, Fe, Mn, and B. However, taking into consideration the values of pH and EC recorded in DT, the application of this vermicompost to crops should carefully carry out. Several legislations of different European countries suggest that a range of pH values between 6.0 and 8.5 is preferable to ensure compatibility with most plants, although mandatory limit values have not been established (Hogg et al., 2002). There are not mandatory limit values for EC in compost but Lasaridi et al. (2006) suggested that their maximum value of EC should be 4 dS m^{-1}, which is considered tolerable by plants with a medium sensitivity to salinity. In view of these recommendations, the high values of pH and EC recorded in DT warns that this vermicompost should be applied to crops taking in consideration the particular crop requirements and the soil pH. However, as DT as the others can be added to soils for restoring its organic matter content without no pollution hazard

according to the thresholds for heavy metals (i.e. Cu, Zn, Ni, Pb, and Cd) established by the Spanish legislation (Gobierno de España, 2005) and those of other countries (Hogg et al., 2002).

Table 6.1. Chemical characteristic of the vermicomposts produced from cattle manure (CM), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and winery wastes (WW).

Composition[1]	CM	DT	OB	WW
pH	7.5 (0.1) c	10.4 (0.2) a	7.4 (0.3) c	8.3 (0.2) b
EC dS m^{-1}	1.4 (0.3) b	4.4 (0.4) a	1.3 (0.2) b	1.4 (0.3) b
TOC g kg^{-1}	98 (2) d	156 (3) c	342 (3) b	422 (2) a
N g kg^{-1}	10.1 (0.1) d	15.8 (0.4) c	17.8 (0.5) b	22.5 (0.2) a
P g kg^{-1}	2.0 (0.1) a	2.0 (0.1) a	1.5 (0.0) b	1.4 (0.1) b
K g kg^{-1}	11.0 (0.8) b	22.7 (1.7) a	8.8 (0.3) b	4.0 (0.2) c
Ca g kg^{-1}	25.8 (1.7) a	18.6 (0.6) b	23.4 (1.6) a	24.5 (1.0) a
Mg g kg^{-1}	5.6 (0.4) b	7.2 (0.3) a	3.6 (0.2) c	3.7 (0.2) c
Na g kg^{-1}	1.2 (0.0) b	1.4 (0.0) a	0.4 (0.0) c	0.5 (0.0) c
Fe g kg^{-1}	7.6 (1.9) ab	8.6 (0.2) a	5.2 (0.1) b	1.6 (0.1) c
Mn mg kg^{-1}	254 (9) a	256 (12) a	134 (1) b	69 (4) c
B mg kg^{-1}	159 (4) b	303 (16) a	163 (6) b	158 (12) b
Cu mg kg^{-1}	49 (2) b	27 (1) c	66 (3) a	66 (6) a
Zn mg kg^{-1}	210 (10) b	99 (1) c	502 (25) a	202 (12) b
Ni mg kg^{-1}	13.8 (0.4) b	15.8 (1.4) b	31.1 (2.5) a	6.8 (0.5) c
Pb mg kg^{-1}	15.4 (2.6) b	<0.2 (0.0) c	34.5 (1.6) a	<0.2 (0.0) c
Cd mg kg^{-1}	<0.2 (0.0) a	<0.2 (0.0) a	<0.2 (0.0) a	<0.2 (0.0) a

Numbers in parenthesis are standard deviations. For each parameter different letters indicate significant differences among the vermicomposts ($p < 0.05$). EC: electrical conductivity; TOC: total organic carbon.

6.3.2. DGGE

DGGE of 16S RNA gene amplified fragments originated a band patterns for each vermicompost sample (Figure 6.1). Bands observed in the DGGE correspond to dominant

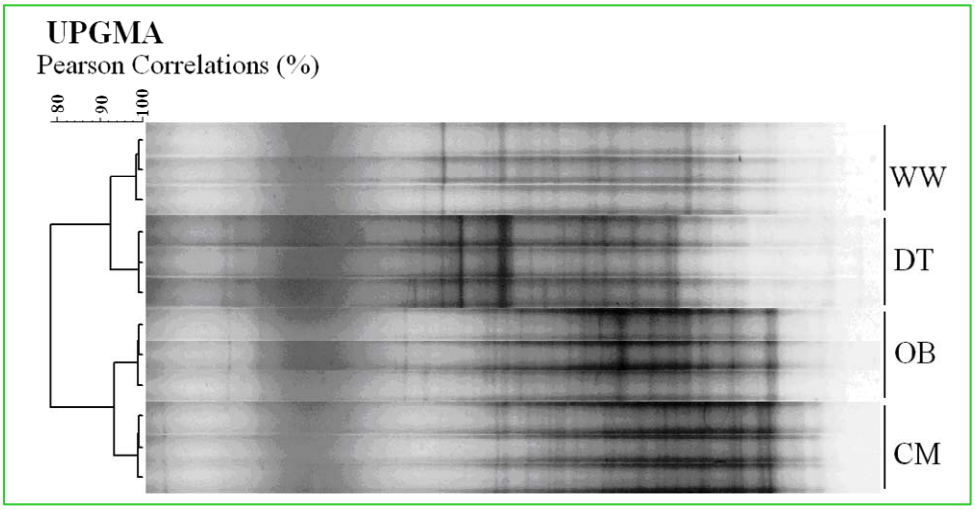


Figure 6.1. Cluster analysis of the DGGE bands patterns of the vermicomposts produced from olive-mill waste and biosolids (OB), winery wastes (WW), damaged tomato fruits (DT), and cattle manure (CM).

rRNA sequences of bacterial taxa whereas the background contains sequences from subdominant bacterial taxa, which can not be displayed. Hence, previous studies have reported that to analyse the band number in DGGE is an inadequate estimation of bacterial diversity in a complex samples, recommending the use of DGGE band patterns as genetic fingerprints of bacterial communities (Loisel et al., 2006). Taking into consideration this recommendation, in the present study, the DGGE band patterns produced from the vermicomposts were used for assessing degrees of similarity among bacterial communities of each vermicompost sample. Thus, cluster analysis of the vermicomposts' DGGE bands patterns showed that the three subsamples collected from each vermicompost had a degree of similarity >95% according the UPGMA similarity coefficient based on Pearson correlation (Figure 6.1). This result suggests the vermicomposts were homogeneous materials which contained a well defined bacterial community. This also confirms the reproducibility of DGGE to obtain fingerprints of vermicompost bacterial community. The comparison among the vermicomposts' DGGE band patterns also revealed an average similarity coefficient near 80% among the bacterial communities contained in vermicomposts produced from different wastes. This may be due to the fact of that all wastes were processed by the same earthworm species (*E. fetida*). Early studies reported that the particular digestive track of each earthworm species digest certain microorganisms whereas others can resist and proliferate, being stimulated to colonized the digested wastes, favouring thus the apparition of a particular bacterial community (Edwards and Fletcher, 1988). Although the bacterial

communities initially contained in different wastes could affect the bacterial communities of their resulting vermicomposts, it has also suggested that earthworm activity strongly transform the physical and chemical state of diverse wastes in a particular way, modifying their bacterial communities in great extent (Domínguez et al., 2010). Supporting this is Fernández-Gómez et al. (2010b), who studied the vermicomposting of different wastes by the same earthworm and revealed that the bacterial communities in various organic substrates had an average similarity coefficient of 42% whereas the bacterial communities of resulting vermicomposts had an average similarity coefficient of 73%.

The cluster analysis of the DGGE band patterns also showed that WW and DT were grouped together with 92.6% of similarity whereas OB and CM were clustered recording 93.2% of similarity (Figure 6.1). Although WW and OB were produced through the same vermicomposting procedure, the fingerprints of their bacterial communities were not clustered together. This could be explained because the bacterial community inhabiting a vermicompost is mainly influenced by its particular chemical features as suggest the significant correlation found between the occurrence of DGGE bands in the vermicomposts and their chemical features ($r = 0.86$, $p = 0.001$). A pH value > 8 recorded in WW and DT whereas OB and CM recorded pH values < 8 (Table 6.1) could be responsible for this grouping pattern. Supporting this idea, pH have been declared as the main environmental factor responsible for differences among bacterial communities inhabiting complex environments like soils (Fierer and Jackson, 2006). These results suggest that various vermicompost can develop analogous bacterial communities as a consequence of they were produced by using the same earthworm species. However, the use of different parental wastes and dissimilar vermicomposting conditions can give rise to vermicomposts with different chemical features which were partially responsible for dissimilarities among their resident bacterial communities.

6.3.3. COMPOCHIP

The analysis of the vermicomposts' bacterial community through COMPOCHIP reported a total of 58 positive probes, corresponding to 40 bacterial taxa (Figure 6.2 and Figure. 6.3). Differences in abundance of these bacterial taxa among the four vermicomposts can be approximately estimated by comparing the intensity of fluorescence hybridisation of each single probe shown in Figure 6.2 (Franke-Whittle et al. 2005).However, comparisons among different probes can not be used to define abundance of bacterial taxa becouse different

COMPOCHIP's probes have different affinity degree for their targets and thus produce unequal hybridisation signals. Given that COMPOCHIP includes multiple probes targeting the same bacterial taxon, the detection of more than one positive probe targeting a particular bacterial taxon in a vermicompost sample is a reliable indicator of the presence of the taxon (Wagner et al., 2007). In addition, in this study, more importance to the presence of a bacterial taxon in a particular vermicompost has been attributed when complementary probes targeting that taxon were detected in the two replicates analysed.

The COMPOCHIP array analysis revealed several bacterial taxa shared by all the vermicomposts. Two probes targeting the genera *Streptomyces* (KO630) and *Sphingobacterium* (KO551) were detected in the both replicates of each vermicompost. Moreover, other three probes targeting these genera were positive, although not in all vermicompost samples. This result indicates that members of these genera are involved in the biostabilization of different organic materials conducted by *E. fetida*. Supporting this idea, Yasir et al. (2009) reported that a mixture of paper sludge and dairy sludge was enriched in *Streptomyce*s spp. after being vermicomposted by *E. fetida*. These authors also found 22 strains, most of them identified as *Streptomyce*s spp, which showed strong antifungal activities against several plant pathogenic fungi. Likewise, Gopalakrishnan et al. (2011) also isolated four species of this generous (*Streptomyces tsusimaensis*, *Streptomyces caviscabies*, *Streptomyces setonii*, and *Streptomyces africanus*) in a vermicompost produced from plant debris by using *E. fetida*, showing the antifungal activity of those bacteria against *Fusarium oxysporum* f. sp. *ciceri*. Hence, the detection of many positive probes targeting *Streptomyce*s spp in OB may indicate the potential usefulness of this vermicompost for being applied in biocontrol of soil-borne plant diseases caused by pathogenic fungi. On the other hand, the presence of the genus *Streptomyces* in all the vermicomposts could indicate that their organic matter had an acceptable degree of maturity since members of this genus have been reported to be involved in mesophilic-maturation phases during composting (Peters et al., 2000). Concerning *Sphingobacterium*, there are important evidences of that the presence of *Sphingobacterium* spp in all the vermicomposts was a particular consequence of using *E. fetida* as several bacterial species belonging to this genus have been found to be characteristic of the *E. fetida* digestive tract (Byzov et al., 2009). Furthermore, Verkhovtseva et al. (2002) reported that members of the genus *Sphingobacterium* play an essential role in the organic matter stabilization in vermicompost samples as they are capable of degrading the simple organic compounds formed after the initial breakdown of complex organic substances. Other positive probes targeting bacterial taxa at higher taxonomic levels, such as

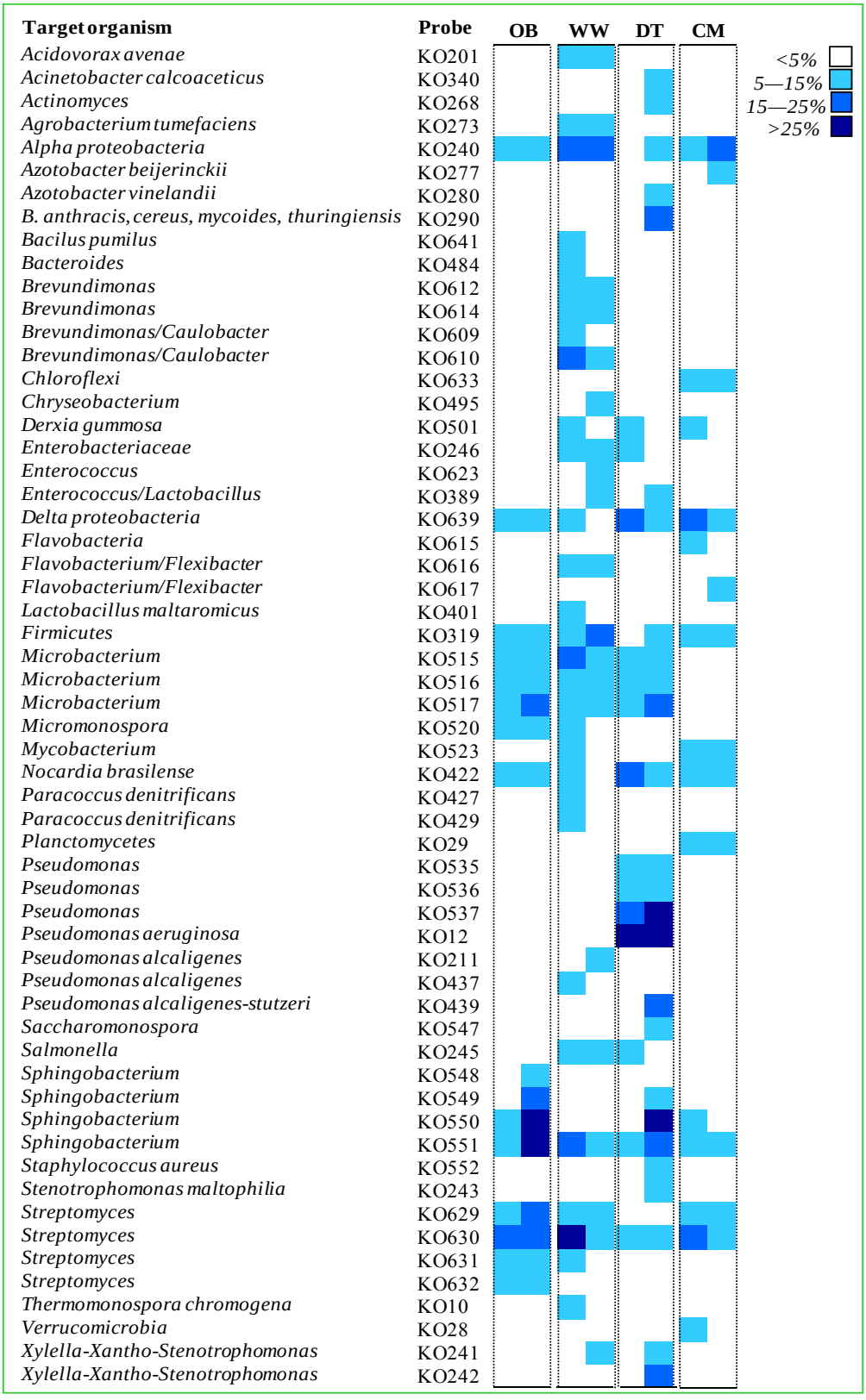


Figure 6.2. Heat map containing the hybridisation results of positive probes recorded in the vermicomposts produced from olive-mill waste and biosolids (OB), winery wastes (WW), damaged tomato fruits (DT), and cattle manure (CM). Hybridisation signal intensity is expressed with respect to the average signal intensity of the positive control probes.

Alpha-Proteobacteria (KO240), Delta-Proteobacteria (KO639), and Firmicutes (KO319) were also observed to be common to all the vermicomposts (except in one replicate of DT and another one of WW). In agreement with this, Fracchia et al. (2006) reported that within the Proteobacteria, the Alpha class was dominant in mature vermicomposts. The presence of Delta-Proteobacteria and Firmicutes in all the vermicomposts could also be explained by the passage of the different wastes through the *E. fetida* digestive track since previous studies revealed greater abundance of populations of these bacterial groups into earthworm casts than in undigested organic substrates (Knapp et al., 2009; Schönholzer et al., 2002). Other positive probes shared by most vermicomposts were three probes targeting *Microbacterium* spp (KO515, KO516, and KO517), which were detected in both replicates of DT, OB and WW. By contrast, this genus was not found in any sample of CM. Given that *Microbacterium* spp has been described as a dominant bacterial taxon characteristic of composts with a relatively high organic matter content (Danon et al., 2008), the absence of this genus in CM could be related to its low TOC content as compared with the others (6.1). This suggests that the presence of *Microbacterium* spp could be used as an indicator of the degree of mineralization of organic matter in vermicomposts.

In contrast to the positive probes detected in all the vermicomposts, certain probes were exclusively detected in WW, DT, and CM. For instance, two probes targeting the genus *Brevundimonas* (KO616 and KO614) and two targeting *Brevundimonas /Caulobacter* (KO609 and KO610) were detected only in WW, whereas medium-high level signals for various *Pseudomonas*-specific probes (KO12, KO535, KO536, and KO537) were found exclusively in DT. Concerning CM, positive signals targeting the phyla Planctomycetes (KO29) and Chloroflexi (KO633) were found only in this vermicompost. In the case of OB, all positive probes recorded were shared with the other vermicomposts. Although any probe was exclusively detected in OB, this vermicompost was characterized by higher signal intensities for the probes KO551 (*Sphingobacterium*) and KO630 (*Streptomyces*) as compared with the others. The distinctive presence of these bacterial taxa in the vermicompost allowed differentiating each particular vermicompost through correspondence analysis (CA) (Figure 6.3). This analysis also highlighted the positive signals that of microarray data helped to detect the positive probes which were shared by all the vermicomposts (probes encircled in Figure 6.3).

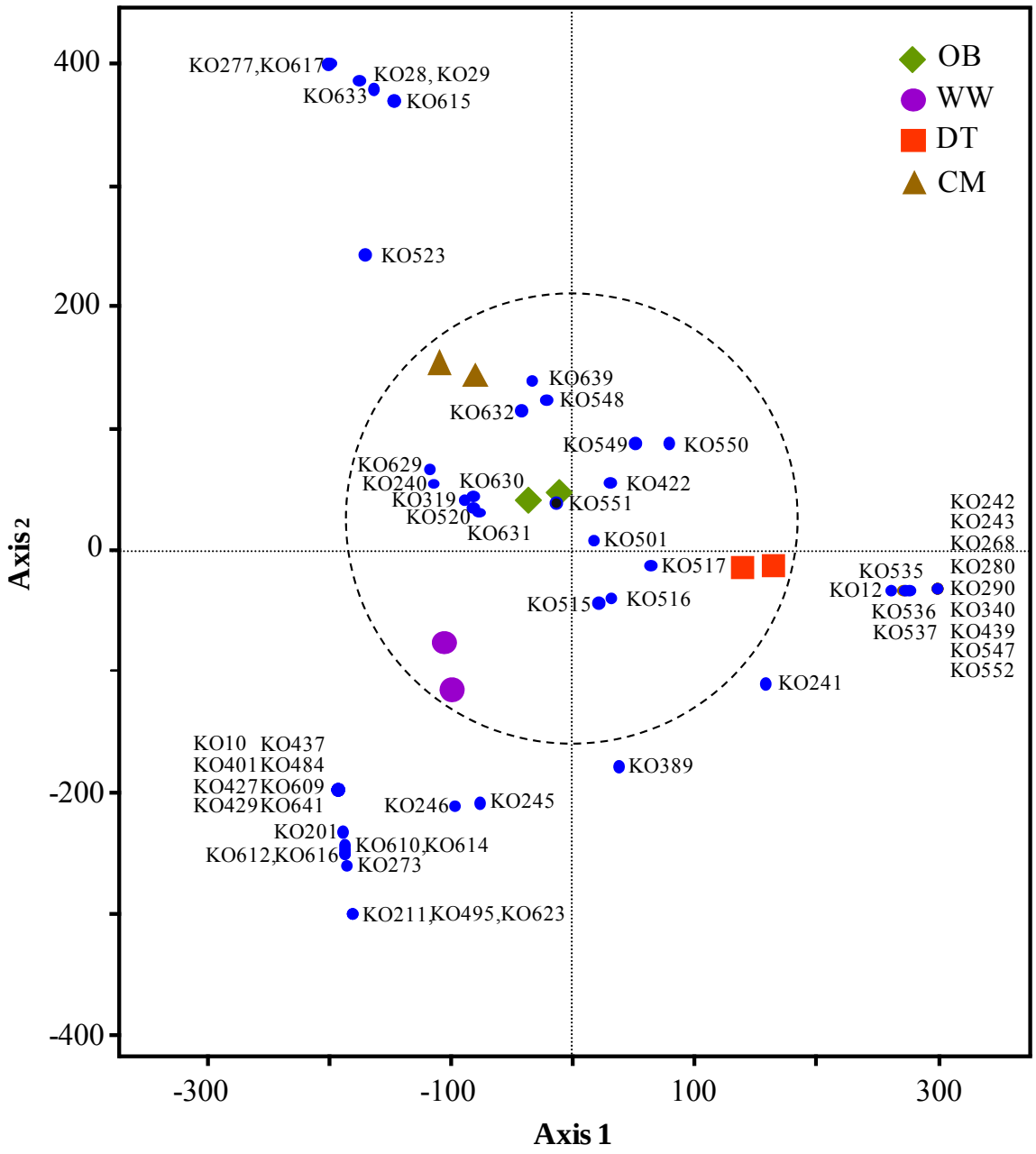


Figure 6.3. Correspondence analysis of community microarray profiles from the vermicompost produced from olive-mill waste and biosolids (OB), winery wastes (WW), damaged tomato fruits (DT), and cattle manure (CM). Probe names are as in Figure 2. Encircled probes showed positive detected in common among all the vermicomposts.

Finally, the COMPCHIP was able to detect the presence of the human pathogen *P. aeruginosa* in DT. Although *P. aeruginosa* is traditionally considered an opportunistic human pathogen, the ability of some strains of this species for degrading pollutants in soil is well known (Das and Mukherjee 2007). In addition, Zambare et al. (2011) isolated a strain of

P. aeruginosa (MCM B-327) from vermicompost pit soil and reported its ability to secrete a novel protease which can be used for recovering dehairing activities in leather industry. Thus, DT could be an interesting resource of *P. aeruginosa* strains with useful abilities for bioremediation or industrial processes. On the other hand, a positive single probe targeting *Nocardia brasiliensis* was found in all the vermicomposts. However, this could be a false positive because only one of the three alternative probes targeting this species gave a positive signal and no positive signals were detected by the *Nocardia* genus probes. Therefore, the specificity of this probe should be further investigated. Other COMPOCHIP's probes targeting human pathogenic bacteria (i.e.: *Campylobacter jejuni*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Vibrio cholerae*, *Helicobacter pylori*, *Streptococcus pyogenes*, *Yersinia enterocolitica*, and pathogenic species belonging to *Clostridium*) did not display positive signals for none of the vermicomposts. Concerning the probes targeting plant pathogenic bacteria, one out of three probes of the COMPOCHIP targeting *Acidovorax avenae* was positive in the two WW replicates. In addition, one out of four probes targeting *Agrobacterium tumefaciens* was positive in the same WW samples. Thus, COMPOCHIP warned that *A. avenae* and *A. tumefaciens* could be present in WW. However, these bacteria may be slightly abundant in WW since the other probes targeting these bacteria displayed negative signals. Nevertheless, the presence of these plant pathogens should be further investigated by culture-plate methods. Franke-Whittle et al. (2005) reported that the detection limit of the COMPOCHIP is 10^3 and 10^5 cells, varying among the different probes targeting the same bacterial taxon as they have not the same affinity degree for their targets. No other COMPOCHIP's probes targeting plant pathogenic bacteria (i.e *Erwinia amylovora*, *Pantoea* sp., *Azospirillum brasilense*, *Ralstonia solanacearum*, *Pseudomonas syzygii*, *Xylella fastidiosa*) were detected as positive. It has been previously reported by many studies that a efficient vermicomposting process performed by using *E. fetida* reduces the abundance of pathogenic microorganisms due to various events: 1. Earthworms modify physical and biochemical properties of organic wastes originating an unfavourable environment for pathogens, which can not compete with other microbes for the limited nutrients (Domínguez et al., 2010; Monroy et al., 2009); 2. Some pathogen are eliminated by the passage of wastes through the gut of earthworms due to they can not survive gut fluids and/or are competitively displaced by the proliferation of other microorganisms (Monroy et al., 2009); and, 3. Enrichment of vermicomposted organic materials in microorganisms with antimicrobial activities against pathogens (Yasir et al., 2009).

6.4. CONCLUSIONS

The present study provides novel information on bacterial communities inhabiting vermicomposts. The results suggest that various vermicomposts produced from different wastes by using the same earthworm species under dissimilar conditions develop analogous bacterial communities with particular differences because of their specific chemical features. Analysing vermicompost bacterial community through COMPOCHIP is a suitable approach for inspecting simultaneously the presence of diverse bacteria which help to define vermicompost quality and usefulness for agricultural practices, suppression of plant pathogen, and other biotechnological applications. Therefore, the joint use of DGGE and COMPOCHIP may become a rapid strategy for comparing among bacterial communities of various vermicomposts, assessing their potential as bioactive organic materials.

ACKNOWLEDGEMENTS

This study was founded by the Junta de Andalucía (P05-AGR-00408) and the Marie Curie Actions (MEIF-CT-2006-041034). Manuel J. Fernández-Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant and his temporary stay at the University of Innsbruck (AP2006-03452). Marta Goberna and Heribert Insam thank support by the Marie Curie Actions (MEIF-CT-2006-041034). The authors also thank David Nesbitt for assisting in the English translation of the manuscript.

REFERENCES

Arancon NQ, Edwards CA, Bierman P, Metzger JD, Lucht C. Effects of vermicomposts produced from cattle manure, food waste and paper waste on the growth and yield of peppers in the field. Pedobiologia 2005a; 49:297-306.

Arancon NQ, Galvis PA, Edwards CA. Suppression of insect pest populations and damage to plants by vermicomposts. Bioresour Technol 2005b; 96:1137-42.

Byzov BA, Nechitaylo TY, Bumazhkin BK, Kurakov AV, Golyshin PN, Zvyagintsev DG. Culturable microorganisms from the earthworm digestive tract. Microbiology 2009; 78:360-8.

Danon M, Franke-Whittle IH, Insam H, Chen Y, Hadar Y. Molecular analysis of bacterial community succession during prolonged compost curing. FEMS Microbiol Ecol 2008; 65:133-44.

Das K, Mukherjee AK. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour Technol 2007; 98:1339-45.

Delgado-Moreno L, Peña A. Compost and vermicompost of olive cake to bioremediate triazines-contaminated soil. Sci Total Environ 2009; 407:1489-95.

Domínguez J, Aira M, Gómez-Brandón M. Vermicomposting: earthworms enhance the work of microbes. In: Insam H, Franke-Whittle I, Goberna M, editors. Microbes at Work. Berlin: Springer; 2010. p. 93-114.

Edwards CA, Arancon NQ, Sherman RL. Vermiculture technology: earthworms, organic wastes, and environmental management. Boca Raton: CRC Press Taylor and Francis Group; 2010a.

Edwards CA, Subler S, Arancon NQ. Quality criteria for vermicomposts. In: Edwards CA, Arancon NQ, Sherman RL, editors. Vermiculture technology: earthworms, organic wastes, and environmental management. Boca Raton: CRC Press Taylor and Francis Group; 2010b. p. 287-301.

Edwards CA, Fletcher KE. Interactions between earthworms and microorganisms in organic-matter breakdown. Agr Ecosyst Environ 1988; 24:235-47.

European Union. Establishing ecological criteria for the award of the community eco-label to soil improvers and growing media. Commission Decision 2001/688/EC 2001.

Fernández-Bayo JD, Nogales R, Romero E. Improved retention of imidacloprid (Confidor) in soils by adding vermicompost from spent grape marc. Sci Total Environ 2007; 378:95-100.

Fernández-Gómez MJ, Nogales R, Insam H, Romero E, Goberna M. Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops. Waste Manage 2010a; 30:2461-8.

Fernández-Gómez MJ, Romero E, Nogales R. Feasibility of vermicomposting for vegetable greenhouse waste recycling. Bioresour Technol 2010b; 101:9654-60.

Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci USA 2006; 103:626-31.

Fracchia L, Dohrmann AB, Martinotti MG, Tebbe CC. Bacterial diversity in a finished compost and vermicompost: Differences revealed by cultivation-independent analyses of PCR-amplified 16S rRNA genes. Appl Microbiol Biotechnol 2006; 71:942-52.

Franke-Whittle IH, Klammer SH, Insam H. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J Microbiol Methods 2005; 62:37-56.

Fu Y, Shao L, Liu H, Tong L, Liu H. Ethylene removal evaluation and bacterial community analysis of vermicompost as biofilter material. J Hazard Mater 2011; 192:658-66.

Gobierno de España. Real Decreto 824/2005 sobre productos fertilizantes. BOE. Madrid: Gobierno de España; 2005. p. 25602-69.

Gopalakrishnan S, Pande S, Sharma M, Humayun P, Kiran BK, Sandeep D, Vidya MS, Deepthi K, Rupela O. Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium* wilt of chickpea. Crop Prot 2011; 30:1070-8.

Gutiérrez-Miceli FA, Santiago-Borraz J, Montes Molina JA, Nafate CC, bud-Archila M, Oliva Llaven MA, Rincón-Rosales R, Dendooven L. Vermicompost as a soil supplement to improve growth, yield and fruit quality of tomato (*Lycopersicum esculentum*). Bioresour Technol 2007; 98:2781-6.

Heuer H, Krsek M, Baker P, Smalla K, Wellington EM. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 1997; 63:3233-41.

Hogg D, Favoino E, Centemero M, Caimi V, Amlinger F, Devliegher W, Brinton W, Antler S. Comparison of compost standards within the EU, North America and Australia. Oxon: The Waste and Resource Action Programme; 2002.

Kale RD, Mallesh BC, Kubra B, Bagyaraj DJ. Influence of vermicompost application on the available macronutrients and selected microbial populations in a paddy field. Soil Biol Biochem 1992; 24:1317-20.

Knapp BA, Podmirseg SM, Seeber J, Meyer E, Insam H. Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. Soil Biol Biochem 2009; 41:2299-307.

Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in Bacterial Systematics. New York: John Wiley & Sons; 1991. p. 115-75.

Lasaridi K, Protopapa I, Kotsou M, Pilidis G, Manios T, Kyriacou A. Quality assessment of composts in the Greek market: The need for standards and quality assurance. J Environ Manage 2006; 80:58-65.

Loisel P, Harmand J, Zemb O, Latrille E, Lobry C, Delgenes JP, Godon JJ. Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ Microbiol 2006; 8:720-31.

Monroy F, Aira M, Domínguez J. Reduction of total coliform numbers during vermicomposting is caused by short-term direct effects of earthworms on microorganisms and depends on the dose of application of pig slurry. Sci Total Environ 2009; 407:5411-6.

Moon KE, Lee SY, Lee SH, Ryu HW, Cho KS. Earthworm cast as a promising filter bed material and its methanotrophic contribution to methane removal. J Hazard Mater 2010; 176:131-8.

Moreno B, Vivas A, Nogales R, Macci C, Masciandaro G, Benítez E. Restoring biochemical activity and bacterial diversity in a trichloroethylene-contaminated soil: The reclamation effect of vermicomposted olive wastes. Environ Sci Pollut Res 2009; 16:253-64.

Peters S, Koschinsky S, Schwieger F, Tebbe CC. Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. Appl Environ Microbiol 2000; 66:930-6.

Schönholzer F, Hahn D, Zarda B, Zeyer J. Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of *Lumbricus terrestris* L. J Microbiol Methods 2002; 48:53-68.

Szczech MM. Suppressiveness of vermicompost against Fusarium wilt of tomato. J Phytopathol 1999; 147:155-61.

Vaz-Moreira I, Silva ME, Manaia CM, Nunes OC. Diversity of bacterial isolates from commercial and homemade composts. Microb Ecol 2008; 55:714-22.

Verkhovtseva NV, Osipov GA, Bolysheva TN, Kasatinov VA, Kuzmina NV, Antsiferova EJ, Alexeeva AS. Comparative investigation of vermicompost microbial communities. In: Insam H, Riddech N, Klammer SH, editors. Microbiology of composting. Berlin: Springer; 2002. p. 99-110.

Wagner M, Smidt H, Loy A, Zhou J. Unravelling microbial communities with DNA-microarrays: challenges and future directions. Microb Ecol 2007; 53:498-506.

Williams S. Official methods of analysis of the Association of Official Analytical Chemists. Arlington, VA, USA: Association of Official Analytical Chemists; 1984.

Yasir M, Aslam Z, Kim SW, Lee SW, Jeon CO, Chung YR. Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity. Bioresour Technol 2009; 100:4396-403.

Zambare VP, Nilegaonkar SS, Kanekar PP. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. New Biotechnol 2011; 28:173-81.

CAPÍTULO 7
CHAPTER 7

PAPEL DE LA COMPOSICIÓN QUÍMICA, DIVERSIDAD FUNCIONAL MICROBIANA, Y ESTRUCTURA DE LA COMUNIDAD FÚNGICA DE VERMICOMPOSTS EN SUS RESPUESTAS RESPIRATORIAS MICROBIANAS A TRES PLAGUICIDAS

ROLE OF VERMICOMPOST CHEMICAL COMPOSITION, MICROBIAL FUNCTIONAL DIVERSITY, AND FUNGAL COMMUNITY STRUCTURE IN THEIR MICROBIAL RESPIRATORY RESPONSE TO THREE PESTICIDES

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M. (2011). Role of vermicompost chemical composition, microbial functional diversity, and fungal community structure in their microbial respiratory response to three pesticides, Bioresource Technology 102, 9638-9645

RESUMEN

Se investigaron las relaciones entre características químicas, actividades enzimáticas, perfiles fisiológicos a nivel de comunidad (CLPPs), y estructuras de comunidades fúngicas del vermicompost, y su respuestas respiratorias microbianas a plaguicidas. La estructura de la comunidad fúngica de vermicomposts producidos a partir de destríos de tomate (DT), residuos vitivinícolas (WW), residuos oleícolas y biosólidos (OB), y estiércol vacuno (CM) se determinaron mediante electroforesis en gel con gradiente desnaturalizante de ADNr 18S. MicroResp™ se utilizó para calcular los CLPPs de los vermicomposts y testar la respuesta microbiana a metalaxyl, imidacloprid y diuron. Las actividades enzimáticas y los CLPPs de los vermicomposts indicaron que WW, OB y DT tuvieron una diversidad funcional microbiana más alta que CM. La microbiota de los primeros toleró los tres plaguicidas mientras que la respiración microbiana en CM fue afectada negativamente por el metalaxil y el imidacloprid. La respuesta de la microbiota de los vermicompost al fungicida metalaxil se correlacionó con la estructura de su comunidad fúngica. Los resultados sugieren que vermicomposts con mayor diversidad funcional pueden ser útiles para el control de la contaminación por plaguicidas en agricultura.

Palabras clave: Diversidad funcional microbiana, plaguicidas, estructura de la comunidad fúngica, actividad enzimática, perfil fisiológico a nivel de comunidad.

ABSTRACT

The relationships between vermicompost chemical features, enzyme activities, community-level physiological profiles (CLPPs), fungal community structures, and its microbial respiratory response to pesticides were investigated. Fungal community structure of vermicomposts produced from damaged tomato fruits (DT), winery wastes (WW), olive-mill waste and biosolids (OB), and cattle manure (CM) were determined by denaturing gradient gel electrophoresis of 18S rDNA. MicroResp™ was used for assessing vermicompost CLPPs and testing the microbial response to metalaxyl, imidacloprid, and diuron. Vermicompost enzyme activities and CLPPs indicated that WW, OB, and DT had higher microbial functional diversity than CM. The microbiota of the former tolerated all three pesticides whereas microbial respiration in CM was negatively affected by metalaxyl and imidacloprid. The response of vermicompost microbiota to the fungicide metalaxyl was correlated to its fungal community structure. The results suggest that vermicomposts with higher microbial functional diversity can be useful for the management of pesticide pollution in agriculture.

Keywords: Microbial functional diversity, pesticides, fungal community structure, enzyme activity, community-level physiological profile.

7.1. INTRODUCTION

Vermicomposting is a low-cost biotechnology which enables the recycling of a variety of wastes from different nature through the combined action of earthworms and microorganisms. Vermicomposts were early reported as bioactive amendments housing microbial communities relevant to improve soil fertility (Kale et al., 1992). Apart from being excellent organic products for agriculture, vermicomposts can be considered useful materials for restoring pesticide contaminated soils as they enhance the adsorption of pesticides reducing the environmental risk of pesticide leaching towards groundwaters (Romero et al., 2006). Fernández-Bayo et al. (2009) reported that amending soils with vermicomposts fosters dissipation of pesticides, such as diuron in agricultural soils. On the other hand, vermicomposts have also been reported as suitable materials for developing bioremediation tools such as biobarriers (Moreno et al., 2009) or biocovers (Moon et al., 2010). In this sense, vermicomposts could be promising organic materials to constitute biomix layers in biobed systems, like thermophilic-composts, which have already been reported to be effective for adsorbing and degrading pesticides in biobeds (Vischetti et al., 2008). Recently, Blaszak et al. (2011) have isolated microorganisms capable of biodegrading the pesticide simazine from a vermicompost produced from manure, suggesting that vermicomposts are a source of pesticide-biodegrading microorganisms. Hence, further knowledge on the resident microbial community in a vermicompost could help to predict its potential utility for pesticide bioremediation.

It is expected that vermicomposts produced from different parental wastes have different chemical compositions and dissimilar autochthonous microbial communities. However, to date, the interrelationships between the chemical features of different vermicomposts and the functional diversity of their resident microbiota are still unclear. Moreover, there are no studies which provide information on the connection between the functional diversity of vermicompost microbiota and its responses to pesticides. Thus, it remains to be elucidated to what extent microbial functional diversity of a vermicompost could be related with its potential utility for pesticide bioremediation. Relevant information on this topic could be inferred by investigating the impact of pesticides on the microbiota of different vermicompost types along with their chemical features and microbial functional diversity. The microbial functional diversity of different vermicomposts can be assessed by determining their community-level physiological profile (CLPP), which captures the ability of the vermicompost microbiota to metabolize single carbon substrates (Campbell et al.,

2003; Mondini and Insam, 2005). Hill et al. (2000) reported that if CLPPs from two samples are clearly separated, then the functional diversity of their resident microbial communities can be considered different. MicroResp™ is a micro-respiration system designed for determining the CLPPs of microbial communities housed in whole-substrates, avoiding the disadvantages of other CLPP approaches based on culturing of microorganisms on plates such as the Biolog system (Campbell et al., 2003). In addition, the great versatility of this system makes it useful for assessing the effect polluting substances, such as pesticides, on the respiratory activity of microbiota inhabiting organic substrates (Campbell et al., 2003). On the other hand, the assessment of enzyme activities, such as oxidoreductases and hydrolases, in vermicomposts has also been reported useful for studying the biochemical functional diversity of vermicompost microbiota (Benítez et al., 1999; Vivas et al., 2009). Recently, Sen and Chandra (2009) reported that the changes in enzyme activities and CLPP occurred during the vermicomposting of sugarcane waste allows assessing the functional diversity of the microbiota involved in vermicomposting. Hence, the joint analysis of enzyme activities and CLPPs seems to be a suitable approach to investigate the functional diversity of microbial communities housed in diverse vermicomposts. On the other hand, the high activity of some hydrolytic enzymes in a vermicompost could be related to the potential ability of its resident microbiota to degrade certain organic substrates and xenobiotics. For instance, Fernández-Bayo et al. (2009) suggested that the high urease activity found in diuron contaminated soils, which had been amended with a vermicompost produced from spent grape marc, could be related to the hydrolysis of this nitrogen-containing herbicide as ureases catalyses the cleavage of N–C bonds in ureic compounds.

Fungal communities play a very important role as an active component of the vermicompost microbiota (Aira et al., 2006). Previous studies reported that vermicomposts house a high diversity of fungi able to degrade a variety of compounds (Anastasi et al., 2005). In addition, most fungi tolerate high concentrations of polluting chemicals, since they have a complex enzymatic machinery able to degrade complex polymers and xenobiotics such as pesticides (Bending et al., 2002). Thereby, the fungal community structure in vermicompost might be related to the response of its microbiota to pesticides. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 18S rRNA gene fragments has been reported as a useful fingerprinting technique for determining the structure of fungal communities inhabiting complex environmental samples (Vainio and Hantula, 2000). Previous studies have highlighted that DGGE is an easy, fast and reproducible technique to

compare the fungal community development during the different phases of vermicomposting (Fernández-Gómez et al., 2010).

In view of the above, this study investigated the chemical features, enzyme activities (dehydrogenase, β-glucosidase, acid phosphatase, and urease), community-level physiological profile, and the fungal community structure of four different vermicomposts, exploring the relationships between these parameters and the respiratory response of the vermicomposts' microbiota to the fungicide metalaxyl, the insecticide imidacloprid, and the herbicide diuron.

7.2. MATERIALS AND METHODS

7.2.1. Vermicompost collection

The vermicomposts analysed in this study were the following: a vermicompost from damaged tomato fruits (DT), one from winery wastes (WW), one from olive-mill waste mixed with biosolids (OB), and one from cattle manure (CM). All these vermicomposts were produced by using the earthworm species *Eisenia fetida*. DT was produced by vermicomposting damaged tomato fruits through an indoor continuous-flow reactor as described by Fernández-Gómez et al. (2010). Briefly, 500 g of earthworms were inoculated in a layer of sheep manure (15 kg) placed in the bottom of the reactor, and 10 kg of damaged tomatoes were applied on that layer every week for five months. Afterwards, the earthworms were removed by hand and the organic substrate was left maturing in the reactor for two months without further waste addition. WW was produced by vermicomposting of spent grape marc mixed with lees cake at a ratio 1:1 (dw:dw) by adding an earthworm biomass equivalent to 10% of the waste mass (dw) contained in the bed. Waste moisture was kept at 80-85% by periodical watering during the vermicomposting process. After six months, the earthworms were removed by hand and the resulting organic substrate was finally matured for two months without further water addition. OB was obtained from wet olive cake mixed with municipal biosolids at a ratio 8:1 (dw:dw) after six months of a vermicomposting process which was similar to that of WW. CM was commercially produced by Lumbricor S.L. (Córdoba, Spain) from cattle manure, which was vermicomposted on a large-scale windrow system for four months plus one month of maturation. All these vermicomposts were homogenised and three samples of 250 g were separately taken and ground (<2 mm).

7.2.2. Chemical analyses

Vermicompost pH and electrical conductivity (EC) were measured with a glass electrode using a 1:10 sample:water (dw:v) ratio. Total organic carbon (TOC) and total nitrogen (N) were determined with a LECO TruSpec CN analyzer (LECO Corporation, St. Joseph, USA). Water soluble carbon (WSC) was extracted by mechanical shaking at 60ºC for 1 h with distilled water (1:10 sample:water; dw:v). Humic acid like (HAL) and fulvic acid like (FAL) compounds were extracted from 2 g of sample by mechanical shaking at 37ºC for 2 h with 40 ml of a solution consisting of 0.1 M $Na_2P_4O_7$ and 0.1 M NaOH. This extract was subsequently acidified to pH≈1 with H_2SO_4 and centrifuged at 3500 rpm to separate the HAL fraction that precipitated from the FAL fraction, which remained in solution. The HAL solution was then obtained by dissolving the precipitate in 10 ml of 0.5 M NaOH. The C content in the WSC, HAL, and FAL solutions was determined by dichromate oxidation followed by titration with ferrous ammonium sulphate.

7.2.3. Enzyme activity analyses

Dehydrogenase activity was determined using iodonitrotetrazolium formazan (INTF) as substrate, as described by García et al. (1997). β-glucosidase and acid phosphatase activities were analysed by determining the amount of p-nitrophenol (PNP) produced from 4-nitrophenyl-β-D glucanopyranoside (PNG) and 4-nitrophenyl phosphate (PNPP) as described by Tabatabai (1982), and Tabatabai and Bremner (1969), respectively. Urease activity was determined using urea as a substrate as described by Kandeler and Gerber (1988). Each enzyme activity was determined per triplicate using 0.2 g of vermicompost sample.

7.2.4. Microresp™ analysis

The community level physiological profiles (CLPPs) were determined by using the micro-respiration system Microresp™ with eleven carbon sources: five carbohydrates (L-arabinose, D-xylose, N-acetyl-D-glucosamine, D-trehalose, D-raffinose), four amino-acids (L-arginine, L-cysteine, D-lysine, glycine), and two organic acids (DL-malic acid and D-galacturonic acid), which are ecologically relevant in soils since they are plant root exudates. Different quantities of each C substrate were dissolved in milliQ-water so that a C concentration

equivalent to 30 mg glucose g^{-1} vermicompost water (11 mg C g^{-1} vermicompost water) was set in each deep-well of the Microresp™ plate after the addition of 0.025 ml of substrate solution (Campbell et al., 2003). In addition to these C substrates, the respiratory response to three pesticides was assayed in the same Microresp™ plate: metalaxyl (acylamino-acid fungicide), imidacloprid (neonicotinoid insecticide), and diuron (phenylurea herbicide). Analytical standard of metalaxyl or imidacloprid were dissolved into milliQ-water and 0.025 ml of each solution was added to vermicompost samples placed in each deep-well (0.20g) to obtain the follow pesticide concentrations: 1, 2, 4 and 8 μg pesticide g^{-1} vermicompost, which are concentrations usually detected in soils as a consequence of pesticide application in conventional agriculture. Assayed concentrations of diuron were: 0.5, 1, 2 and 4 μg pesticide g^{-1} vermicompost. The low solubility of this pesticide (35 mg l^{-1}) prevents to test diuron concentrations over 4 μg pesticide g^{-1} vermicompost since Microresp™ is a miniature applies a small volume of solutions to analyse samples. A volume over 0.025 ml is unadvised because its can cause anaerobic conditions in the tested material (Campbell et al., 2003). Together with the solutions containing the C-substrate and the pesticides one control with deionized water was also included in the Microresp™ deep-well plate. Thus, samples from all four vermicomposts (each being incubated with eleven substrates, three pesticides with four concentrations each, plus a control) were placed together into the same Microresp™ 96 deep-well plate. The assay was reproduced in triplicate.

The patterns of C substrate oxidation in each vermicompost type (CLPPs) were determined by measuring the absorbance (590 nm) in each well of the detection plates at time zero and after 6 hours of incubation with a Zenyth 3100 multimode detector (Anthos, Eugendorf, Austria) spectrophotometer, following the recommendations as described by Campbell et al. (2003). A respiration index (RI) was defined as the difference in absorbance between zero hours and 6 hours of incubation in each well containing each C substrate divided by the difference in absorbance between zero hours and 6 hours recorded in wells containing deionized water. RI values obtained from C substrates and pesticides were expressed per g vermicompost placed into each deep well. The RI values of each C substrate reflect the ability of the vermicompost microbiota to oxidize the substrate. In the case of pesticides, RI values >1 indicate a stimulation of vermicompost microbial respiration as compared with its basal respiration determined by addition of deionized water. Conversely, RI values <1 indicate an inhibition of the basal respiration of the vermicompost microbiota.
The Shannon's diversity index (*H'*) (Shannon and Weaver, 1963) was used as an index of potential metabolic diversity since this was calculated based on the RI values of single C

substrates. This index was measured for each vermicompost as follows: $H' = -\Sigma\ pi \log pi$, where *pi* is the ratio of the respiration index for each single C substrate to the sum of the respiration indices for all carbon substrates. The catabolic versatility index (*CV*) (Sharma et al., 1998) was also calculated using the RI values of single C substrates registered in each vermicomposts as follows: $CV = M/SD$, where *M* is the average RI value from all RI values of every single C substrates measured in each vermicompost, and *SD* is the standard deviation of *M*.

7.2.5. DNA extraction and PCR-DGGE analysis

Total DNA was separately extracted from 250 mg of each one of the three samples taken from the four vermicompost types by means of the PowerSoil™ DNA Isolation kit (MO BIO Laboratories, Solana Beach, USA). DNA solutions were checked for quality by electrophoresis in 1% agarose gels stained with ethidium bromide.

A DNA extract from each sample of the four vermicomposts was subjected to PCR amplification using the primer set FR1 (5'-AICCATTCAATCGGTAIT- 3', I = inosin) and FF390 (5'- CGATAACGAACGAGACCT-3') in order to amplify fragments of 390 bp of the fungal 18S rRNA gene (Vainio and Hantula, 2000). PCR reactions were performed using 2 µl of DNA solution in a total volume of 25 µl, containing 0.2 µM each primer, 1 X reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20], 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin, and 0.025 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany). PCR reactions without a DNA template were included in parallel as control. Thermal cycling was performed as described by Fernández-Gómez et al. (2010), using a PCR Express cycler (ThermoHybaid, Ulm, Germany). Proper sizes of amplification products were verified by electrophoresis on 1.5% agarose gels stained with ethidium bromide and inspected under a UV-transilluminator. PCR product concentrations were determined with the PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, USA). Fluorescence was measured with an Anthos Zenyth 3100 multimode detector (Anthos, Eugendorf, Austria).

The denaturing gradient gel electrophoresis (DGGE) was conducted in an INGENYphorU System (Ingeny International, Goes, The Netherlands), after loading 100 ng of PCR products into a 8% (w/v) polyacrylamide gel in 1 X TAE (20 mM Tris-Cl, 10 mM acetate, 0.5 mM Na_2EDTA). The gel contained a denaturing gradient of 30% to 60% (100% denaturants consisting of 40% [v/v] formamide and 7 M urea) as described by Fernández-

Gómez et al. (2010). A 100 bp DNA ladder (Genecraft®, Germany) was used as marker. Gels were stained with silver nitrate using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Freiburg, Germany) and photographed for subsequent image analysis as described below.

7.2.6. Data analysis

Significant differences among the vermicomposts concerning chemical parameters, enzyme activities, and the indices *H'* and *CV*, were determined by one-way analysis of variance (ANOVA) with mean separation based on the *post hoc* Duncan's multiple-ranged test conducted at a confidence level >95% ($p < 0.05$). The correlations between these parameters were examined using Pearson correlation coefficients, and significant results are given at a high confidence level ($p < 0.01$). A two-way ANOVA was performed for assessing the effect of the pesticides on the RI values registered in the vermicomposts. When significant differences were observed for either the type of vermicomposts or the assayed concentrations of each pesticide, the *post hoc* Duncan's multiple-ranged test was conducted at a confidence level >95% ($p < 0.05$). These statistical analyses were carried out using the software SPSS® Windows Version 13.0 (IBM, Chicago, USA).

The RI values registered for every single C substrate in each vermicompost, which as a whole determine the vermicompost CLPP, were subjected to principal component analysis (PCA) using the PC-ORD program Version 5.0 (MjM Software Design, Gleneden Beach, USA). Multivariate ANOVA of PCA-axes values was applied using SPSS® Windows Version 13.0 for the statistical testing of the separation of vermicomposts along each PC. When a significant F-statistic was noted, the *post hoc* Duncan's multiple-ranged test was conducted at a confidence level >95% ($p < 0.05$) to compare samples' scores for the PCA-axis.

Comparison of DGGE patterns was carried out with the GelCompar II software (Applied Maths, Kortrijk, Belgium). After the conversion of the scanned gel, DGGE band patterns were normalized using reference positions defined by the molecular ladder to align the bands for proper comparison. Similarity values among the band patterns recorded in the vermicompost samples were calculated by comparing their densitometric curves using the Pearson correlation coefficient. The dendrogram was calculated with the unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm setting a position tolerance of 1% for band matching. The binary matrix representing the occurrence of DGGE

bands (band presence/absence) in the vermicompost samples was exported. Statistical correlations between the matrix of occurrence of DGGE bands and the matrices corresponding to the chemical parameters, the enzyme activities, the RI of the C substrates, and the RI of the pesticides measured in the vermicomposts were evaluated by applying a Mantel tests with 999 iterations. Mantel test was performed using Euclidean distances for calculating the distance matrices of the chemical parameters, the enzyme activities, the RI of the C substrates, and the RI of the pesticides; and binary distance for the matrix of occurrence of DGGE bands was used. Significant matrix correlations are given at a high confidence level ($p < 0.01$). This analysis was performed using the Vegan package for R 2.12.1 (R Development Core Team, 2007).

7.3. RESULTS AND DISCUSSION

7.3.1 Chemical features of the vermicomposts

Significant differences were observed for the chemical features examined in the vermicomposts (Table 7.1). The TOC and N contents in the vermicomposts were in the order of WW > OB > DT > CM. Vermicomposts with a higher organic carbon content are more adequate for pesticide bioremediation as they are capable of adsorbing more pesticides as

Table 7.1. Chemical features of vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM).

Properties[1]	DT	WW	OB	CM
TOC (g kg^{-1})	156 (2.6) c	422 (1.5) a	342 (3.1) b	98 (1.7) d
WSC (g kg^{-1})	19.1 (0.4) a	7.2 (0.6) c	10.4 (0.2) b	3.4 (0.9) d
HAL (g kg^{-1})	30.6 (0.7) b	53.9 (9.0) a	11.2 (0.4) c	15.3 (0.2) c
FAL (g kg^{-1})	17.0 (0.9) b	44.5 (2.1) a	9.2 (0.4) c	8.7 (0.5) c
N (g kg^{-1})	15.8 (0.4) c	22.5 (0.2) a	17.8 (0.5) b	10.1 (0.1) d
pH	10.4 (0.2) a	8.3 (0.2) b	7.4 (0.3) c	7.5 (0.1) c

TOC: total organic carbon; WSC: water soluble carbon; HAL: humic acid like compounds; FAL: fulvic acid like compounds; N: total nitrogen. For each property (standard deviations are given in brackets for n=3), different letters indicate significant differences among vermicomposts ($p < 0.05$).

well as providing greater amounts of decomposable carbon compounds that can facilitate the microbial pesticide degradation by co-metabolic processes (Tsui and Roy, 2007). On the other hand, Castillo et al. (2008) reported that high levels of N contents in organic materials can promote pesticide degradation since the greater availability of this nutrient may stimulate the growth and/or activity of autochthonous microorganisms capable of degrading pesticides. These authors also reported that low levels of N in organic materials can activate the fungal lignin-degrading enzymatic system, thus promoting pesticide biodegradation by fungi. The pH value of the vermicomposts is another parameter which could have relevant influence on processes of pesticide biodegradation. The neutral pH of OB and CM can favour the activity of bacteria involved in pesticide biodegradation (Castillo et al., 2008).

The greatest water soluble C (WSC) content was recorder in DT and the lowest in CM (Table 7.1). Given that this fraction of organic-matter comprises the C compounds which are easily available for microorganisms (Benítez et al., 1999), then a high WSC content in vermicompost could allow the microbial pesticide degradation by co-metabolic processes. Other fractions of organic matter in vermicompost such as humic acid-like (HAL) and fulvic acid-like (FAL) compounds have been reported to be capable of adsorbing pesticides from an aqueous solution (Romero et al., 2006). In this sense, the higher HAL and FAL contents in WW and DT suggest that these vermicomposts would be the most effective in adsorbing pesticides from soil solution (Table 7.1). Previous studies reported that improving the adsorption of pesticides in agricultural soils through addition of vermicomposts with a high ability to adsorb pollutants is a successful strategy for decontamination purposes (Fernández-Bayo et al., 2009). Despite the binding of pesticides to vermicomposts is able to decrease their availability for microorganisms, this does not necessarily constitute a barrier against their mineralization since part of a pesticide's sorbed fraction could be still degraded through extracellular enzymes and other microbial mechanisms (Aislabie and Lloyd-Jones, 1995). On the other hand, previous studies have also reported that there is an inversely proportional dependence between the adsorption of a given substance and its solubility in water, so that pesticides having low solubility are likely adsorbed on organic materials (Ignatowicz, 2011).

7.3.2. Enzyme activities of the vermicomposts

A visual comparison among the enzyme activities measured in the vermicomposts is shown in the Figure 7.1. Therein, the values of the four enzyme activities define a Sun-ray plot. The Sun-ray plots has been proposed as a visual fingerprint of biochemical activity in organic

materials, considering the area and shape of this kind of plot reflect the potential functional diversity of the inhabiting microorganisms (Vivas et al., 2009). The Sun-ray plot displayed by DT was characterized by a high value of dehydrogenase activity, which was significantly higher than those of other vermicomposts. This suggests that DT contains the most active

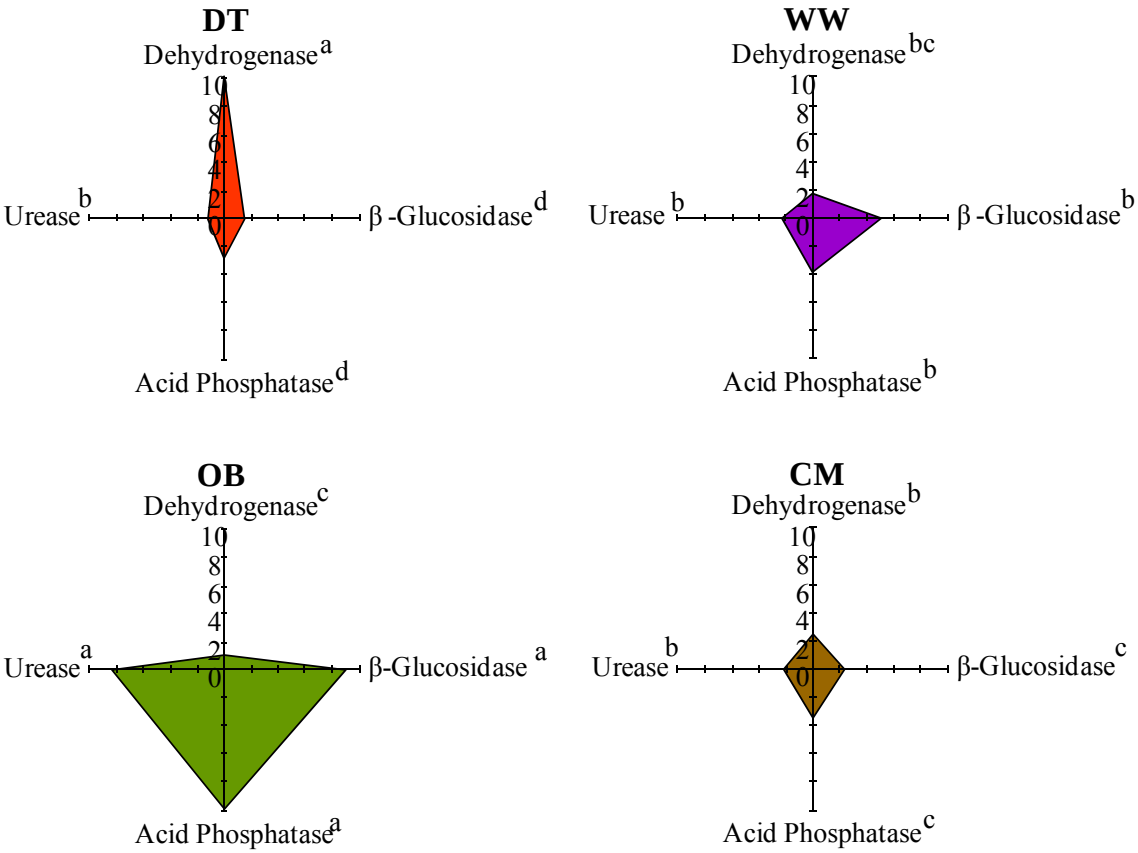


Figure 7.1. Enzyme activities in the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). Dehydrogenase activity is expressed as 7 x (μg INTF g^{-1} h^{-1}), β-glucosidase activity as 75^{-1} x (μg PNP g^{-1} h^{-1}), acid phosphatase activity as 250^{-1} x (μg PNP g^{-1} h^{-1}), and urease activity as 20^{-1} x (μg NH_4^+ g^{-1} h^{-1}). For each enzyme activity, different letters indicate significant differences among the vermicomposts ($p < 0.05$).

microbiota since dehydrogenase activity is considered as a measure of the overall microbial activity (García et al., 1997). This enzyme activity was positively correlated with the content in WSC of the vermicomposts ($r = 0.83$, $p < 0.01$), suggesting that the high microbial activity of DT was supported by its great pool of easily available carbon compounds for the resident microbiota. This is in line with the results reported by Benítez et al. (1999), who

also found a significant positive correlation between dehydrogenase activity and WSC during vermicomposting. With regard to WW, this was characterized by a higher level of β-glucosidase activity as compared with its other enzyme activities. However, among all vermicompost, the greatest value of β-glucosidase activity was observed in OB. β-glucosidases are enzymes involved in the mineralization of organic materials as they catalyse the hydrolysis of terminations of the β-D-glucose chain releasing β-glucose that becomes available for microorganisms. This enzyme activity was positively correlated to the content in TOC of the vermicompost ($r = 0.73$, $p < 0.01$). Therefore, the greater β-glucosidase activity in WW and OB may be a consequence of the higher TOC content in these vermicomposts as compared with that in DT and CM. The β-glucosidase activity was also positively correlated with the acid phosphatase ($r = 0.93$, $p < 0.01$) and urease activities ($r = 0.91$, $p < 0.01$) recorded in the vermicomposts. The latter activities were also positively correlated to each other ($r = 0.99$, $p < 0.01$). Acid phosphatase and urease activities are involved in the mineralization of organic phosphomonoester and ureic compounds, respectively. Like β-glucosidases, acid phosphatases and ureases are inducible enzymes involved in the breaking down of the organic matter during vermicomposting (Benítez et al., 1999). Taking into account that a low microbial activity was found in OB, as its dehydrogenase activity value indicated, the high activity of these hydrolytic enzyme evidenced in OB might be due to the existence of a pool of extracellular enzymes. This is in agreement with Benítez et al. (2005), who reported a high concentration of active ureases immobilized in a vermicompost from olive-cake wastes.

Given that the potential activity of hydrolytic enzymes measured in organic amendments is indicative of the biochemical functional diversity of its resident microbiota (Vivas et al., 2009), then the highest β-glucosidase, acid phosphatase and urease activities recorded in OB suggest that this vermicompost might have the largest microbial functional diversity. Conversely, the poor activity of these hydrolases recorded in DT and CM suggest a low biochemical functional diversity of their resident microbiota. In addition, the smallest Sun-ray plot area recorded in CM indicates that its microbiota was scarcely active, indicating an even lower biochemical functionality than that of DT.

7.3.3 Community-level physiological profiles of the vermicomposts

Principal component analysis (PCA) of the CLPPs of the vermicomposts is shown in the Figure 7.2. The first and second principal components (PC 1 and PC 2) explained 80.9 and

13.6 % of the variance, respectively (Figure 7.2). PC 1 was inversely correlated to L-cysteine (r = - 0.99) and DL-malic acid (r = - 0.97) whereas PC 2 was directly correlated to the carbohydrates D-trehalose (r = 0.93) and D-rafinose (r = 0.92). Statistical analysis of the samples' scores for each PCA-axis demonstrated a significant separation of the vermicomposts in two groups along PC 1 ($F = 37.7$, $p < 0.05$): a group comprising the CLPPs of WW, OB, and DT, and another constituted of the CLPPs of CM. This indicated that the microbial functional diversity of CM is significantly different from that of the other vermicomposts. Mondini and Insam (2005) reported that PCA on CLPPs of samples from different phases of composting was a sensitive method for distinguishing among the microbial communities with different functional abilities involved in each composting phase.

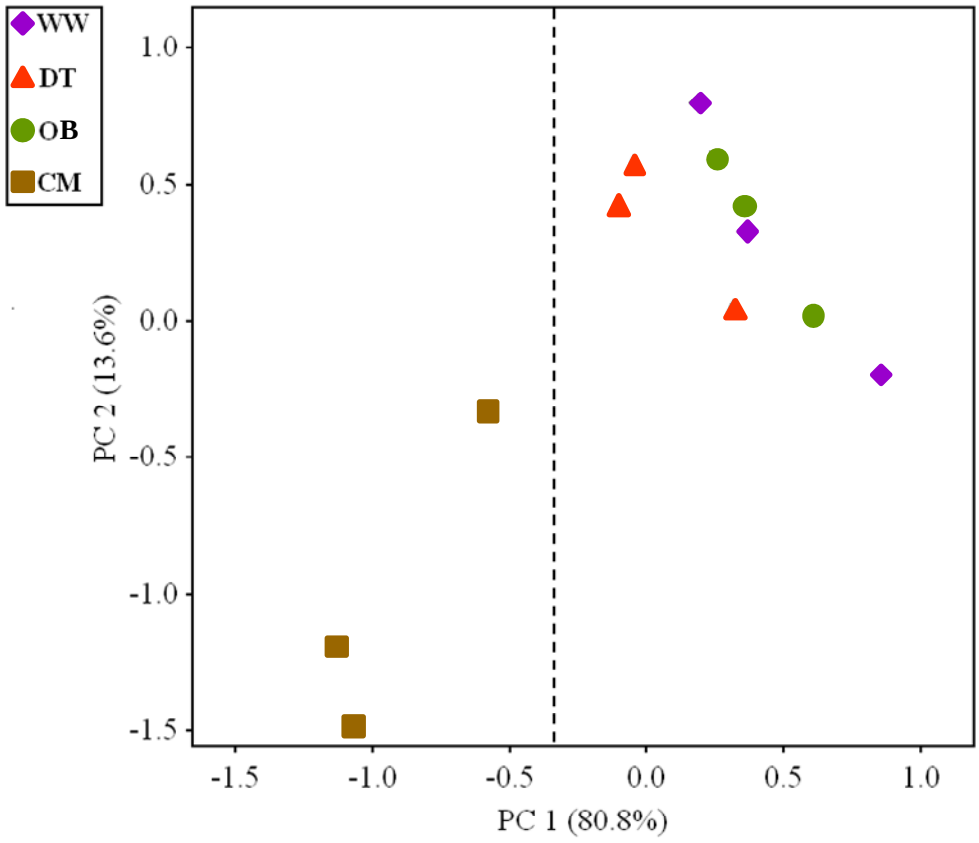


Figure 7.2. Principal component analysis of the community-level physiological profile of the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). The dotted line indicates a significant separation of the vermicomposts along PC 1 ($p < 0.05$).

Concerning the functional diversity indices determined in the vermicomposts, the value of Shannon's index (*H'*) was the lowest in CM (Table 7.2). This indicates that the resident microbiota in CM had comparatively the poorest ability to oxidize the diverse C substrates tested. Campbell et al. (2003) reported that a low ability of a microbial community to oxidize diverse C substrates is indicative of its limited functional diversity. Hence, according to *H'*, CM had less microbial functional diversity as compared to that of other vermicomposts. With regard to the catabolic versatility (*CV*) index, the vermicomposts were ranked as follows: WW and OB > DT > CM (Table 7.2). This indicates that the microbiota in WW and OB was able to oxidise every C substrate with a similar strength (Sharma et al., 1998). Conversely, the microbiota housed in DT and CM preferentially oxidised certain C substrates. Both indices were positively correlated with the vermicompost TOC (*H'*: r = 0.75, p <0.01; *CV*: r = 0.94, p <0.01) and N content (*H'*: r = 0.85, p <0.01; *CV*: r = 0.94, p <0.01), suggesting that the functional and catabolic diversity of the resident microbiota in the vermicomposts could be influenced by these chemical parameters. Thus, the low contents of TOC and N in CM might be partly responsible for the little capability of its resident microbiota to oxidize different C substrates. These results are in accordance with the observations on enzyme activities that pointed out the lowest biochemical functionality of the resident microbiota in CM (see section 7.3.2).

Table 7.2. Shannon's (*H'*) and catabolic versatility (*CV*) indices calculated from the respiration of single C substrates in vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM).

Vermicompost[1]	*H'*	*CV*
WW	1.035 (0.001) a	11.178 (0.877) a
DT	1.024 (0.005) a	6.985 (0.940) b
OB	1.034 (0.000) a	10.298 (0.248) a
CM	0.958 (0.019) b	3.135 (0.523) c

[1]Different letters for each index (standard deviations are given in brackets; n=3) indicate significant differences among vermicomposts (p < 0.05).

7.3.4. Fungal community structure of the vermicomposts

The DGGE corresponding to the resident fungal community in each vermicompost is shown in Figure 7.3a. From this picture, discrete bands were associated to dominant rRNA sequences of fungal taxa whereas the background contains subdominant sequences from rare fungal taxa, that were generally omitted from the analysis of DGGE profile (Loisel et al., 2006). Earlier studies have demonstrated that a single band may contain rRNA sequences from more than one species, besides several bands can be generated from a single species (Sekiguchi et al., 2001). In addition, Loisel et al. (2006) reported that the number of bands is usually stabilized rapidly around 35 despite an increase in the number of sequences belonging to different microorganisms, thus revealing that the analysis of the DGGE bands is inadequate for estimating the true diversity of microbial communities. Therefore, in the present study, DGGE band patterns of the vermicomposts were mainly considered as genetic fingerprints of their fungal community structure.

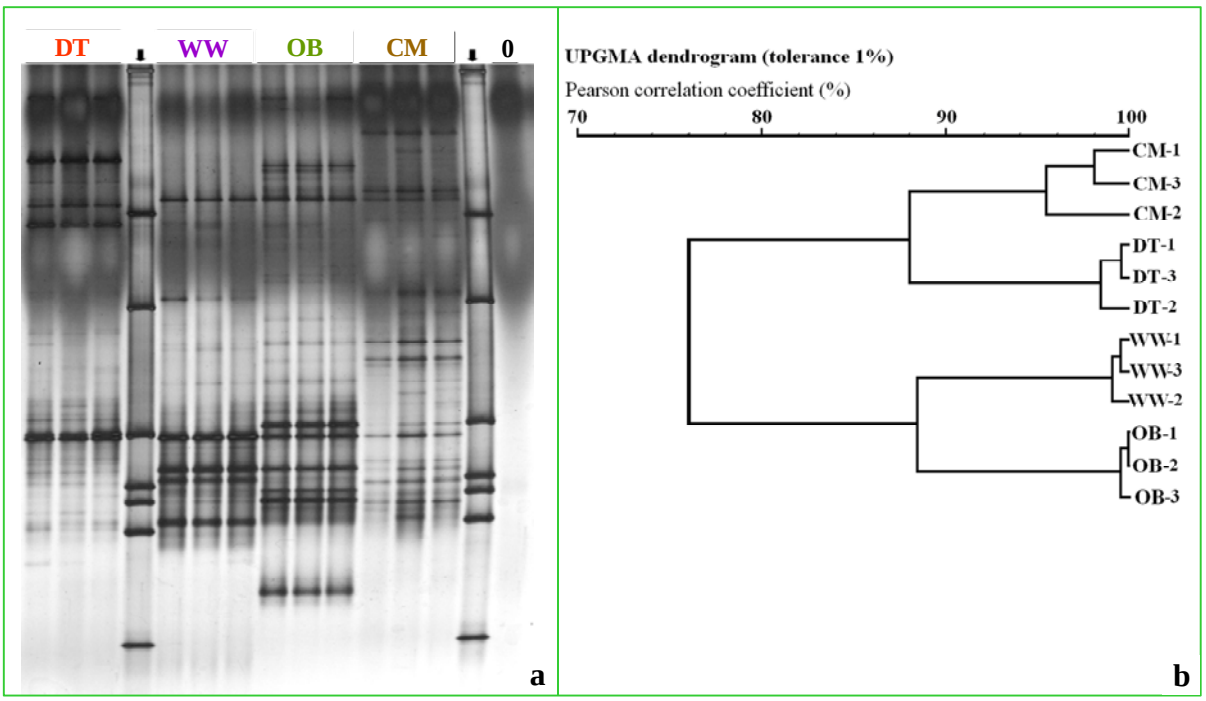


Figure 7.3. Picture of the DGGE of fungal 18S rRNA gene fragments (a) and dendrogram depicting the relatedness among the band patterns (b) in the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). Black arrows indicate lanes with a molecular ladder. Lane defined as 0 includes the PCR product from control reaction without DNA template.

The cluster analysis of the DGGE profiles reported similarities (Pearson correlation coefficient) >95% among the three samples collected from each vermicompost (Figure 7.3b). This suggests that the vermicomposts were homogeneous materials with a well defined fungal community. Furthermore, this confirms the reproducibility of this analysis. The comparison among the DGGE bands patterns also revealed that the fungal communities in the different vermicomposts had analogous structures (similarity ≥ 75%) but with relevant dissimilarities, which were responsible for the formation of two vermicompost clusters. A group was constituted of WW and OB, which clustered together with 88.3 % similarity, whereas DT and CM constituted another group with 87.6 % similarity. This grouping pattern could be influenced by the chemical features of the vermicomposts since a significant correlation was detected between the occurrence of DGGE bands in the vermicomposts and their chemical features, according to the Mantel test ($r = 0.88$, $p < 0.01$). Among the chemical features examined, the strongest predictor of the DGGE band patterns of the vermicomposts was their TOC content ($r = 0.72$, $p < 0.01$), greater in WW and OB than that in DT and CM (Table 7.1). The occurrence of DGGE bands in the vermicomposts was also correlated with their enzyme activities ($r = 0.60$, $p < 0.01$), recording the β-glucosidase activity alone the highest correlation value ($r = 0.60$, $p < 0.01$). This indicates that the chemical characteristics of each vermicompost conditioned its fungal community structure, which may be responsible for part of the hydrolase activities in the vermicomposts, mainly for the β-glucosidase activity. In agreement with this, Lazcano et al. (2008) reported a significant positive correlation between the β-glucosidase activity in a vermicompost from cattle manure and its content in ergosterol, a molecule used as a biomarker for measuring fungal biomass. By contrast, a comparatively lower correlation coefficient, but significant at lower confidence level, was found between the occurrence of DGGE bands in the vermicomposts and their CLPPs ($r = 0.20$, $p = 0.038$). This suggests that other resident microorganisms in the vermicomposts such as bacteria, protozoa, or non-dominant fungi may be considerably involved in the profile of the consumption obtained from the C substrates.

7.3.5 Response of the vermicomposts' microbiota to pesticides

All the assayed concentrations of metalaxyl caused RI values >1 in WW, DT, and OB, whereas CM registered RI values < 1, which were significantly different from those in the former vermicomposts ($F = 18.5$, $p < 0.05$) (Figure 7.4a). The differences observed among

the RI value of the vermicomposts treated with the different concentrations of metalaxyl were not statistically significant for any vermicompost type ($F = 1.6$, $p = 0.21$). In addition, the interaction between the vermicompost type and the metalaxyl concentration was not significant ($F = 0.2$, $p = 0.99$). The RI values of the vermicomposts were affected by the assayed concentrations of imidacloprid in a similar way similar to that observed in the case of the metalaxyl (Figure 7.4b). Thus, the RI values registered in CM were significantly lower than those in the other vermicomposts ($F = 5.8$, $p < 0.05$), whereas the RI values of the vermicomposts were not significantly different among the assayed concentrations of imidacloprid ($F = 0.9$, $p = 0.43$). Likewise, the interaction between both factors was not significant ($F = 0.1$, $p = 0.99$). Concerning diuron, all types of vermicomposts recorded RI values ≥ 1 for all the assayed concentrations of diuron, except in the case of CM treated with 4 μg diuron g^{-1} vermicompost (Figure 7.4 c). No significant differences were found either among the vermicompost types ($F = 1.5$, $p = 0.23$) or among the assayed concentrations of diuron ($F = 0.9$, $p < 0.45$). No significant interaction was recorded between both factors ($F = 0.33$, $p = 0.96$).

A whole interpretation of these results suggests that the resident microbiota in WW, DT, and OB was able to maintain or even increase its basal respiration despite the presence of these pesticides at all the concentrations assayed. The increment in the microbial basal respiration of these vermicomposts could be due to various events: a) the pesticides were directly used as a carbon resource by the vermicompost microbiota; b) the pesticides stimulated, probably by microbial co-metabolic processes, the consumption of other carbon substrates contained in the vermicompost; and/or c) pesticide toxicity stressed the vermicompost microbiota. However, the particular response of the autochthonous microbiota in each vermicompost and the presumable pesticide degradation cannot be assessed in details because the small quantities analysed by using MicroResp™ hinders to study other parameters. Hence, further studies in depth should be conducted to confirm whether the microbiota inhabiting WW, DT, or OB is able to use these pesticides as carbon source and even degrade them to CO_2. In contrast to WW, DT, and OB, the resident microbiota in CM showed a different response to the pesticides metalaxyl and imidacloprid, decreasing its basal respiration for all the assayed concentrations. Nevertheless, the microbial basal respiration of CM did not decrease by the assayed concentration of diuron. This may be due to the lowest solubility of this pesticide as compared with that of metalaxyl and imidacloprid. Aislabie and Lloyd-Jones (1995) reported that the solubility of a pesticide can conditioned its toxicity for microorganisms inhabiting soils.

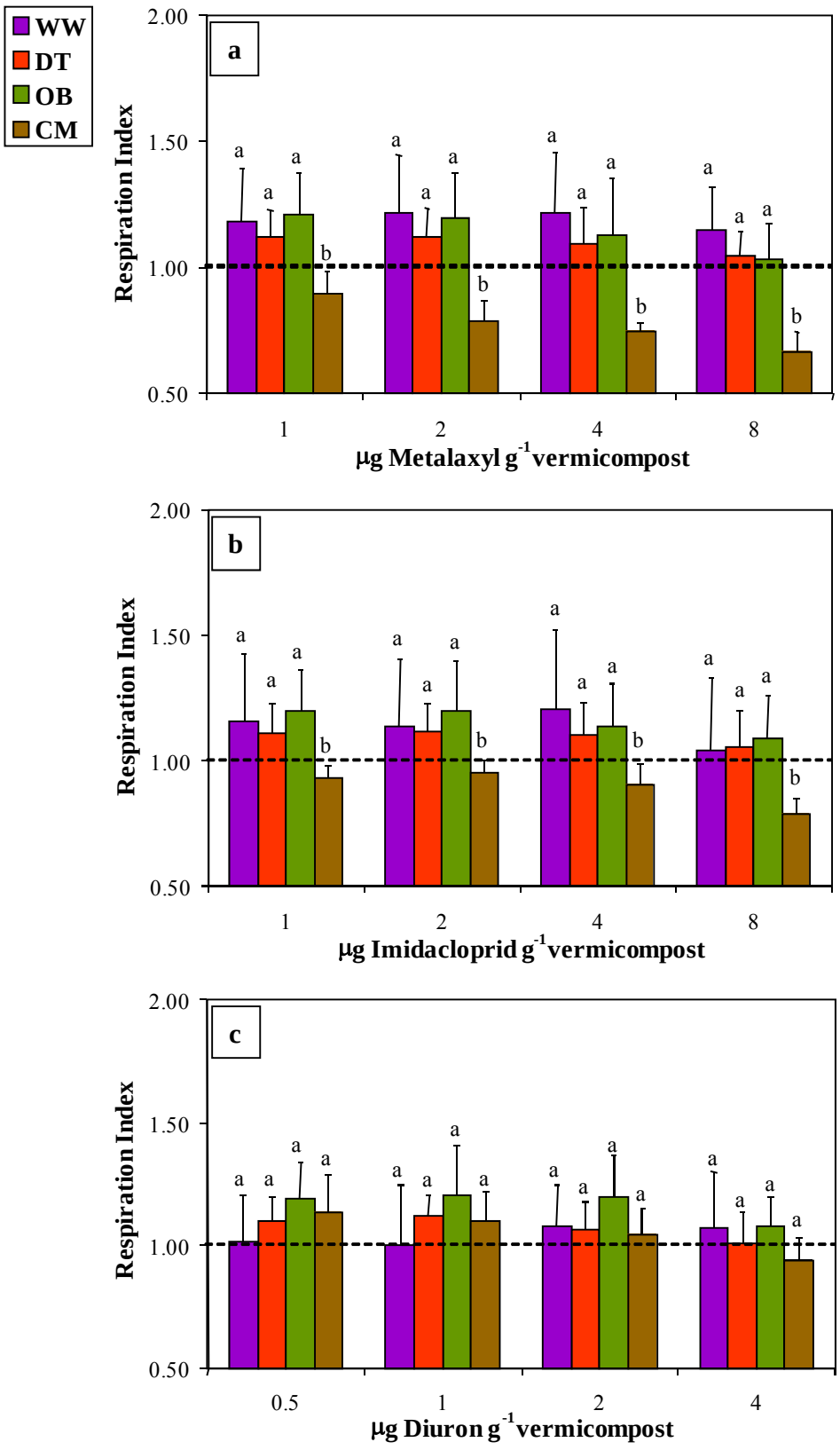


Figure 7.4. Effect of pesticides on the respiration index (RI) of the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). Different letters indicate significant differences among the RI values ($p < 0.05$).

The negative effect of metalaxyl and imidacloprid on the basal microbial respiration of CM could be related to the lowest microbial functional diversity of this vermicompost as compared with that of WW, DT, and OB (see discussion above). Thus, the resident microbiota in WW, DT, and OB could endure the pesticide presence better than that in CM probably due to their greater functional diversity. This assumption is consistent with the idea proposed by Degens et al. (2001), who found that soil microbial communities with a reduced functional diversity are less resistant to stress or disturbance by abiotic factors such as pH, heavy metals, or wet-dry or freeze-thaw cycles.

In the case of the fungicide metalaxyl, the RI values were significantly correlated with the vermicompost fungal community structure ($r = 0.25$ $p = 0.02$). Even a more robust was the correlation found for the highest concentration of metalaxyl alone ($r = 0.32$, $p < 0.01$). This indicates that the impact of this fungicide on the basal respiration of the vermicompost microbiota was partly related to its particular fungal community. Thus, the reduction in the RI observed in CM after metalaxyl might be due to the lesser tolerance of the fungal community inhabiting this vermicompost as compared to the fungal communities of the other vermicomposts. On the other hand, earlier studies reported that as bacteria as fungi are capable of degrading metalaxyl in soil (Bailey and Coffey, 1986). Indeed, recent studies have also reported that some fungi (i.e. *Coriolus versicolor*, *Hypholoma fasciculare* and *Stereum hirsutum*) inhabiting the organic matrix of a biobed can effectively degrade metalaxyl and other pesticides (Bending et al., 2002). Thus, vermicomposts with an increased activity after metalaxyl addition, such as WW, DT, and OB, may be adequate materials to construct the organic matrix of biobeds. Furthermore, the presence in these vermicomposts of fungal strains capable of degrading metalaxyl should be investigated by further studies. Hence, the results indicate that MicroResp™ can be used as a rapid, simple, and inexpensive technique to asses the respiratory response of vermicompost microbiota to pesticides, allowing the fast screening and selection of those vermicomposts whose microbial communities deserve in-depth analyses.

7.4. CONCLUSIONS

This study provides novel information about the relationships between chemical composition, microbial metabolic diversity, and fungal community structure of different vermicomposts with respect to the respiratory response of their resident microbiota to pesticides. The results suggest that vermicomposts with high functional diversity could be

used as organic soil conditioner for diminishing pesticide pollution in agriculture. Moreover, WW, DT, and OB are recommended for conducting further in-depth studies in order to understand the mechanisms of tolerance and presumable degradation of these pesticides by their autochthonous microorganisms. These vermicomposts are also useful for seeking microorganisms capable of degrading the assayed pesticides.

ACKNOWLEDGEMENTS

This study was founded by the Junta de Andalucía (P05-AGR-00408) and the Marie Curie Actions (MEIF-CT-2006-041034). Manuel J. Fernández Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant and his temporary stay at the University of Innsbruck (AP2006-03452). Marta Goberna and Heribert Insam thank support by the Marie Curie Actions (MEIF-CT-2006-041034).

REFERENCES

Aira, M., Monroy, F., Domínguez, J., 2006. *Eisenia fetida* (Oligochaeta, Lumbricidae) activates fungal growth, triggering cellulose decomposition during vermicomposting. Microb. Ecol. 52, 738-747.

Aislabie, J., Lloyd-Jones, G., 1995. A review of bacterial-degradation of pesticides. J. Soil Res. 33, 925-942.

Anastasi, A., Varese, G.C., Filipello Marchisio, V., 2005. Isolation and identification of fungal communities in compost and vermicompost. Mycologia 97, 33-44.

Bailey, A.M., Coffey, M.D., 1986. Characterization of microorganisms involved in accelerated biodegradation of metalaxyl and metolachlor in soils. Canadian J. Microbiol. 32, 562-569.

Bending, G.D., Friloux, M., Walker, A., 2002. Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS Microbiol. Lett. 212, 59-63.

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol. 67, 297-303.

Benítez, E., Sainz, H., Nogales, R., 2005. Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresour. Technol. 96, 785-790.

Blaszak, M., Pelech, R., Graczyk, P., 2011. Screening of microorganisms for biodegradation of simazine pollution (obsolete pesticide Azotop 50 WP). Water Air Soil Pollut., 1-13.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl. Environ. Microbiol. 69, 3593-3599.

Castillo, M.d.P., Torstensson, L., Stenström, J., 2008. Biobeds for environmental protection from pesticide use-a review. J. Agric. Food Chem. 56, 6206-6219.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biol. Biochem. 33, 1143-1153.

Fernández-Bayo, J.D., Nogales, R., Romero, E., 2009. Effect of vermicomposts from wastes of the wine and alcohol industries in the persistence and distribution of lmidacloprid and diuron on agricultural soils. J. Agric. Food Chem. 57, 5435-5442.

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M., 2010. Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops. Waste Manage. 30, 2461-2468.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan. 28, 123-134.

Hill, G.T., Mitkowski, N.A., drich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramírez, S., Lynch, S.T., Nelson, E.B., 2000. Methods for assessing the composition and diversity of soil microbial communities. Appl. Soil Ecol. 15, 25-36.

Ignatowicz, K., 2011. Selection of sorbent for removing pesticides during water treatment. J. Hazard. Mater. 169, 953-957.

Kale, R.D., Mallesh, B.C., Kubra, B., Bagyaraj, D.J., 1992. Influence of vermicompost application on the available macronutrients and selected microbial populations in a paddy field. Soil Biol. Biochem. 24, 1317-1320.

Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils 6, 68-72.

Lazcano, C., Gómez-Brandón, M., Domínguez, J., 2008. Comparison of the effectiveness of composting and vermicomposting for the biological stabilization of cattle manure. Chemosphere 72, 1013-1019.

Loisel, P., Harmand, J., Zemb, O., Latrille, E., Lobry, C., Delgenes, J.P., Godon, J.J., 2006. Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ. Microbiol. 8, 720-731.

Mondini, C., Insam, H., 2005. Effect of inoculum standardization on community level physiological profiles of compost samples. Compost Sci. Util. 13, 27-33.

Moon, K.E., Lee, S.Y., Lee, S.H., Ryu, H.W., Cho, K.S., 2010. Earthworm cast as a promising filter bed material and its methanotrophic contribution to methane removal. J. Hazard. Mater. 176, 131-138.

Moreno, B., Vivas, A., Nogales, R., Macci, C., Masciandaro, G., Benítez, E., 2009. Restoring biochemical activity and bacterial diversity in a trichloroethylene-contaminated soil: The reclamation effect of vermicomposted olive wastes. Environ. Sci. Pollut. Res. 16, 253-264.

R Development Core Team, 2007. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Viena. Available from: http://www.R-project.org.

Romero, E., Salido, A., Cifuentes, C., Fernández-Bayo, J.D., Nogales, R., 2006. Effect of vermicomposting process on pesticide sorption capability using agro-industrial wastes. Int. J. Environ. Anal. Chem. 86, 289-297.

Sekiguchi, H., Tomioka, N., Nakahara, T., Uchiyama, H., 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnol. Lett. 23, 1205-1208.

Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresour. Technol. 100, 804-811.

Shannon, C.E., Weaver, W., 1963. The Mathematical Theory of Communication. University of Illinois Press, Urbana.

Sharma, S., Rangger, A., von Lützow, M., Insam, H., 1998. Functional diversity of soil bacterial communities increases after maize litter amendment. Eur. J. Soil Biol. 34, 53-60.

Tabatabai, M.A., 1982. Soil enzymes, in: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. ASA and SSSA, Madison, pp. 907-943.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301-307.

Tsui, L., Roy, W.R., 2007. Effect of compost age and composition on the atrazine removal from solution. J. Hazard. Mater. 139, 79-85.

Vainio, E., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104, 927-936.

Vischetti, C., Monaci, E., Cardinali, A., Casucci, C., Perucci, P., 2008. The effect of initial concentration, co-application and repeated applications on pesticide degradation in a biobed mixture. Chemosphere 72, 1739-1743.

Vivas, A., Moreno, B., García-Rodríguez, S., Benítez, E., 2009. Assessing the impact of composting and vermicomposting on bacterial community size and structure, and microbial functional diversity of an olive-mill waste. Bioresour. Technol. 100, 1319-1326.

CAPÍTULO 8

CHAPTER 8

EFECTOS A CORTO PLAZO DE COMPOST TERMOFÍLICO Y VERMICOMPOST PRODUCIDOS IDEPENDIENTEMENTE A PARTIR DE RESIDUOS ALIMENTICIOS DE SUPERMERCADO SOBRE LAS PROPEDADES BIOLÓGICAS DE UN SUELO

SHORT-TERM EFFECTS OF THERMOPHILIC-COMPOST AND VEMICOMPOST INDEPENDENTLY PRODUCED FROM GROCERY-FOOD WASTES ON BIOLOGICAL SOIL PROPERTIES

Fernández-Gómez, M.J., Dick, R.P., Edwards, C.A., Romero, E., Nogales, R. (2011). Short-term effects of thermophilic-compost vs vemicompost produced from food wastes on soil enzyme activities and microbial community structure. Geoderma. Enviado/Sent. (GEODER6422)

RESUMEN

Existe un interés creciente en la aplicación de composts como enmiendas orgánicas para el mantenimiento y la mejora de la calidad del suelo. El objetivo de este estudio fue comparar los efectos a corto plazo sobre las propiedades biológicas del suelo que causa la aplicación de dos tipos de enmiendas orgánicas diferentes, un compost termofílico y un vermicompost, que fueron producidos de forma independiente a partir del mismo tipo de material: destríos residuos alimenticios de supermercado. Un suelo agrícola se enmendó con compost termifílico, vermicompost, o una mezcla de ambos en proporción 4:1 usando razones equivalentes a 0, 10 y 30 Tm ha^{-1}. La respiración basal, las actividades β-glucosidasa, ureasa, fosfatasa ácida, y arilsulfatasa, y la estructura de la comunidad microbiana basad en el perfil de ácidos grasos microbianos (FAME) se determinó a lo largo de 28 días de incubación. Después de 28 días, ambas tipos de enmiendas orgáncias aumentaron la producción de CO_2-C sobre la del suelo cuando se aplicaron a la razón de 30 Tm ha^{-1}. Ambos tipos de composts aumentaron en términos generales las actividades enzimáticas, pero la aplicación de 30 Tm ha^{-1} de compost termifílico provocó valores más altos de actividad β-glucosidasa y fosfatasa ácida en comparación con el vermicompost. Los perfiles FAME dejaron patente que únicamente la mayor proporción de cada enmienda orgánica aumentó la biomasa microbiana del suelo, fundamentalmente debido a una mayor biomasa bacteriana. El análisis principal de componentes de los FAME mostró la separación de los dos tipos de composts en términos de comunidades microbianas. En conclusión, el compost termofílico y el vermicompost tuvieron un efecto similar sobre la respiración basal del suelo debido a sus propiedades químicas similares, aunque causaron un efecto diferente sobre algunas actividades enzimáticas del suelo, posiblemente a causa de que las estructuras de la comunidad microbianas desarrolladas en el suelo enmendado con el compost termofílico y en el suelo enmendado con el vermicompost fueron diferentes.

Palabras clave: Enmienda orgánica de suelo, residuos alimenticios de supermercado, actividades enzimáticas, FAME, comunidad microbiana.

ABSTRACT

There is growing interest in the application of composts as organic amendments for the maintenance and improvement of soil quality. The objective of this study was to compare the short-term effects on biological soil properties caused by the application of two different types of organic amendments as thermophilic-compost or vermicompost, which were produced independently by recycling of the same type of material: grocery-food wastes. A cropland soil was amended with thermophilic-compost, vermicompost, or a mixture of both in 4:1ratio by using rates equivalent of 0, 10 or 30 Tm ha^{-1}. Basal respiration, β-glucosidase, urease, acid phosphatase, and arylsulphatase activities, and microbial community structure based on fatty acid methyl esters (FAME) profile were determined along 28 days of incubation. After 28 days, both amendments increased the CO_2-C production over that of the control soil when were applied at the rate of 30 Tm ha^{-1}. Both types of organic amendments increased overall enzyme activity, but 30 Tm ha^{-1} of thermophilic-compost caused higher values for β-glucosidase and acid phosphatase activity as compared with vermicompost at 30 Tm ha^{-1}. FAME profiles showed that only the highest rates of each compost amendments increased soil microbial biomass, primarily due to greater bacterial biomass. A principal component analysis of FAME showed separation of the two compost types in terms of microbial communities. In conclusion, thermophilic-compost and vermicompost had similar effect on basal soil respiration due to their similar chemical properties, although they impacted on soil enzyme activities differently, possibly because the microbial community structure developed in soil amended with the thermophilic-compost was different from that in soil amended with vermicompost..

Keywords: Soil organic amendment, grocery-food wastes, enzyme activities, FAME, microbial community.

8.1. INTRODUCTION

Agricultural applications of organic products produced by wastes-recycling technologies, such as thermophilic-composting and vermicomposting, is gaining importance all over the world in integrated and biological agriculture soil management. This is because such organic amendments are often considered beneficial for the soil and at the same time disposal problems of organic wastes are resolved. The incorporation of organic amendments into the soil may, directly, increase the soil organic matter (SOM) content as well as activate the soil microbial biomass and, indirectly, stimulate the biogeochemical cycles (Pascual et al., 1998), providing nutrients as N, P, K, and micronutrients which are essential for plant nutrition.

Thermophilic-composts and vermicomposts are stabilized organic wastes through two different biooxidative processes, which are considered to be ecological organic materials useful for amending soils. Composting and vermicomposting differ concerning the mechanism responsible for waste degradation and stabilization during each process. Composting includes a thermophilic phase (45 to 65ºC), during which labile organic matter degradation is improved, whereas vermicomposting is a mesophilic process, without thermophilic phase, in which organic matter degradation is due to the combined action of earthworms and microorganisms. Hence, the microbiota involved in each technology may be different. Supporting this is Lazcano et al. (2008), who reported a different microbiological transformation of cattle manure when this waste was composted or vermicomposted. Likewise, Vivas et al. (2009) reported that each technology modified the original bacterial community of a same waste in a diverse way, giving rise to organic product containing different bacterial communities. Therefore, resident microbiota in thermophilic-composts and vermicomposts may be different even if both organic amendments are produced by recycling the same waste type.

Several reports dealing with application of thermophilic-composts or vermicomposts have been published in the last decade. Most these studies have focused on the effect of adding those organic materials on physico-chemical soil properties (García-Gil et al., 2004; Celik et al., 2010) or plant yield (Atiyeh et al., 2002; Arancon et al., 2003). There are few studies about how each compost type impact on soil microbial community. On the other hand, the available studies reported the effects of amending soils with compost or vermicompost, which were produced from different waste sources (Albiach et al., 2000), obtained after processing a same waste by using jointly both technologies, thermophilic composting prior to earthworm inoculation (Tognetti et al., 2005). By contrast, there are not

studies aimed at comparing the effects on soil microbial properties of adding the same waste which was separately biostabilized by thermophilic-composting or vermicomposting.

To know short-term effects of different types of organic amendments on soil biochemical and microbiological properties becomes essential when considering, for example, the conservation of soil quality. Soil biochemical and microbiological properties are considered the most useful tools to assess the soil quality status. In recent years, soil analysis based on enzyme activities or microbial biomass, activity and community structure, have been seen to be early and sensitive indicators of soil stress and could be used to predict soil quality (Dick, 1994). In this context, soil microbial biomass and soil enzymatic activities respond much more quickly to changes in soil management practices compared to other variables and therefore may be useful as early indicators of biological soil changes (Bandick and Dick, 1999; Benítez et al., 2004). On the other hand, fatty acid methylester (FAME) analysis is a fingerprinting technique that provides qualitative and quantitative information on soil microbial community since several signature fatty acids can be used as indicators for certain microbial groups, reflecting soil microbial community structure, and also the sum of microbial signature fatty acids in a given sample can be used as a measure of microbial biomass (Marschner, 2007).

In view of the above issues, the objective of this study was to compare the short-term effects on soil microbiological properties, such soil microbial biomass and activity, soil enzyme activities and soil microbial community structure, caused by the application of two different organic amendments as thermophilic-compost or vermicomposts, which were produced using a similar food waste. A mixture of 80% compost and 20% vermicompost was also assayed as organic soil amended for assessing interactive effect of both composts.

8.2. MATERIALS AND METHODS

8.2.1. Soil sampling and amendments preparation

The soil used in this study was a Crosby silt loam taken (0-10 cm depth) from The Ohio State University Waterman Farm located in Columbus (Ohio, USA). The soil was sieved to pass through a 2 mm screen. Some chemical and physical characteristics of the soil are as follows: sand, 290 g kg^{-1}; silt, 490 g kg^{-1}; clay 220 g kg^{-1}; pH (1:2.5), 7.6; electrical conductivity (1:5 soil:water; dw:dw), 0.13 dS m^{-1}; total organic C (TOC), 8.8 g kg^{-1}; total N, 1.3 g kg^{-1}; $NaHCO_3$-extractable P, 1.0 g kg^{-1}; and $NaHCO_3$-extractable K, 3.2 g kg^{-1}.

Both compost types were produced commercially from grocery store food wastes (fruit and vegetables) which were collected from a chain of supermarkets in Portland Oregon. The thermophilic-compost was produced in windrows by an Oregon company, Natures Needs®. The food waste passed through a thermophilic phase involving 55º-70ºC temperatures for at least 72 hours. The vermicompost was produced in an automated, continuous flow vermicomposting reactor system, which added thin layers of food wastes daily to the top of the system and harvested them from the bottom of the system. Residence time in the reactor was about 60 days. The reactors, which used the species *Eisenia fetida* to process the wastes, were operated by Oregon Soil Corporation (Portland Oregon). The analytical characterization of both organic amendments is shown in Table 8.1.

Table 8.1. Chemical properties of organic amendment applied to the soil.

Composition	Thermophilic-compost	Vermicompost
TOC *	123 (6)	155 (6)
N *	9.9 (0.2)	11.4 (0.3)
P *	1.5 (0.1)	1.9 (0.1)
K *	6.8 (0.2)	7.4 (0.5)
Ca *	7.3 (0.4)	9.8 (0.1)
Mg *	3.5 (0.2)	3.9 (0.1)
Fe *	22 (3)	17 (0.1)
Na **	728 (27)	525(36)
Mn **	675 (29)	539 (48)
Cu **	50 (2)	41 (1)
Zn **	157 (6)	149 (6)
B **	9.2 (0.1)	7.8 (0.9)
Ni **	12 (1)	16 (1)
Pb **	56 (2)	42 (2)
Cd **	<0.2	<0.2

Numbers in parenthesis are standard deviations. *: g kg^{-1}, **: mg kg^{-1}.

8.2.2. Soil incubations trial

Three replicate amounts of soil (100 g) were mixed thoroughly with thermophilic-compost, vermicompost, or a mixture of thermophilic-compost and vermicompost in 4:1 ratio (dw:dw) at two rates: 10 Tm ha^{-1} (1% dw) and 30 Tm ha^{-1} (3% dw), and placed in 250 ml plastic containers. Three containers filled with 100 g of non-amended soil were used as control treatment. The treatment abbreviations used were: control soil (S), soil amended with thermophilic-compost at 1% (C1) and 3% (C3), soil amended with vermicompost at 1% (V1) and 3% (V3), and soil amended with the mixture of both compost types at 1% (CV1) and 3% (CV3). All treatments were brought to 60% field moisture capacity with deionized water, placed in canning jars, and incubated at 25ºC in the dark for 4 weeks. On a weekly basis, soil moisture was adjusted gravimetrically and the microcosms were ventilated for 1 h to maintain aerobic conditions. Microcosms were sampled destructively at 1, 3, 7, 14 or 28 days to assess soil basal respiration, enzyme activity and microbial community structure.

8.2.3. Chemical analyses

Total organic carbon (TOC) and total nitrogen (N) content in the soil, thermophilic-compost, and vermicompost was determined using a CN analyzer. Total P was measured by the nitrovanadomolybdate method, total K and Na by photometry, and total Ca, Mg, Fe, Mn, Cu, and Zn, by atomic-absorption spectrometry after digestion of the samples with HNO_3:$HClO_4$ (Williams, 1984).

8.2.4. Basal respiration and enzyme activities analyses

Basal respiration was assessed as CO_2-C evolution that was trapped in 1 M NaOH, and titrated to neutral pH with 0.5 M HCl after addition of 1 M $BaCl_2$. NaOH traps were placed with soil containers in the canning jars and replaced every 7 days. Basal respiration was expressed as cumulative mg CO_2-C 100 g^{-1}.

Enzyme activities were determined on 1 g soil samples from each replicate. The enzymatic activities β-glucosidase, acid phosphatase and arylsulphatase were quantified by determining the amount of p-nitrophenol (PNP) produced from the substrates 4-nitrophenyl-β-D glucanopyranoside (PNG), 4-nitrophenyl phosphate (PNPP), and 4-nitrophenyl sulphate (PNPS), respectively, as described by Tabatabai and Bremner (1969) and Tabatabai (1982).

Urease activity was determined using urea as substrate following the method described by Kandeler and Gerber (1988).

8.2.5. Soil microbial community structure

Soil microbial community structure was determined by FAME analysis according to the ester-linked FAME method by using a Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA) equipped with a 25 m × 0.2 mm fused silica capillary column (5% diphenyl-95% dimethylpolysiloxane) and a flame ionization detector as described by Schutter and Dick, 2000). According to previous research (Zelles, 1999; Kaur et al., 2005; Marschner, 2007; Moore-Kucera and Dick, 2008), the sum of the following fatty acids was used as biomarkers for Gram-positive bacteria: i15:0, a15:0, i16:0, i17:0, a17:0, and 10Me16:0; for Gram-negative bacteria: 16:1ω5c, 16:1w7c, cy17:0, 18:1ω7c, and cy19:0; for actinomycetes: 10Me16:0, 10Me17:0, and 10Me18:0; and for fungi: 18:1ω9c, 18:2ω6c and 18:3 w6c. The sum of the fatty acids indicative of Gram-positive bacteria, Gram-negative bacteria, and actinomycetes, plus the following three additional fatty acids: 14:0, 15:0, 17:0 were used as a measure of total bacterial biomass. Total amount of FAME extracted (nmol FAME g^{-1} soil) was used as an index of the total microbial biomass.

8.2.6. Statistical analyses

A one-way ANOVA was used to analyze the significant differences between treatments with mean separation based on Tukey's test. Statistical analyses were conducted at >95% confidence level ($p < 0.05$) using SPSS® Windows Version 13.0 (Chicago, Illinois, USA). FAME profiles of soil microbial community at the end of the incubation period were analyzed by principal components analysis (PCA) using the PC-ORD program Version 5.0 (McCune and Mefford, 1999).

8.3. RESULTS AND DISCUSSION

8.3.1. Characteristic of the organic amendments

Although the thermophilic-compost and vermicompost were produced separately from the same waste type, they showed similar elemental composition (Table 8.1). However, some

differences could be observed such as a higher TOC content in the vermicompost than in the thermophilic-compost. The vermicompost also contained more plant macronutrients such as N, P, K, Ca and Mg than the thermophilic compost. Conversely, the thermophilic-compost had higher levels of Na, Fe, Mn, Cu, and B, than the vermicompost. On the other hand, the amounts of heavy metals, i.e. Zn, Ni, and Pb, contained in both amendments were below the limits established by the European Union to grant and eco-label growing media (Commission Decision 2001/688/EC).

Table 8.2. Microbial community of organic amendment applied to the soil.

FAME (nmol g^{-1})	Thermophilic-compost	Vermicompost
Total	1792 (103)	460 (24)
Bacteria	1062 (75)	264 (23)
Gram positive	540 (41)	104 (10)
Gram negative	393 (26)	130 (10)
Actinomycetes	184 (14)	27 (3)
Fungi	282 (6)	60 (8)

Numbers in parenthesis are standard deviations.

FAME analyses revealed more microbial biomass in the thermophilic-compost than in the vermicompost (Table 8.2). Although thermophilic-compost and vermicompost had similar fungi:bacteria ratios, two important differences were observed between them. First, the vermicompost contained a higher percentage of FAME biomarkers for gram negative bacteria (49%) than the thermophilic compost (37%), whereas the percentage of FAME for gram positive were higher in the thermophilic-compost (51%) than in the vermicompost (39%). This could be due to earthworms involved in vermicomposting. Toyota and Kimura (2000) reported that earthworms contain gram-negative bacteria in their intestinal canal, which can be pushed out along with digested organic materials. Second, actinomycetes, which are facultative thermophilic bacteria tolerant to high temperatures, were more abundant in the thermophilic-compost (17%) than in the vermicompost (10%) as indicated by their FAME biomarkers. It also well known that the high temperature developed during thermophilic phase improves spore-forming actinomycetes population, which becomes dominant and increases as composting proceeded (Amner et al., 1988). Lazcano et al. (2008)

reported that vermicomposting produced from cattle manure showed abundances of actinomycetes 100 times smaller than thermophilic composting

8.3.2. Soil basal respiration and enzyme activities

The CO_2-C produced by the amended soil at the low rate was not different significantly from that of the unamended soil (Figure 8.1). Conversely, from the 2nd week on, amended soil with 3% of different organic amendments (C3, V3, CV3) produced significantly more CO_2-C than the control soil. There was no evidence that the type of compost affected respiration rather only the amount added. These results suggest that a high rate of organic amendment is necessary to rise up the total microbial activity of this soil.

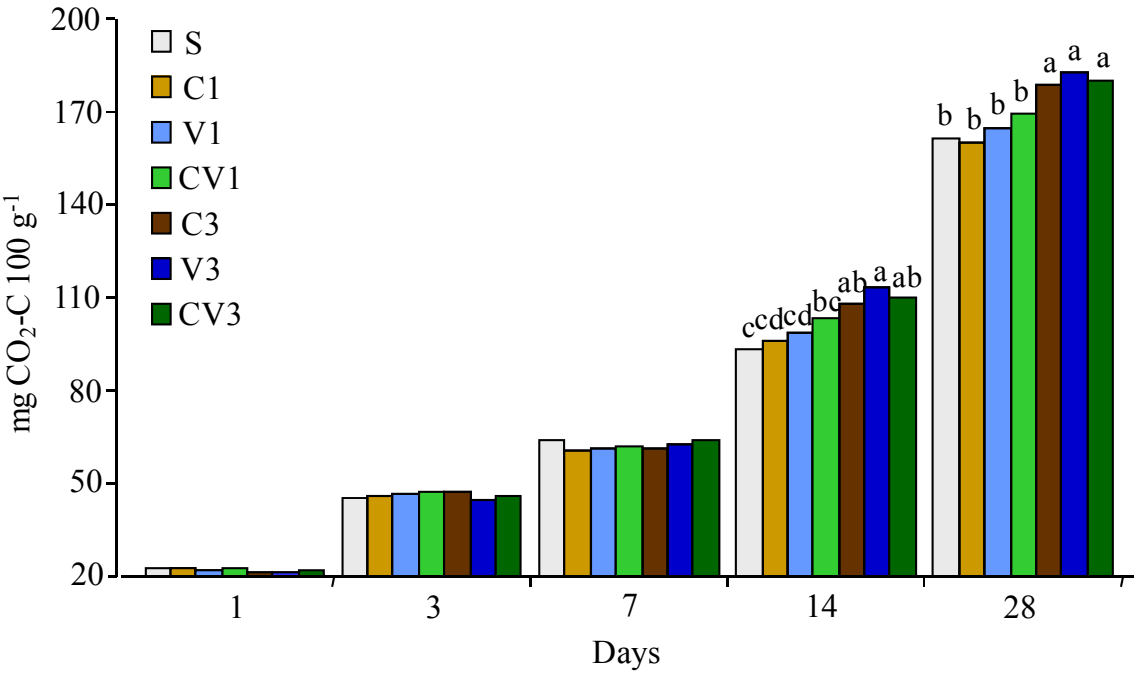


Figure 8.1. Cumulative CO_2-C production during 28-day incubation of a cropland soil (S), which was amended with thermophilic-compost (C), vermicompost (V), or a mixture of thermophilic-compost and vermicompost in 4:1 ratio (CV) at two different rates (1% and 3%). At each given time (days), bars with the same letters are not significantly different (p <0.05).

β-glucosidase activity catalyzes the hydrolysis of cellobiose and other disaccharides playing a major role in organic matter decomposition and provides glucose as a readily available C source for microorganisms. Although the pattern of this enzyme activity varied

widely among the treatments, β-glucosidase activity did not increase or even decreased in all treatments compared with those values observed at day 1. A exception was the soil amended with thermophilic-compost at the high rate (C3) in which β-glucosidase activity increased significantly from the 7th to 28th day of incubation (Figure 8.2a), recording a activity value significantly higher that in the control soil at the end time of the incubation. This suggests that the thermophilic-compost had a greater amount of labile C compounds than the vermicompost, thus induced the synthesis of β-glucosidases. Supporting this idea, Pascual et al. (1998) found that an increase in β-glucosidase activity was observed in soils amended with high rates of a composts which had great easily metabolisable C compounds.

Urease acts on carbon nitrogen (C–N) bonds other than the peptide linkage and are involved in the hydrolysis of urea to carbon dioxide and ammonium. Urease activity increased in all treatments after 3-7 days of incubation and decreased subsequently at the 14th day (Figure 8.2b) down to activity values which were similar to initial values. No differences were found among amended and unamended soils. However, from 14th day to end of the incubation period, the urease activity increased in amended soils while remained at the same level in unamended soils. This indicates that the application of these organic amendments may stimulate the N-cycle, although this enzyme activity seems to not be affected by the different compost type or application rates.

Acid phosphatases are enzymes of agricultural value because they catalyse the hydrolysis of organic phosphomonoester to plant available inorganic phosphate. From 1st day of incubation on, amended soils recorded values of acid-phosphatase activity higher than those in control soil, remaining this activity more or less stable over time during the incubation period (Figure 8.3a). In contrast to urease activity, acid phosphatase activity was sensitive to the rate of organic amendment as indicated by higher values of this activity in soil amended at 3% rate, particularly in the case of C3-amended soil. This suggests that the thermophilic-composts incorporated a great amount of organic-phosphate into the soil that stimulated the microbial community to produce phoshatases. In agreement with this Chang et al. (2007) reported that acid phosphatase increased with linearly increasing rate of compost added to a agricultural soil.

Arylsulphatases are enzymes that catalyze the hydrolysis of aromatic sulphate esters to phenols and sulphate, which is a plant-nutrient. Initial value of arylsulphatase activity decreased initially in all treatments after 3 days of incubation, but increased subsequently peaking at 14th day (Figure 8.3b). From that day on, values of this enzyme activity remained high in amended soil at 3% rate, without differences among the amendment type applied. By

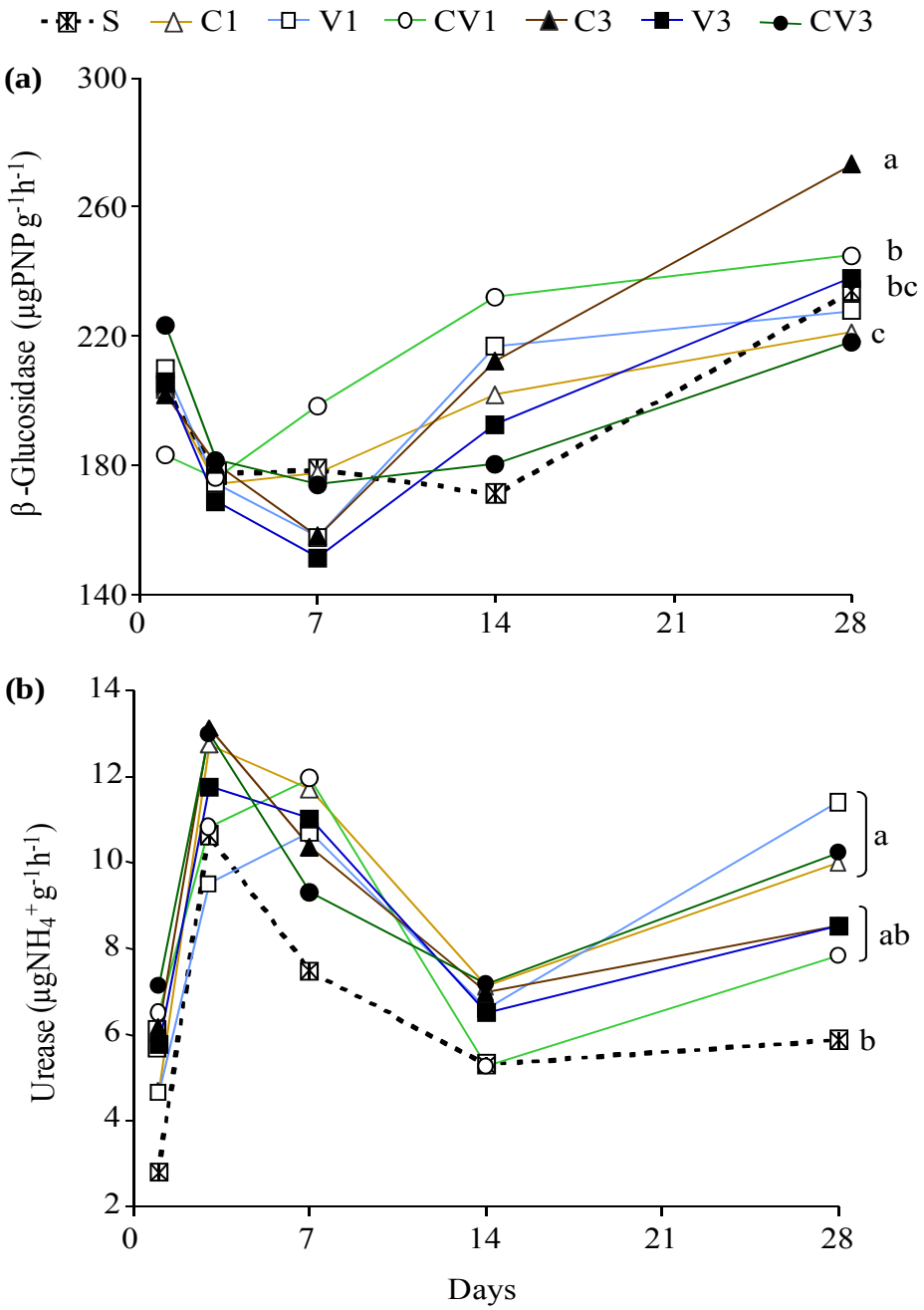


Figure 8.2. β-glucosidase (a) and urease (b) activities during 28-day incubation of a cropland soil (S), which was amended with thermophilic-compost (C), vermicompost (V), or a mixture of thermophilic-compost and vermicompost in 4:1 ratio (CV) at two different rates (1% and 3%). Data with the same letter are not significantly different (p <0.05) at 28 days incubation.

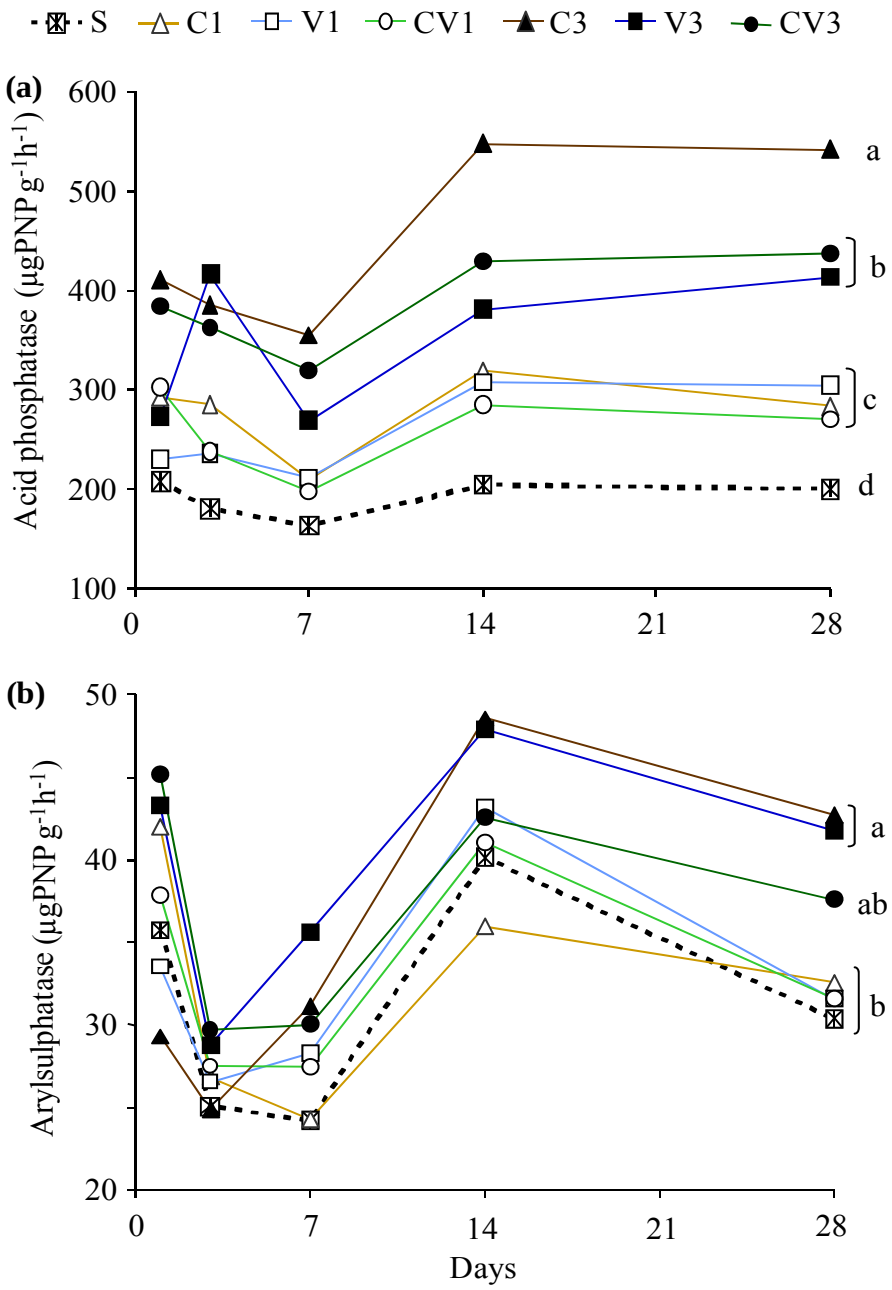


Figure 8.3. Acid phosphatase (a) and arylsuphatase (B) activities during 28-day incubation of a cropland soil (S), which was amended with thermophilic-compost (C), vermicompost (V), or a mixture of thermophilic-compost and vermicompost in 4:1 ratio (CV) at two different rates (1% and 3%). Data with the same letter are not significantly different (p <0.05) at 28 days incubation.

contrast, this activity decreased in amended soil at low rate and control soil. This could be due to arylsulphatase activity is conditional on the soil microbial activity, which was higher in amended soil at 3% rate. This is supported by the significantly high correlation found between this enzyme activity and soil basal respiration at the end time of incubation ($r = 0.56$, $p < 0.01$).

8.3.3. Soil microbial community structure

After the incubation period, total FAME concentrations decreased significantly in the unamended soil (S) and 1%-amended soil (C1, V1, CV1) as compared to day 1 (Table 8.3). Conversely, total FAME concentrations remained more or less constant in the 3%- amended soil (C3, V3, CV3) along the period of incubation. At the end time of the incubation, total FAME concentrations in the 3%-amended soil were significantly greater than those recorded in the soil amended at the low application rate (C1, V1, CV1) and in the control soil. In addition, the amount of total bacterial FAME concentration was also higher in the 3%-amended soil than in the soil amended at 1% and unamended soil whereas that the fungal FAME showed no significant differences between unamended and amended soil. This is reflected in amount of for gram-positive, gram-negative, and actinomycetes in 3%-amended soil which were higher than in 1%-amended and unamended soil (Table 8.4). These results indicate that a high rate of compost amending is able to increase soil bacterial biomass but this has not affect on soil fungi. In agree with these results, Araújo and Monteiro (2006) reported that a high application of composted sludge (1.90 g per 100 g of soil) increases the microbial biomass significantly and also bacterial counts in soil.

After 28 days of incubation, no significant differences concerning total amount of FAME (soil microbial biomass) or FAME concentration for the different microbial groups (Tables 8.3 and 8.4) were found in the soil when was amended with different organic materials at the same rate. However, principal component analysis (PCA) of FAME profiles from the treatments at the end time of incubation separated 3%-amended soil from unamended soil from, and also distinguished between the soil amended with 3% of vermicompost (V3) and the soil amended with 3% of thermophilic-compost (C3) or the mixture constituted of thermophilic-compost plus vermicompost (CV3) (Figure 8.4).

Table 8.3. Total FAME and sum of bacterial and fungal FAME biomarkers (nmol g^{-1}) during 28-day incubation of a cropland soil (S), which was amended with thermophilic-compost (C), vermicompost (V), or a mixture of thermophilic-compost and vermicompost in 4:1 ratio (CV) at two different rates (1% and 3%).

Treatment	Total						Bacteria						Fungi					
	1	3	7	14	28		1	3	7	14	28		1	3	7	14	28	
S	227	200	183	161	163	c	109	91	83	80	78	c	56	54	50	43	44	ab
C1	181	223	183	191	174	bc	88	104	88	96	86	bc	43	57	48	48	46	ab
V1	197	231	178	154	158	c	95	100	86	79	77	c	48	66	48	37	41	b
CV1	181	220	179	180	160	c	85	99	87	89	77	c	47	60	50	47	43	ab
C3	184	269	267	172	187	ab	90	129	128	89	92	ab	43	69	74	42	49	ab
V3	199	176	175	208	197	ab	93	80	86	106	101	a	46	43	45	50	44	ab
CV3	207	229	190	207	202	ab	102	106	92	104	99	a	47	57	50	52	53	a

Data with the same letter are not significantly different ($p < 0.05$) at the end time of the period of incubation.

Table 8.4. Sum of bacterial gram positive, bacterial gram negative and actinomycetes FAME biomarkers (nmol g^{-1}) during 28-day incubation of a cropland soil (S), which was amended with thermophilic-compost (C), vermicompost (V), or a mixture of thermophilic-compost and vermicompost in 4:1 ratio (CV) at two different rates (1% and 3%).

Treatment	Gram positive						Gram negative						Actinomycetes					
	1	3	7	14	28		1	3	7	14	28		1	3	7	14	28	
S	56	45	42	38	36	b	44	38	35	30	31	c	20	17	16	14	12	c
C1	46	53	43	46	41	ab	36	43	35	36	32	c	17	19	16	16	14	bc
V1	48	48	40	37	36	b	40	45	33	30	30	c	18	18	15	13	13	c
CV1	43	49	40	42	37	b	35	42	34	35	29	c	16	18	15	15	13	c
C3	47	60	60	43	43	a	37	54	51	34	35	ab	17	28	21	14	16	ab
V3	47	39	39	48	47	a	42	37	35	43	40	a	16	14	14	17	16	ab
CV3	53	54	42	49	47	a	43	46	37	40	37	ab	19	19	16	17	17	a

Data with the same letter are not significantly different ($p < 0.05$) at the end time of the period of incubation.

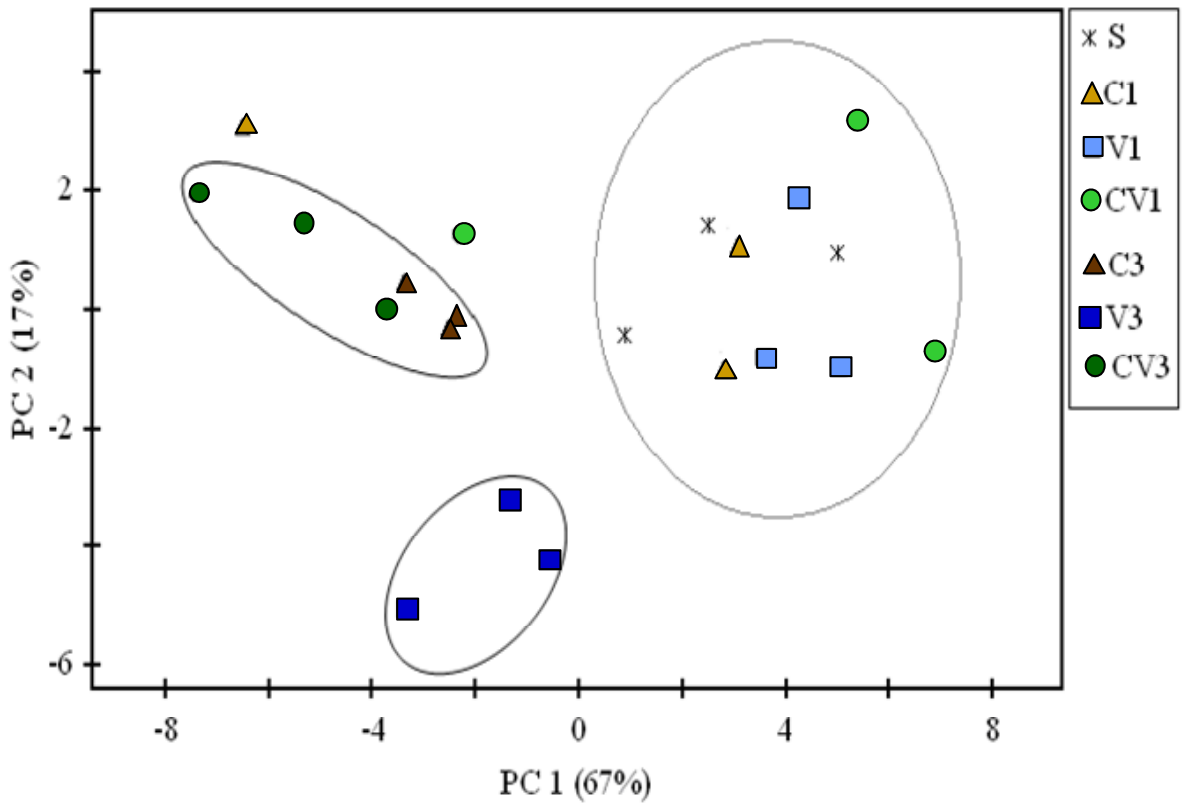


Figure 8.4. Principal components analysis of FAME profiles from microbial communities developed after 28 days of incubation in a cropland soil (S), which was amended with thermophilic-compost (C), vermicompost (V), or a mixture of thermophilic-compost with vermicompost in 4:1 ratio (CV) by using two different rates (1% and 3%). The variance explained by the each principal component (PC) axis is shown in parentheses.

Given that FAME profiles analyzed in soils can be considered as a measure of soil microbial community structure (Marschner, 2007), the result of PCA anlalysis allows to propose two ideas. First, high rate of organic amendment is needed to shift the soil microbial community structure. This is consistent with the results of Saison et al. (2006) who reported that the succession in a microbial community structure was strongly dependent on the compost application rates and only a high rate of compost affected soil microbial community structure. These authors also concluded that changes observed in soil microbial characteristics after compost application were due to the inputs of organic matter and only marginally to the inputs of compost-borne microorganisms. The second idea is that amending a soil with thermophilic-compost or vermicompost can modify its microbial community differently if the organic amendment is applied at high rate; although both organic amendments can affect other above-mentioned soil properties, such as basal respiration, urease and arylsulphatase

enzyme activity, and total microbial biomass, by similar way. In agree with this Pérez-Piqueres et al. (2006) concluded that the impact of the organic amendments on the soil microbiota was dependent on the type of compost used. Other previous studies which were conducted by using FAME analysis demonstrated the usefulness of comparing soil FAME patterns for distinguishing among soil microbial communities subjected to different tillage practices (Schutter and Dick, 2000; Ritchie et al., 2000).

8.4. CONCLUSIONS

A rate of 3% of thermophilic-compost and/or vermicompost increased the basal respiration of a cropland soil and improved its biochemical properties in short-term time. No positive interactive-effects were found when thermophilic-compost and vermicompost were jointly added to the soil. In spite of both organic had similar chemical composition, thermophilic-compost produced a greater stimulation of β-glucosidase and acid phosphatase activities in soil compared with the application of vermicompost at the same rate. Differences concerning enzyme activity temporal pattern among soil amended with each compost type could be explained because each compost type modified the soil microbial community differently despite both affect soil total microbial biomass in a similar way. PCA analysis of FAME profiles is simple and fast method for distinguishing among soil microbial communities developed as a result of adding different organic amendment inhabited by dissimilar microbial communities.

ACKNOWLEDGMENTS

Manuel J. Fernández-Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant (AP2006-03452) that supported this research. We thank Dr. Nicola Lorenz for the support given using the gas chromatography for FAME analyses.

REFERENCES

Albiach, R., Canet, R., Pomares, F., Ingelmo, F., 2000. Microbial biomass content and enzymatic activities after the application of organic amendments to a horticultural soil. Bioresour. Technol. 75, 43-48.

Amner, W., McCarthy, A.J., Edwards, C., 1988. Quantitative assessment of factors affecting the recovery of indigenous and released thermophilic bacteria from compost. Appl. Environ. Microbiol. 54, 3107-3112.

Arancon, N.Q., Edwards, C.A., Bierman, P., Metzger, J.D., Lee, S., Welch, C., 2003. Effects of vermicomposts on growth and marketable fruits of field-grown tomatoes, peppers and strawberries. Pedobiologia 47, 731-735.

Araújo, A.S.F., Monteiro, R.T.R., 2006. Microbial biomass and activity in a Brazilian soil amended with untreated and composted textile sludge. Chemosphere 64, 1043-1046.

Atiyeh, R.M., Lee, S., Edwards, C.A., Arancon, N.Q., Metzger, J.D., 2002. The influence of humic acids derived from earthworm-processed organic wastes on plant growth. Bioresour. Technol. 84, 7-14.

Bandick, A.K., Dick, R.P., 1999. Field management effects on soil enzyme activities. Soil Biol. Biochem. 31, 1471-1479.

Benítez, E., Melgar, R., Nogales, R., 2004. Estimating soil resilience to a toxic organic waste by measuring enzyme activities. Soil Biol. Biochem. 36, 1615-1623.

Celik, I., Gunal, H., Budak, M., Akpinar, C., 2010. Effects of long-term organic and mineral fertilizers on bulk density and penetration resistance in semi-arid Mediterranean soil conditions. Geoderma 160, 236-243.

Chang, E.H., Chung, R.S., Tsai, Y.H., 2007. Effect of different application rates of organic fertilizer on soil enzyme activity and microbial population. Soil Sci. Plant Nutr. 53, 132-140.

Commission Decision 2001/688/EC, 2001. Establishing Ecological Criteria for the Award of the Community Eco-label to Soil Improvers and Growing Media

Dick, R.P., 1994. Soil enzyme activities as indicators of soil quality, in: Doran, J.W., Coleman, D.C., Bedicek, D.F., Stinner, B.R. (Eds.), Defining soil quality for a sustainable environment. SSSA and ASA, Madison, pp. 107-124.

García-Gil, J.C., Ceppi, S.B., Velasco, M.I., Polo, A., Senesi, N., 2004. Long-term effects of amendment with municipal solid waste compost on the elemental and acidic functional group composition and pH-buffer capacity of soil humic acids. Geoderma 121, 135-142.

Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils 6, 68-72.

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., Kaushik, R., 2005. Phospholipid fatty acid - A bioindicator of environment monitoring and assessment in soil ecosystem. Current Sci. 89, 1103-1112.

Lazcano, C., Gómez-Brandón, M., Domínguez, J., 2008. Comparison of the effectiveness of composting and vermicomposting for the biological stabilization of cattle manure. Chemosphere 72, 1013-1019.

Marschner, P., 2007. Soil microbial community structure and function assessed by FAME, PLFA and DGGE-Advantages and Limitations, in: Varma, A., Oelmüller, R. (Eds.), Advanced Techniques in Soil Microbiology. Springer, Berlin, pp. 181-200.

McCune, B., Mefford, M.J., 1999. Multivariate analysis of ecological data, version 5.0. MjM Software Design, Gleneden Beach.

Moore-Kucera, J., Dick, R.P., 2008. PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. Microb. Ecol. 55, 500-511.

Pascual, J.A., Hernández, T., García, C., Ayuso, M., 1998. Enzymatic activities in an arid soil amended with urban organic wastes: Laboratory experiment. Bioresour. Technol. 64, 131-138.

Pérez-Piqueres, A., Edel-Hermann, V., Alabouvette, C., Steinberg, C., 2006. Response of soil microbial communities to compost amendments. Soil Biol. Biochem. 38, 460-470.

Ritchie, N.J., Schutter, M.E., Dick, R.P., Myrold, D.D., 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. Appl. Environ. Microbiol. 66, 1668-1675.

Saison, C., Degrange, V., Oliver, R., Millard, P., Commeaux, C., Montange, D., Le Roux, X., 2006. Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. Environ. Microbiol. 8, 247-257.

Schutter, M.E., Dick, R.P., 2000. Comparison of Fatty Acid Methyl Ester (FAME) Methods for Characterizing Microbial Communities. Soil Sci. Soc. Am. J. 64, 1659-1668.

Tabatabai, M.A., 1982. Soil enzymes, in: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. ASA and SSSA, Madison, pp. 907-943.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301-307.

Tognetti, C., Laos, F., Mazzarino, M.J., Hernández, M.T., 2005. Composting vs. vermicomposting: A comparison of end product quality. Compost Sci. Util. 13, 6-13.

Toyota, K., Kimura, M., 2000. Microbial community indigenous to the earthworm *Eisenia foetida*. Biol. Fertil. Soils 31, 187-190.

Vivas, A., Moreno, B., García-Rodríguez, S., Benítez, E., 2009. Assessing the impact of composting and vermicomposting on bacterial community size and structure, and microbial functional diversity of an olive-mill waste. Bioresour. Technol. 100, 1319-1326.

Williams, S., 1984. Official methods of analysis of the Association of Official Analytical Chemists., 14th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A review. Biol Fertil. Soils 29, 111-129.

CAPÍTULO 9
CHAPTER 9

VERMICOMPOSTS Y/O INOCULACIÓN FÚNGICA MICORRÍCICA EN RELACIÓN A LA DISPONIBILIDAD DE METALES Y LA CALIDAD BIOQUÍMICA DE UN SUELO CONTAMINADO CON METALES PESADOS

VERMICOMPOSTS AND/OR ARBUSCULAR MYCORRHIZAL FUNGAL INOCULATION IN RELATION TO METAL AVAILABILITY AND BIOCHEMICAL QUALITY OF A SOIL CONTAMINATED WITH HEAVY METALS

Fernández-Gómez, M.J., Quirantes, M., Vivas, A., Nogales, R. (2011). Vermicomposts and/or arbuscular mycorrhizal fungal inoculation in relation to metal availability and biochemical quality of a soil contaminated with heavy metals. Water, Air and Soil Pollution, Enviado/*Sent (*WATE-S-11-01076).

RESUMEN

Se realizó un experimento en macetas bajo invernadero para investigar como la adición de dos vermicompost (comercial o producido a partir de destríos de tomates de invernadero) y/o la inoculación con hongos micorrícicos arbusculares (AM) afectó a la disponibilidad y extrabilidad de P, K y metales, y a la calidad bioquímica de un suelo contaminado con metales pesados. Las macetas se sembraron con *Trifolium repens*, el cual se cosechó 40 días después de su germinación. La materia seca de los brotes y raíces de *T. repens* aumentó por la adición de ambos vermicomposts. La absorción de P, K, Fe, Mn, Cu, y Zn por parte de *T. repens* aumentó después de la adición de vermicompost, mientras que no se detectaron concentraciones de Ni, Pb y Cd. Después de la cosecha, las concentraciones de Fe, Cu, Zn, Cd, y Pb extraíble con AB-DTPA disminuyeron en el suelo enmendado orgánicamente; mientras el P, K, y Mn AB-DTPA aumentaron. El aporte de ambos vermicomposts, especialmente del obtenido a partir de destríos de tomate, estimuló las actividades deshidrogenasa, β-glucosidasa y ureasa en el suelo post-cosecha, implicando una diversidad funcional microbiana y una calidad bioquímica más alta en este suelo enmendado. Aunque, las actividades fosfatasas fueron mayores en los suelos post-cosecha con niveles más altos de metales extraíbles con AB-DTPA, las otras actividades enzimáticas estuvieron negativamente afectadas. La inoculación del suelo con hongos AM tuvo escasos efectos sobre el crecimiento vegetal así como sobre la disponibilidad y extrabilidad de metales y las actividades enzimáticas en comparación con la no inoculación.

Palabras clave: vermicomposts, micorriza, suelo multicontaminado, *Trifolium repens*, disponibilidad y extrabilidad de metales pesados, actividades enzimáticas.

ABSTRACT

A greenhouse pot experiment was conducted to investigate how the addition of two vermicomposts (commercial or produced from damaged greenhouse tomatoes) and/or inoculation with arbuscular mycorrhizal (AM) fungi affected availability and extractability of P, K, and metals, and biochemical quality of a soil contaminated with heavy metals. The pots were planted with *Trifolium repens*, which was harvested 40 days after germination. Shoot and root dry matter of *T. repens* increased by the addition of both vermicomposts. P, K, Fe, Mn, Cu, and Zn uptake by *T. repens* increased after vermicompost addition, whereas Ni, Pb, and Cd concentrations were not detected. After the harvest, AB-DTPA extractable Fe, Cu, Zn, Cd, and Pb decreased in the organically amended soil; whereas AB-DTPA P, K, and Mn increased. The addition of both vermicomposts,particularly that made from damaged tomatoes boosted dehydrogenase, β-glucosidase, and urease activities in the postharvest soil, implying a higher microbial functional diversity and biochemical quality in this amended soil. Although, phosphatase activities were greater in the postharvest soils with higher levels of AB-DTPA extractable metals, the other enzyme activities were negatively affected. The inoculation of the soils with AM fungi had weak effects on plant growth as well as on the availability and extractability of metals and enzyme activities compared to non-inoculation.

Keywords: vermicomposts, mycorrhyza, multicontaminated soil, *Trifolium repens*, availability and extractability heavy metals, enzyme activities

8.1. INTRODUCTION

The heavy-metal (HM) pollution of soils is an ever-growing problem worldwide, causing long-term risks to ecosystems and humans. Although heavy metals are released in varying quantities into soil by geochemical weathering of rocks, the increasing environment contamination is caused mainly by human activities (metal smelting, coal and fuel combustion, solid-waste disposal, and agricultural and industrial activities).

Despite the natural capacity of the soil to reduce solubility and bioavailability of heavy metals, at many polluted sites environmental risks persist, requiring remedial action. Remediation technologies of HM-polluted soils are based primarily on engineering techniques of decontamination. These technologies are expensive, environmentally invasive as well as labour intensive, and in general technically limited to relatively small areas. Natural remediation of HM-polluted soils can be promoted by *in situ* inactivation through the use of soil amendments. A great number of inorganic natural and synthetic materials (zeolites, phosphate rocks, ashes, lime, clay minerals, and iron and manganese oxides) have been assayed to evaluate their potential to reduce the solubility and bioavailability of heavy metals in contaminated soils (Basta and McGowen, 2004; Chirenje et al., 2006; Madrid et al., 2009; Ruttens et al., 2010). Organic amendments such as composts, manures, and biosolids can also contribute to metal immobilisation through the formation of stable complexes with OH or COOH groups on the solid surfaces of the organic polymers (Park et al., 2011; Tandy et al., 2009). However, some organic amendments can add soluble organic ligands which could increase the availability and the mobility of the heavy metals into the soil (Shuman, 1998).

Furthermore, *in situ* immobilization of HM-polluted soils can be combined with the use of plants to cover the soil in order to prevent contaminant migration via wind and water erosion, leaching, and soil dispersion (Padmavathiamma and Li, 2007). Establishing plant covers on HM-polluted soils often requires microorganisms to facilitate the revegetation. Arbuscular mycorrhiza fungi (AM fungi) enhance plant growth and improve plant reproduction (Smith and Read, 1997), often protecting plants against high concentrations of heavy metals in their shoots by augmenting metal retention into the roots (Leyval and Jones, 2001). Therefore, the symbiosis with AM fungi has been proposed as one of the plant mechanisms of HM tolerance and water stress avoidance, could possibly help in revegetation

or phytostabilization of metal-contaminated sites which could help in revegetation of HM-polluted soils (Adriano et al., 2004; Medina and Azcon, 2010)

Vermicompost, the end product of the biotransformation of organic wastes through the joint action of epigeic earthworms and aerobic microorganisms in a non-thermophilic process, is a well-known inexpensive organic soil amendment, which has traditionally been used for agricultural purposes, because it contains humus-like compounds as well as a wide range of nutrients, and it improves physical, chemical, and biological soil properties, increasing and/or restoring soil fertility (Arancon et al., 2006; Benítez et al., 2000). Vermicomposts can also be used to immobilize soil heavy metals, because, like other organic amendments, the humic substances contained in vermicomposts have high metal-adsorption capacity, due to the presence of negatively charged functional groups, such as carboxylic acids, phenolic and alcoholic hydroxyls (Plaza et al., 2007). In addition, these composts contain active microorganisms and enzymes which can greatly enhance the biochemical fertility of soils degraded by pollution (Fernández-Gómez et al., 2010a). However, little information is available regarding the use of vermicomposts in HM-polluted soils (Carrasquero et al., 2006; Jadia and Fulekar, 2008). In addition, no information is available on the combined use of vermicomposts and AM fungi as a strategy for the revegetation and rehabilitation of HM-polluted soils.

The aim of this study was to evaluate the effects of adding two vermicomposts (commercial or produced from damaged greenhouse tomatoes) and/or inoculation with arbuscular micorrhizal (AM) fungi on availability and extactability of P, K, and metals, and biochemical quality of a multicontaminated soil with heavy metals. Therefore, a greenhouse pot experiment was performed using white clover (*Trifolium repens*) as a plant to cover the soil. Biochemical postharvest soil quality was assessed by analysing four enzyme activities: dehydrogenase, β-glucosidase, urease, and acid phosphatase.

9.2. MATERIAL AND METHODS

9.2.1. Soil, vermicomposts and experimental treatments

Bulk samples (0-20 cm depth) of a non-calcareous, coarse-loamy HM-contaminated soil were collected of a mining area located in the Cartagena-La Union Mining District (south-

eastern Spain). The HM-contaminated soil was air dried, sieved through 2 mm mesh, and mixed with quart sand (<1 mm) to reach 1:1 soil-to-sand ratio (v:v).

Table 9.1. General properties of the HM-contaminated soil mixed with sand (S), commercial vermicompost (CM) and vermicompost from damaged greenhouse tomato fruits (DT).

	S	CM	DT
pH	8.1	8.3	10
EC (dS m^{-1})	1.3	1.4	4.4
TOC (g kg^{-1})	2.2	112	148
TKN (g kg^{-1})	0.16	17	13
P (g kg^{-1})	0.7	2	2.1
K (g kg^{-1})	9.6	11	23
Fe (mg kg^{-1})	78400	7570	8590
Mn (mg kg^{-1})	5400	254	256
Cu (mg kg^{-1})	105	49	27
Zn (mg kg^{-1})	27500	211	93
Cd (mg kg^{-1})	34	<0.2	<0.2
Ni (mg kg^{-1})	26	14	16
Pb (mg kg^{-1})	6425	15	<0.2
Dehydrogenase (INTF $g^{-1}h^{-1}$)	2.2	18	71
ß-Glucosidase (µgPNP $g^{-1}h^{-1}$)	25	178	115
Acid Phosphatase (µgPNP $g^{-1}h^{-1}$)	2140	877	691
Urease (µgNH_4^+ $g^{-1}h^{-1}$)	7.3	44	25

EC: electrical conductivity; TOC: total organic carbon; TKN: total Kjeldahl nitrogen.

Two vermicomposts produced from different organic wastes (cattle manure and damaged greenhouse tomato fruits) were used. Vermicompost from cattle manure (CM) was produced by Lumbricor S.L. (Córdoba, Spain). This vermicompost was produced on a large-scale wind-row system using cattle manure, which was vermicomposted for four months and matured for one month. Vermicompost from damaged greenhouse tomato fruits (DT) was produced using an indoor continuous-flow vermicomposting reactor as described by Fernández-Gómez et al. (2010b). This system was initially provided with a layer of sheep

manure (15 kg) placed in the bottom of the reactor. Then, 500 g of *E. fetida* earthworms were inoculated, and the vermireactor was continuously fed with damaged tomatoes every week (10 kg/week) for five months. Afterwards, the earthworms were removed and the pre-processed organic substrate was matured for two months. Some chemical and biochemical characteristics of the HM-contaminated soil mixed with sand (1:1) and the vermicomposts are given in Table 9.1.

The indigenous AM fungal inoculum was isolated from a Cd-contaminated soil (Nagyhorcsock, Hungary) and was identified by morphological examination as a *Glomus mosseae* strain (Vivas et al., 2003). Bulked in an open-pot culture of red clover, the mixture consisted of soil, spores, mycelia and infected root fragments with overall fungal colonization of 70%.

The greenhouse study consisted of 6 treatments arranged in a factorial, completely randomized block design with untreated HM-contaminated soil/sand mixture (S), HM-contaminated soil/sand mixture amended with vermicompost of cattle manure (+CM) and HM-contaminated soil/sand mixture amended with vermicompost from damaged greenhouse tomato fruits (+DT). Each vermicompost was mixed at the rate of 5% with the soil/sand mixture and left for equilibration for 10 days at room temperature. The subsequent treatments were identical to the ones just described, but contained the indigenous mycorrhizal inoculum. For this, 10 g of inoculum (1 % v/v) were added to the soil/sand mixture, alone or amended with both vermicomposts at sowing time just below the white clover seeds. Each treatment was replicated five times.

9.2.2. Experimental layout

Five hundred grams of soil/sand mixture alone or previously mixed both vermicomposts or/ and inoculated with indigenous mycorrhiza were placed in 0.5 L pots. Four seeds of white clover (*Trifolium repens* L.) were sown in each pot. A suspension of the diazotrophic bacterium *Rhizobium leguminosarum* bv. *trifolii* (10^8 cell mL^{-1}) was sprinkled over the seeds of all pots at the time of sowing. The plants were grown in a greenhouse under a day/night cycle of 16-8 h, 21-15°C and 50% relative humidity, and all the pots were irrigated daily to field capacity with distilled water. To prevent the leaching of nutrients and trace elements from the pots, plastic trays were placed under each pot and the leachates collected were put back into the respective pots. At harvest (40 days after planting), white clover was harvested

and separated into shoots and roots. The shoots and roots, after analysis of symbiotic development, were oven dried at 60°C for 48 h and weighed. In addition, after plant material was harvested, postharvest soil/sand samples were collected from each pot. Samples of each pot were divided into two subsamples, of which one was stored at -20°C for enzyme-activity analyses, while the other was air dried and finely ground for chemical analyses.

9.2.3. Symbiotic development

The percentage of mycorrhizal root length infected was estimated by observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v) (Phillips and Hayman, 1970). Quantification was performed using the grid-line intersect method (Giovannetti and Mosse, 1980).

9.2.4. Plant-chemical analysis

The shoots of white clover were digested with HNO_3-$HClO_4$ (2:1) (A.O.A.C., 1984). P was measured by the nitrovanadomolybdate method, total K was measured by photometry (C.I.I., 1969), and Fe, Mn, Cu, Zn, Cd, Ni, and Pb with an atomic absorption spectrophotometer (GBC 932 plus).

9.2.5. Soil and vermicompost chemical analysis

Texture was determined by the pipette method (M.A.P.A., 1986), and pH (in water) was measured with a glass electrode using a 1:2.5 sample-to-water ratio. Total organic C (TOC) and total Kjeldahl N (TKN) were determined using the dichromate oxidation and Kjeldahl method, respectively (M.A.P.A., 1986). For the determination of $CaCO_3$, CO_2 released by addition of HCl was measured (M.A.P.A., 1986). The concentrations of total elements (P, K, Fe, Mn, Cu, Zn, Cd, Ni, and Pb) in the soil/sand and vermicomposts were determined by agua regia digestion as measured by McGrath and Cunliffe (1985). In addition, these elements were extracted using 1M NH_4HCO_3-0.005M DTPA, pH 7.6 (AB-DTPA) in a 1:2 sample: extractant ratio (Soltanpour and Schwab, 1977). In the digests and extracts, P was measured using the ammonium vanadomolybdate method; K by flame-photometry; and

metals (Fe, Mn, Cu, Zn, Cd, Ni and Pb) with atomic-absorption spectrophotometer (GBC 932 plus).

9.2.6. Analysis of enzyme activities in soils and vermicomposts

Dehydrogenase activity was determined by incubating 1 g of sample for 20 h at 25°C with 0.5 ml of 0.4% 2-*p*-iodophenyl-3*p*-nitrophenyl-5 tetrazolium chloride (INT) as a substrate. The iodonitrotetrazolium formazan (INTF) produced in the reduction of INT was extracted with a mixture of acetone:tetrachloroetene (1.5:1) and measured in a spectrophotometer at 490 nm (García et al., 1997).

To determine *β*-glucosidase and phosphatase activity, 2 ml of 0.05M 4-nitrophenyl-*β*-D-glucanopyranoside (PNG) and 0.115M 4-nitrophenyl phosphate (PNPP) were used as the substrate, respectively (Tabatabai, 1982), and 1 g of each sample was incubated at 37°C for 2h with 2 ml of maleate buffer at pH 6.5. The samples were then kept at 2ºC for 15 min to stop the reaction, and the p-nitrophenol (PNP) produced in the enzyme reactions was extracted and determined at 398 nm (Nannipieri et al., 1982).

For the determination of the urease activity, 2 ml of 6.4% urea and 2 ml of 0.1 M phosphate buffer at pH 7.0 were added to 1 g of sample, incubated at 37°C for 2 h and immediately afterwards the reaction was stopped by cooling at 2°C for 15 min, as described by Nannipieri et al. (1980). The NH_4^+ produced by both types of enzymes was measured after extraction with 2 M KCl using a modified salicylate-nitroprusside colorimetric method (Kandeler and Gerber, 1988). Assays for *β*-glucosidase, phosphatase urease, adding destilled water instead of reaction substrate were conducted simultaneously as control.

9.2.7. Statistical analysis

All results are the means of three replicates. The data were subjected to an analysis of variance (ANOVA) using STATGRAPHICS Plus statistical software (Statistical Graphics Corp., Princeton, NJ), and Duncan's Multiple-Range Test was used to discriminate the means. AB-DTPA extractable metals and enzyme-activity values recorded in the postharvest soils were analysed by principal-components analysis (PCA) using the PC-ORD program Version 5.0 (McCune and Mefford, 1999).

9.3. RESULTS AND DISCUSSION

9.3.1. Shoot and root biomass and symbiotic development

The application of both vermicomposts significantly increased the shoot and root biomass of *T. repens* (Figure 9.1). Comparatively, commercial vermicompost (+CM) was more effective (179%) than the vermicompost from damaged greenhouse tomato fruits (+DT) (146%) to increase the aerial biomass of *T. repens*. The single AM fungal inoculation or in combination

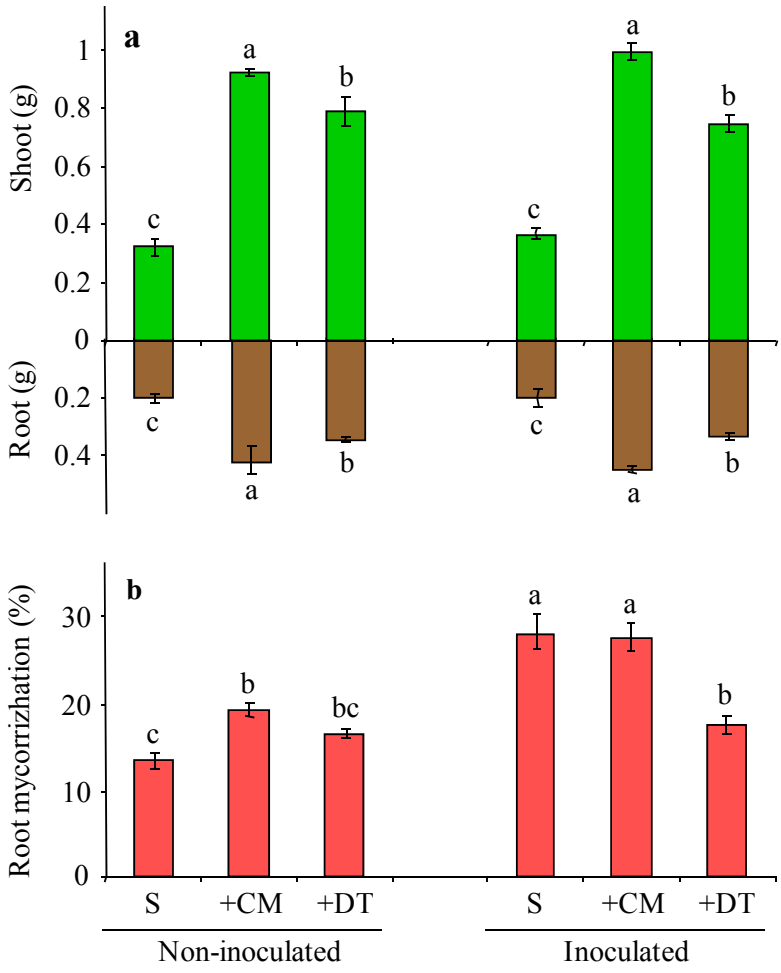


Figure 9.1. Dry-matter yield of root and shoot of *Trifolium repens* (a) and percentage of root mycorrhization (b) in a HM-contaminated soil/sand mixture, alone (S) or amended with commercial vermicompost (+CM) or vermicompost from damaged greenhouse tomato fruits (+DT), not inoculated or inoculated with AM fungi. Error bars represent the mean standard error. For each parameter, means followed by the same letter do not significantly differ ($P < 0.05$).

with both vermicomposts had no significant effects on the shoot and root biomass compared to the corresponding treatments without inoculation. Increases in shoot and root biomass after the addition of both vermicomposts was attributed to the ability of those organic amendments to stimulate plant growth due to the supply of available nutrients for the crop. In other studies, a similar effect was observed when different vermicomposts were added to the soil (Arancon et al. 2005; Nogales and Benítez, 2006). However, the slight increases recorded after the AM fungal inoculation contrast with those reported by Azcon et al. (2009) showing increased growth and nutrition of *T. repens* growing in HM-contaminated soils.

As expected, there were a significantly higher percentage of mycorrhizal roots in the HM-contaminated soil that had received the AM fungal inoculum (Figure 9.1). These increases were less pronounced when the soil had been amended with vermicompost from damaged greenhouse tomato fruits (+DT). This was ascribed to the higher organic carbon in the vermicompost of damaged fruit than in the commercial vermicompost (9.1), implying poorer mycorrhizal development in the roots. Previous studies have shown that the application of organic amendments provides the soil easily assimilable nutrients that reduce the need for plant mycorrhization. Sáinz et al. (1998) noted a significant reduction in mycorrhizal colonization of clover roots in a soil amended with vermicompost from urban wastes. In contrast, Cavender et al. (2003) and Azcon et al. (2009) reported the opposite effect, when the soil has been amended, respectively, with vermicompost from swine waste or beet waste treated with *Aspergillus niger*.

9.3.2. P and K in plant and postharvest soil

Regardless of AM fungal inoculation of the soil, the application of the two vermicomposts significantly boosted the P and K concentrations in the shoot of *T. repens* and the ABDTPA-extractable P and K from the soil after harvest (Table 9.2). Comparatively higher increases were found in treatments made with the vermicompost from damaged tomato fruits (+DT). Greater P in plant and postharvest soil after application of vermicomposts may be attributed to higher soil microbial activity after the application of those amendments and consequently, release of P during organic-matter decomposition. In addition, the application of vermicomposted materials is expected to stimulate organic P mineralizing bacteria and inorganic P by solubilizing bacteria, which would increase the availability of P in the soil (Saha et al. 2010). Besides increasing soil biological activity, vermicompost and its

degradation products could form either complexes with active Al and Fe (resulting in a reduction in P fixation) or chelate complexes with Ca from the insoluble Ca-phosphates, following the release of phosphate to available forms (Stevenson, 1994). As with other organic amendments, potassium must be mainly in mineral form in vermicomposts (Benitez et al., 2000). Therefore, vermicompost application increases both the concentration in the plant and the available forms in the soil; such increases would correlate with the total K content in vermicomposts, which was greater in the case of those from damaged greenhouse tomatoes fruits (Table 9.1).

Table 9.2. Phosphorus and potassium concentrations in shoots, uptake by *T. repens*, and AB-DTPA extractable concentrations in postharvest soil samples of different treatments assayed.

	P			K		
	Shoot	Plant uptake	AB-DTPA	Shoot	Plant uptake	AB-DTPA
	g kg^{-1}	mg pot^{-1}	g kg^{-1}	g kg^{-1}	g pot^{-1}	mg kg^{-1}
	Non-inoculated					
S	0.11±0.01d	36±4e	0.71±0.7c	7.9±0.1d	2.5±0.21d	11±0.4d
+CM	2.1±0.08bc	1933±64c	8.5±0.6b	29±1.4c	27±1.7c	155±10c
+DT	1.9±0.07c	1495±92d	11±0.3b	48±0.9b	35±2.9b	608±19b
	Inoculated					
S	0.20±0.01d	73±3e	0.81±0.03c	12±0.3d	4.4±0.28d	15±0.5d
+CM	2.2±0.08b	2225±135b	9.7±0.2b	27±0.6c	28±1c	136±8c
+DT	3.5±0.15a	2599±140a	13±0.3a	63±1.5a	50±1.1a	759±38a

Means in the same column followed by the same letter are not significantly different ($P > 0.05$).

Although the improved P nutrition in plants by the mycorrhizal inoculation is emphasized in the literature, both in polluted and non-polluted soils (Christie et al., 2004; Turnau et al., 2010) in our study, no significant differences were detected in the P concentration in shoots of plants grown in the single AM fungus-inoculated soil or in combination with the commercial vermicompost (+CM) (Table 9.2). Only significant increases in P and K were noted when the plants were grown in the inoculated and amended soil with vermicompost from damaged tomatoes (+DT).

9.3.3. Metals in plant and postharvest soil

The Fe, Mn, Cu, and Zn concentrations in the *T. repens* shoot were affected by soil application of both vermicomposts and/or inoculation with AM fungi (Table 9.3). Inoculation with AM fungi lowered the Fe and Mn concentrations, while the vermicompost application, and particularly that from damaged greenhouse tomatoes increased the Fe, Mn, and Zn concentrations. For the other heavy metals analysed (Cd, Ni, and Pb), the levels recorded were below the reliable detection limit (0.02, 0.06, and 0.1 mg kg^{-1}), respectively, of the analytical procedure.

Soil inoculation with AM fungi was ineffective in augmenting metal uptake by *T. repens*, which in the case of Fe and Mn tended to decrease, while for Cu and Zn the increases were not significant (between 22 and 35%; Table 9.3). By contrast, the application of the two vermicomposts proved effective to increase the plant uptake of those metals. Comparatively, the vermicompost from damaged greenhouse tomato fruits (+DT), when added to the soil, increased Mn uptake (202%) and especially Fe uptake (385%), whereas the commercial vermicompost was more effective at increasing Cu uptake (186%) and Zn uptake by *T. repens* (348%). The combined use of vermicomposts and soil inoculation with AM fungi had little effect or even lowered metal uptake by the plant compared with the single application of vermicomposts. In any case, despite increases in the Fe, Mn, Cu, and Zn plant uptake by the application of both vermicomposts, the results did not clearly demonstrate a phytoextraction effect by *T. repens*, and this crop also proved ineffective at extracting Cd, Ni, and Pb from the soil. Several factors may have influenced these results, such as insufficient exposure time, as the plants were collected 40 days after sowing, although the effect of exposure time on the metal uptake has not been previously elucidated in this crop. Vivas et al. (2003) reported a more pronounced effect of this crop on the metal uptake, being harvested after 12 weeks of growth. Generally, the uptake of the most metals depends on the exposure time and plant species (Grant et al. 1998). Bidar et al. (2007) registered a lower accumulation of Pb in roots of *T. repens* compared with *Lolium perenne* and also less accumulation of Cd, Pb, and Zn in the shoot. Kohler et al. (2000) showed that, after a short exposure time, dicotyledonous species accumulate more Pb in their roots than do monocotyledons, although the opposite was found after 2 weeks of growth. In our study, the HM concentrations in the roots were not analysed, because the roots were stained to

Table 9.3. Iron, manganese, copper and zinc concentrations in shoots and uptake by *T. repens* in the different assayed treatments.

	Fe		Mn		Cu		Zn	
	Shoot g kg^{-1}	Uptake mg pot^{-1}	Shoot mg kg^{-1}	Uptake mg pot^{-1}	Shoot mg kg^{-1}	Uptake mg pot^{-1}	Shoot mg kg^{-1}	Uptake mg pot^{-1}
				Non-inoculated				
S	2.8±0.07c	896±88c	173±7b	54±5b	11±0.8c	3.7±0.5c	208±6c	67±8d
+CM	2.2±0.05d	2067±63b	164±3b	151±4a	11±0.5c	11±0.4b	325±8a	300±10a
+DT	5.5±0.07a	4346±217a	208±8a	163±12a	10±0.4c	8.1±0.8c	347±12a	273±18ab
				Inoculated				
S	1.9±0.05e	690±41c	115±5c	42±3b	14±0.7b	5±0.5c	228±9bc	82±5d
+CM	2.2±0.10d	2177±77b	159±8b	158±7a	11±0.6c	11±0.8b	247±12b	245±10bc
+DT	4.5±0.11b	2242±99b	209±8a	154±5a	18±0.6a	13±0.5a	320±11a	236±5c

Means in the same column followed by the same letter are not significantly different ($P > 0.05$).

analyse symbiotic development, and then dried and weighed. Bidar et al (2007) observed in *T. repens* that the roots were the preferential metal-storage organs, suggesting therefore the usefulness of this plant species for the phytostabilization of heavy metals in contaminated soils.

Table 9.4 shows the results of AB-DTPA extractable Fe, Mn, Cu, Zn, Cd, and Pb in the contaminated soil after harvesting. AB-DTPA extractable Ni were below the detection limit in all the soil samples. Inoculation with the AM fungi consortium had no significant effects on AB-DTPA extractable metal concentrations in postharvest soils. The addition of both vermicomposts, alone or in combination with the AM fungi significantly decreased the AB-DTPA extractable Fe, Cu, Zn, Cd, and Pb in the postharvest soil. Comparatively, the greatest decreases of AB-DTPA extractable metals, except Zn, occurred when the contaminated soil was amended with vermicompost from damaged greenhouse tomato fruits (+DT). Closely negative correlations ($P < 0.05$) were recorded between the extractable forms of Fe, Cu, and Zn in the soil after harvest and the uptake of those metals by the shoot of *T. repens* (Fe r= -0,7620; Cu r= -0,4670; Zn r= -0,5401). On the contrary, the AB-DTPA extractable Mn increased due to the addition of both vermicomposts, and therefore a positive correlation was found with the Mn uptake by the plant (r = 0.4346).

Table 9.4. AB-DTPA extractable metals in the postharvest soil samples of the different treatments assayed.

	Fe	Mn	Cu	Zn	Cd	Pb
	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
			Non-inoculated			
S	14±1a	5.6±0.6b	5.7±0.2a	337±14a	2±0.08a	246±5a
+CM	12±0.5b	6.2±0.5b	5.4±0.1ab	293±16bc	1.6±0.0.6b	215±12b
+DT	9±0.4c	9.0±0.6a	4.9±0.1bc	298±8bc	1.5±0.0.05b	201±3bc
			Inoculated			
S	16±0.8a	5.5±0.5b	5.2±0.2ac	325±22ab	1.9±0.05a	233±4a
+CM	12±10b	6.4±0.4b	5.3±0.1ab	277±6c	1.6±0.0.05b	211±3b
+DT	8±0.8c	8.6±1.4a	4.5±0.5c	287±10c	1.1±0.1c	189±5c

Means in the same column followed by the same letter are not significantly different ($P > 0.05$).

Other studies have shown that the application of organic amendments generally decreased HM extractability and bioavailability in contaminated soils, although the effects on soil-metal availability are greatly influenced by the stability and maturity, humic quality, and content in metals and dissolved organic matter of the applied organic amendment as well as by the intrinsic properties of the soils (pH, organic matter, clay and iron oxide content, redox potential, carbonates). The decreases recorded in AB-DTPA extractable Fe, Cu, Zn, and Cd in the postharvest soils could be due to different interdependent factors: i) processes of chelation, complexation, and adsorption between metals in the soil and the most humified organic-matter content in both vermicomposts, ii) increase in soil microorganisms with immobilizating potential, iii) dilution effect due to the incorporation of those materials (Adriano et al., 2004; Fageria et al., 2004; Romero et al., 2005; Shuman et al., 2001).

Increases in AB-DTPA extractable Mn could be due to a possible decrease of the redox potential of the postharvest soil as a result of mineralization of both vermicomposts, which, besides depleting the oxygen through intense microbial activity, would produce organic compounds that can dissolve Mn, increasing the availability of this metal (Hue et al., 2001).

9.3.4. Enzyme activities in postharvest soil

Figure 9.2 shows, in the postharvest soils of each treatment, the sun-ray diagrams plotting the assayed four enzyme activities (dehydrogenase, β-glucosidase, urease, and phosphatase) along different radial axes. The Sun-ray plots have been proposed as a visual fingerprint of biochemical activity in soils and organic materials, in which the area and shape of these types of plots can be used as indicators of functional diversity of the microorganisms and biological quality in those environments (Mijangos et al., 2006, Nannipieri et al., 2002).
Dehydrogenase activity, which has been considered an indicator of overall microbial activity because it occurs intracellularly in all living microbial cells (Nannipieri et al., 2002), generally tends to increase when the soil is organically amended (Varennes e, 2010; Romero et al., 2010). In our study, significant increases of dehydrogenase activity were observed only when the soil had been amended with the vermicompost from damaged tomatoes, alone or in combination with the AM fungi; increases were not found when the soil had been amended with the commercial vermicompost (+CM). These differences were attributed to the fact that the vermicompost from damaged tomatoes had a higher C:N ratio and dehydrogenase activity than did the commercial v ermicomposts (Table 9.1), and therefore lower stability (Tiquia, 2005). This would cause a further increase in soil microbial biomass,

due either to the addition of microorganisms and enzymes in the vermicompost or indirectly to the additional supply of labile C, which would promote the growth of microorganisms indigenous to the soil (García et al., 1994; Sahni et al., 2008).

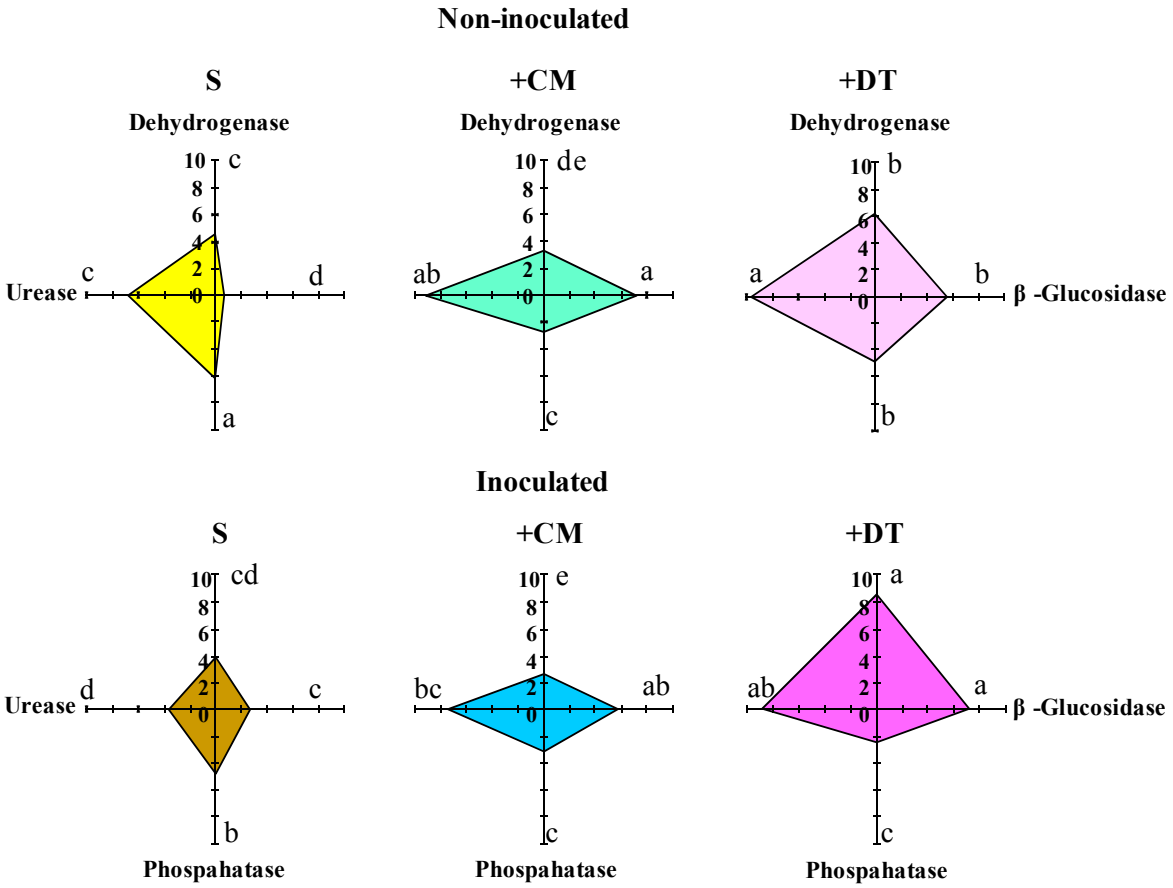


Figure 9.2. Enzyme activities in the postharvest soil samples of different assayed treatments. Dehydrogenase activity is expressed as µg INTF g^{-1} h^{-1}, β-glucosidase activity as 500^{-1} x (µg PNP g^{-1} h^{-1}), acid phosphatase activity as 500^{-1} x (µg PNP g^{-1} h^{-1}), and urease activity as 2^{-1} x (µg NH_4^+ g^{-1} h^{-1}). For each enzyme activity, different letters indicate significant differences among the treatments ($p < 0.05$).

β-glucosidase, an extracellular enzyme related to the C-cycle, which represent a key role in organic-matter degradation, was significantly increased by the application of both vermicomposts, alone or in combination with the AM fungal inoculum. This was because both organic amendments showed high β -glucosidase activities (Table 9.1) and/or sufficient organic C compounds, which during the mineralization of the vermicompsts in the soil, are available to microorganisms, thereby stimulating the synthesis of this enzyme (García-Gil et al., 2000; Varennes and Cunha-Queda, 2010).

Urease is an extracellular enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia, the activity of which is inhibited in HM-polluted soils (Marzadori et al. 2000). The application of both vermicomposts to contaminated soils significantly increased the urease activity, whereas the single mycorrhizal inoculation or in combination with both vermicomposts had no significant effects or was reduced compared to the corresponding treatments without inoculation. Although the commercial vermicompost (+CM) had higher TKN content and urease activity than the vermicompost from damaged tomatoes (Table 9.1), the increases of urease activities in the postharvest soils amended with both vermicomposts were similar, implying that they contained sufficient organic N compounds available to microorganisms, thereby stimulating the synthesis of these enzymes.

Acid phosphatases, enzymes with relatively broad specificity, can hydrolyse various organic and inorganic phosphate esters and are involved in the P-cycle. Previous studies have reported that acid phosphatase activity is increased by the addition of organic amendments to the soil (García-Gil *et al.*, 2000; Romero et al., 2005). However, in our study, in contrast to other enzyme activities, phosphatase activity was reduced by the application of both vermicomposts or/and the inoculation with the mycorrhizal consortium. Mineralization of organic P compounds contained in the vermicomposts together the solubilization of soil inorganic phosphates by the AM fungi could increase the available P content in the soil solution, which has been recognized to reduce phosphatase activity by feedback inhibition (Harrison, 1983; Nanniperi et al., 1979). This finding was confirmed by the closely negative correlation (r= -0.6675) recorded between the phosphatase activities and the AB-DTPA extractable P in the soil after harvest.

As a result of the changes detected in enzyme activities in post-harvest soils, the integrated area of the sun-ray plots varied between the different treatments assayed. Application of the vermicompost from damaged tomatoes sharply increased the area of the plots, approximately 95% of the unamended soil; whereas the increase induced by the commercial vermicompost was lower (22%). These increases imply a higher microbial functional diversity and biochemical quality in the organically amended soils (Andersson, 2003). Whether alone or combined with vermicompost inoculation of the soil with the AM fungi, in general, the treatment had a weak effect on the plot area, and therefore on biolochemical quality of soils in relation with the non-inoculation.

The relationships between the enzyme-activity values and the concentration of AB-DTPA metals recorded in the postharvest soil of different treatments were investigated by principal-components analysis (Figure 8.3). Of total variance, 69.1% was included by the

first and second principal components (PC 1 and PC 2), explaining 55.3 and 13.8% of the variance, respectively.

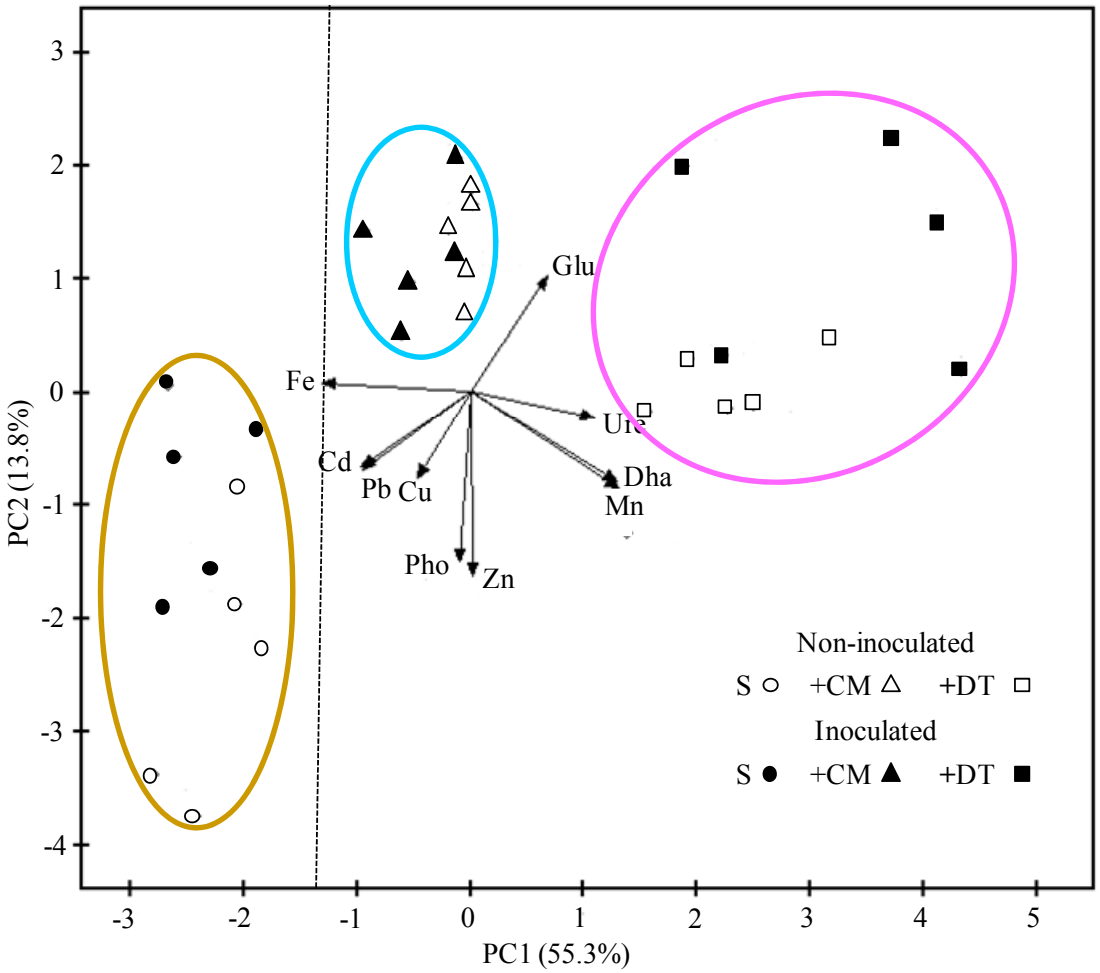


Figure 9.3. Principal component analysis for assessing relationships between AB-DTPA extractable metals and enzyme activities recorded in the postharvest soil of the different treatments. Dotted line indicates a significant separation between the unamended soil and the soils amended with vermicomposts according to PC 1 ($p < 0.05$).

This analysis revealed closely negative correlations ($p< 0.001$) between the dehydrogenase, β-glucosidase, and urease activities and the AB-DTPA extractable Fe,Cd and Pb concentrations in the postharvest soils. By contrast, the phosphatase activities measured in the postharvest soils were positively correlated ($p< 0.001$) with the AB-DTPA extractable Zn, Cd, Pb concentration. These results appear to indicate that the phosphatase activities were greater in the postharvest soils with higher AB-DTPA extractable metals, whereas the activities of the other enzymes were negatively affected. On the other hand, PCA separated the unamended soil (S), inoculated and non-inoculated, from the amended

soils with each of the vermicomposts assayed (+CM, +DT). This confirms that each vermicompost exerted a different effect on the enzyme activities and the available metals in the postharvest soils. However, the inoculation with AM fungi appeared to have no effect on enzyme activities and metal availability in the postharvest soils.

9.4. CONCLUSIONS

The application of two different vermicomposts (one commercial and another from damaged greenhouse tomato fruits) to a HM-contaminated soil increased the dry matter of *T. repens* shoots and roots. Likewise, the P, K, Fe, Mn, Cu, and Zn uptake by the plant increased in the organically amended soils, whereas other metals as Ni, Pb, and Cd, were not detected in the shoots. On the contrary, AB-DTPA extractable Fe, Cu, Zn, Cd, and Pb decreased in the postharvest soils amended with both vermicomposts, whereas AB-DTPA P, K, and Mn increased. Comparatively, no marked differences appeared in plant and soil, between the two vermicomposts assayed.

The biochemical quality of the postharvest soils, determined by enzyme-activity analysis, was enhanced by applying both vermicomposts, this effect being more apparent when the vermicompost from damaged greenhouse tomatoes fruits was used. In addition, dehydrogenase, β-glucosidase, and urease activities were negatively affected in the postharvest soils with higher AB-DTPA extractable metals, while the phosphatase activity tended to increase.

The AM fungal inoculation alone or in combination with both vermicomposts of the soil had no significant effects on plant yield, metal uptake by the plant, soil-metal extractability or soil-enzyme activities compared with the corresponding treatments without inoculation.

Therefore, the use of vermicompost as soil organic amendments may be a promising, suitable and low-cost technology applicable *in situ* to remediate HM-contaminated soils, helping also to establish vegetation cover in those areas. However, long-term studies using plants with greater capacity for HM accumulation are needed to confirm this fact.

ACKNOWLEDGEMENTS

This study was financed by ‘‘Junta de Andalucía” project P05-AGR-00408. Manuel J. Fernández Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant (AP2006-03452). We thank the vermicomposting treatment plant Lombricor S.C.A

(Algallarin, Córdoba, Spain) for providing the commercial vermicompost. The authors thank Fernando Calvo for his technical support and David Nesbitt for assisting in the translation of the manuscript into English.

REFERENCES

A.O.A.C. 1984. Official Methods of Analysis, 14th ed. Arlington: Association of Official Analytical Chemists.

Adriano, D.C, Wenzel, W.W., Vangronsveld, J., Bolan, N.S. 2004. Role of assisted natural remediation in environmental cleanup. Geoderma 122, 121-142.

Anderson, T., 2003. Microbial eco-physiological indicators to asses soil quality. Agriculture, Ecosystems and Environment, 98, 285–293.

Arancon, N.Q., Edwards, C.A., Bierman, P., 2006. Influences of vermicomposts on field strawberries Part 2. Effects on soil microbiological and chemical properties. Biores. Technol. 97, 831-840.

Azcón, R., Medina, A., Roldán, A., Biró, B., Vivas, A., 2009. Significance of treated agrowaste residue and autochthonous inoculates (Arbuscular mycorrhizal fungi and Bacillus cereus) on bacterial community structure and phytoextraction to remediate soils contaminated with heavy metals. Chemosphere 75, 327-334.

Basta, N.T., McGowen, S.L., 2004. Evaluation of chemical immobilization treatments for reducing heavy metal transport in a smelter-contaminated soil. Environ. Pollut. 127, 73–82.

Benítez, E., Melgar, R., Sainz, H., Gómez, M., Nogales, R., 2000. Enzyme activities in the rhizosphere of pepper (Capsicum annuum, L.) grown with olive cake mulches. Soil Biol. Biochem. 32, 829-1835.

Bidar, G., Garton, G., Pruvot, C., Dewaele, D., Cazier, F., Douay, F., Shirali, P., 2007. Behavior of Trifolium repens and Lolium perenne growing in a heavy metal contaminated field Plant metal concentration and phytotoxicity. Environ. Pollut. 147, 546-553.

C.I.I. 1969. Métodos de referencia para la determinación de elementos minerales en vegetales. I. N, P, K, Na, Ca y Mg. Anal. Edaf. Agrobiol. 28, 409–430.

Carrasquero, A., Flores, I., Perozo, C., Pernalete, Z., 2006. Immobilization of lead by a vermicompost and its effect on white bean (*Vigna Sinenis var. Apure*) uptake. Inter. J. Environ. Sci. Technol. 3, 203-212.

Cavender, N. D., Atiyeh, R. M., Knee, M., 2003. Vermicompost stimulates mycorrhizal colonization of roots of Sorghum bicolor at the expense of plant growth. Pedobiol. 47, 85-89.

Chirenje, T., Ma, L.Q., Lu, L., 2006. Retention of Cd, Cu, Pb and Zn by wood ash, lime and fume dust. Water Air Soil Pollut. 171, 301–314

Christie, P., Li, X., Chen, B., 2004. Arbuscular mycorrhiza can depress translocation of zinc to shoots of host plants in soils moderately polluted with zinc. Plant Soil 261, 209-217.

Fageria, N. K., Baligar, V.C., Clark, R.B., 2002. Micronutrientes in crop production. Adv. Agron. 77, 185-268.

Fernández-Gómez, M.J., Romero, E., Nogales, R., 2010a. Feasibility of vermicomposting for vegetable greenhouse waste recycling. Biores. Technol. 101, 9654-9660.

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M., 2010b. Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops. Waste Manag. 30, 2461-2468.

García, C., Hernández, T., Costa, F., Ceccanti, B., 1994. Biochemical parameters in soils regenerated by the addition of organic wastes. Waste Manag. Res. 12, 457-466.

García, C., Hernández, T., Costa, F., 1997. Potencial use of dehydrogenase activity as an indexof microbial activity in degraded soils. Commun. Soil Sci. Plant Anal. 28, 123–134.

García-Gil, J. C., Plaza, C., Soler-Rovira, P., Polo, A., 2000. Long-term effects of municipal solid waste compost application on soil enzyme activities and microbial biomass. Soil Biol. Biochem. 32, 1907-1913.

Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489–500.

Grant, C. A., Buckley, W. T., Bailey, L. D., Selles, F., 1998. Cadmium accumulation in crops. Canadian J. Plant Sci. 78, 1-17.

Harrison, A.F., 1983. Relationship between intensity of phosphatise activity and physic-chemical properties in the woodland soils. Soil Biol. Biochem. 15, 93–99.

Hue, N.V., Vega, S., Silva, J.A., 2001. Manganese toxicity in a Hawaiian Oxisol affected by soil pH and organic amendments. Soil Science Society American Journal, 65, 153-160

Kandeler, E., & Gerber, H. (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fert. Soils 6, 68-72.

Kohler, M., Gleisberg, B., Niese, S., 2000. Investigation of the soil-plant transfer of primordial radionuclides in tomatoes by low-level gamma-ray spectrometry. App. Rad. Isotop. 53, 203-208.

Leyval, C., Joner, E.J., del Val, C., Haselwandter, K., 2002. Potential of arbuscular mycorrhizal fungi for bioremediation. In S. Gianinazzi, H. Schüepp, J.M. Barea and K. Haselwandter (Eds), Mycorrhizal technology in agriculture, (pp. 175-186). Switzerland: Birkhäuser Verlag.

M.A.P.A., 1986. Métodos oficiales de análisis. Tomo III. Plantas, productos orgánicos fertilizantes, suelos, agua, productos fitosanitarios y fertilizantes inorgánicos. Madrid: Publicaciones del Ministerio de Agricultura, Pesca y Alimentación.

Madrid, F., Florido, M.C., Madrid, L., 2009. Trace metal availability in soils amended with metal-fixing inorganic materials. Water Air Soil Pollut. 200, 15-24

Marzadori, C., Francioso, O., Ciavatta, C., Gessa, C., 2000. Influence of the content of heavy metals and molecular weight of humic acids fractions on the activity and stability of urease. Soil Biol. .Biochem. 32, 1893-1898.

McCune, B., Mefford, M.J., 1999. Multivariate analysis of ecological data, version 5.0 Gleneden Beach: MjM Software Design.

McGrath, S. P., Cunliffe, C. H., 1985. A simplified method for the extraction of the metals Fe, Zn, Cu, Ni, Cd, Pb, Cr, Co and Mn from soils and sewage sludge. J. Sci. Food Agricul. 36, 794–798.

Medina, A., Azcón, R., 2010. Effectiveness of the application of arbuscular mycorrhiza fungi and organic amendments to improve soil quality and plant performance under stress conditions. J. Soil Sci. . Plant Nut. 10, 354-372

Mijangos, I., Perez, R., Albizu, I., Garbis, C., 2006. Effects of fertilization and tillage on soil biological parameters. Enzyme Microbial Technol. 40, 100-106

Nannipieri, P., Pedrazzini, F., Arcara, P. G., Piovanelli, C., 1979. Changes in amino acids, enzyme activities, and biomasses during soil microbial growth. Soil Sci. 127, 26-34.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci. Soc. Amer. J., 44, 1011–1016.

Nannipieri, P., Ceccanti, B., Conti, C., Bianchi, D., 1982. Hydrolases extracted from soil Their properties and activities. Soil Biol. Biochem., 14, 257–263.

Nannipieri, P., Kandeler, E., Ruggiero, P., 2002. Enzyme activities and microbiological and biochemical processes in soils. In R.G. Burns and R.P. Dick (Eds.), Enzymes in the

Environment Activity, Ecology, and Application. (pp. 1–33). New York: Dekker Marcel.

Nogales, R.. Benítez, E., 2006. Absorption of zinc and lead to Dittrichia viscosa grown in a contaminated soil amended with olive-derived wastes. Bull. Environ. Contam. Toxicol. 76, 538-544.

Padmavathiamma, P.K., Li, L.Y., 2007. Phytoremediation technology hyper-accumulation metals in plants. Water Air Soil Pollut. 184, 105-126

Park, J.H., Lamba, D., Paneerselvama, P., Choppala, G., Bolana,N., Chung, J., 2011. Role of organic amendments on enhanced bioremediation of heavy metal(loid) contaminated soils. J. Hazard. Mater. 185, 549-574

Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. British Mycol. Soc. 55, 159–161.

Romero, E., Benítez, E., Nogales, R., 2005. Suitability of wastes from olive-oil industry for initial reclamation of a Pb/Zn mine tailing. Water Air Soil Pollut. 165, 153-165.

Romero, E., Plaza, C., Senesi, N., Nogales, R., Polo, A., 2007. Humic acid-like fractions in raw and vermicomposted winery and distillery wastes. Geoderma 139, 397-406.

Romero, E., Fernández-Bayo J.D., Castillo-Díaz, J.M., Nogales, R., 2010. Enzyme activities and diuron persistence in soil amended with vermicompost derived from spent grape marc and treated with urea. App. Soil Ecol. 44, 198-205.

Saha, N., Chattrejee, P., Sharma, A., Metiya, G., Halder, M., Mukherjee, D., 2010. Vermicomposting influences phosphorus microbiology leading to phosphorus enrichment in end product. Intern. J. Global Environ. Issues, 10, 310-326

Sahni, S., Sarma, B. K., Singh, D. P., Singh, H. B., Singh, K. P., 2008. Vermicompost enhances performance of plant growth-promoting rhizobacteria in Cicer arietinum rhizosphere against Sclerotium rolfsii. Crop Prot. 27, 369-376.

Sainz, M. J., Taboada-Castro, M.T., Vilariño, A., 1998. Growth, mineral nutrition and mycorrhizal colonization of red clover and cucumber plants grown in a soil amended with composted urban wastes. Plant Soil, 205, 85-92.

Shuman, L.M., 1998. Effect of organic waste amendments on cadmium and lead in soil fractions of two soils. Commun. Soil Sci. Plant Anal. 29, 2939-2952.

Shuman, L. M., Dudka, S., Das, K., 2001. Zinc forms and plant availability in a compost amended soil. Water Air Soil Pollut. 128, 1-11

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis. San Diego, Academic Press

Soltanpour, P. N., Schwab, A. P. 1977. A new soil test for simultaneous extraction of macro and micronutrients in alkaline soils. Commun. Soil Sci. .Plant Anal. 8, 195–207.

Stevenson, F.J., 1994. Humus Chemistry genesis, composition, reactions, 2nd ed. New York: Wiley & Sons.

Tabatabai, M. A., 1982. Soil enzymes. In A.L. Page, R.H. Miller and D.R. Keeney (Eds), Methods of soil analysis Part 2. Chemical and Microbiologycal Properties, (pp. 903–948). Madison: Soil Science Society of America.

Tandy, S., Healey, J.R., Nason, M.A., Williamson, J.C., Jones, D.L., 2009. Remediation of metal polluted mine soil with compost: co-composting versus incorporation. Environ. Pollut. 157, 690–697

Tiquia, S.M., 2005. Microbiological parameters as indicators of compost maturity. J. App. Microbiol. 99, 816–828.

Turnau, K., Ryszka, P., Wojtczak, G., 2010. Metal tolerant mycorrhizal plants: A Review from the perspective on industrial waste in temperate region. In H. Koltai and Y. Kapulnik (Eds.), Arbuscular Mycorrhizas Physiology and Function, (pp. 257-276) Dordrecht: Springer Science + Business Media B.V.

Varennes, A., Cunha-Queda, C., Qu, G., 2010. Amendment of an acid mine soil with compost and polyacrylate polymers enhances enzymatic activities but may change the distribution of plant species. Water Air Soil Pollut. 208, 91-100.

Vivas, A., Vörös, A., Biró, B., Barea, J. M., Ruiz-Lozano, J.M., & Azcón, R. (2003) Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated with a Cd-adapted strain of *Brevibacillus sp*. in improving plant tolerance to Cd contamination. Appl. Soil Ecol. 24, 177-186.

DISCUSIÓN GENERAL

La viabilidad de los distintos residuos vegetales producidos por los cultivos en invernadero (matas de plantas y frutos de destríos) para ser vermicompostados no había sido investigada hasta ahora. En este sentido, los capítulos 1, 2, 3, 4 y 5 proporcionan una exhaustiva información sobre el vermicompostaje de los restos de plantas cultivadas y de los frutos de destríos procedentes de cultivos bajo plástico o en invernadero como método de reciclaje y valorización; información que es especialmente relevante para la gestión de los restos vegetales generados por los cultivos de tomate bajo plástico y en invernadero, el cultivo hortícola mayoritario en estos sistemas agrícolas a nivel mundial debido a que esta hortaliza presenta la mayor significación económica (Food and Agriculture Organization, 2002).

En relación con la viabilidad de los distintos tipos de residuos vegetales de invernadero para ser biotransformados mediante vermicompostaje, los resultados expuestos en el capítulo 1 indicaron que las matas de plantas desechadas tras las cosechas son residuos orgánicos inviables para ser vermicompostados, según muestra el hecho de que todas las lombrices que fueron inoculadas en el residuo heterogéneo de diferentes plantas (HP) y en el residuo de planta de tomate (P) murieron en menos de 24 horas. La inviabilidad de este tipo de residuo vegetal para ser biotransformado por lombrices puede ser debida a las particulares características físico-químicas que presentan las matas de plantas de invernadero (Tabla 1.1), las cuales son una como consecuencia de los procedimientos de manejo agrícolas que se aplican en estos sistemas de producción intensiva, donde se utilizan una gran cantidad de insumos a fin de obtener altas productividades en las cosechas (Yu et al., 2010). Entre estas características, los bajos valores de relación C/N y el alto contenido en sales de las matas de plantas desechadas por los cultivos en invernadero se encuentra fuera del rango que requieren las lombrices de las especies *E. fetida* o *E. andrei* para poder sobrevivir en los residuos orgánicos (Edwards,1988; Nogales et al., 2008). Puesto que a pesar de aumentar el valor de la relación C/N de HP y P, mezclando cada residuo con paja o estiércol vacuno, igualmente se observó un 100% de mortalidad en las lombrices inoculadas en esas mezclas, se puede afirmar que la inviabilidad de HP y P para ser vermicompostados se debe a su elevada concentración, reflejada en altos valores de conductividad eléctrica (EC).

La importancia del contenido en sales de las matas de plantas desechadas por los cultivos invernaderos en invernadero como factor limitante del proceso de vermicompostaje fue confirmada en el capítulo 4, donde se consiguió vermicompostar los restos de las plantas producidos por cultivos de tomates en invernadero (P) mediante su acondicionamiento con un lodo procedente de la fabricación de papel (S), un residuo de escasa salinidad (Tabla 4.2). En la investigación expuesta en ese capítulo se muestra que mezclando los restos de plantas

de tomate con el lodo de industria papelera en proporción 2:1 y 1:1 (ps:ps) es posible obtener un sustrato orgánico con un contenido en sales que permita la supervivencia de lombrices de la especie *E. fetida*. De esta forma, las lombrices pudieron desarrollarse y vermicompostar las diferentes mezclas de estos dos tipos de residuos, mostrando un patrón de crecimiento y reproducción similar a que se observó durante el vermicompostaje de estiércol vacuno, un residuo considerado idóneo para ser procesado por las especies de lombrices *E. andrei* y *E. fetida* (Figura 4.1). Además, en comparación con el vermicompostaje de lodo de papelera como único material, la mezcla de este residuo con matas de plantas de tomate mejoró el desarrollo y la reproducción de las lombrices, por lo que de esta manera se posibilita no sólo el reciclado de las matas de plantas procedentes de cultivos de invernadero sino que también se mejora el de los lodos de papelera (Figura 4.1).

Teniendo en cuenta que el desarrollo y reproducción de las lombrices durante el vermicompostaje de matas de plantas procedentes de cultivos en invernadero puede verse también afectado por el contenido variable en plaguicidas que estos residuos vegetales pueden presentar, el objetivo del capítulo 5 fue evaluar como diferentes concentraciones de imidacloprid (IMD), un insecticida frecuentemente aplicado en los cultivos de invernadero, pueden afectar al desarrollo de *E. fetida* durante el vermicompostaje de matas de plantas procedentes de invernadero. Inicialmente, en este capítulo se evaluó el efecto que tiene el IMD sobre *E. fetida* cuando esta especie de lombriz se emplea en vermicompostaje de un residuo que le permite alcanzar un desarrollo óptimo (estiércol vacuno). Los resultados de este ensayo mostraron que una concentración de imidacloprid superior a 2 mg kg^{-1} de estiércol dificulta el crecimiento de las lombrices, mientras que concentraciones de imidacloprid de 10 mg kg^{-1} impiden el vermicompostaje debido a que provocan un 80% de mortalidad en las lombrices. Este ensayo inicial permitió determinar el rango de concentraciones de imidacloprid en matas de plantas que permitirían la supervivencia de las lombrices aunque podría afectar a su crecimiento durante el vermicompostaje de estos residuos vegetales de invernadero. De esta forma, en un segundo ensayo se evaluó el efecto que tienen 2, 4 y 8 mg de IMD kg^{-1} mata de planta en el desarrollo de lombrices de la especie *E. fetida* durante el vermicompostaje de estos residuos vegetales acondicionados con lodos de papelera. Los resultados obtenidos señalaron que las matas de plantas de tomate desechadas por los cultivos bajo plástico o en invernadero son solamente viables para ser vermicompostadas si la concentración de imidacloprid no supera los 2 mg IMD kg^{-1} de residuo vegetal. No obstante, una concentración de 2 mg IMD kg^{-1} de mata de planta no permite la reproducción de las lombrices *E. fetida* durante el proceso de vermicompostaje,

por lo que este residuo puede ser biodegradado mediante las lombrices pero no sería adecuado para sistemas de vermicompostaje donde que persiga la producción de biomasa de lombriz a la vez que se bioestabilizan los residuos orgánicos (Edwards et al., 2010).

En el caso de los destríos de tomate, los resultados mostrados en el capitulo 1 indican que el vermicompostaje de destríos de tomate que se encuentran parcialmente deshidratados, por su acumulación en plantas de almacenaje, es viable, ya que tiene características que permiten a las lombrices alimentarse y desarrollarse (Tabla 1.1). Sin embargo, durante el vermicompostaje de los destríos de tomate deshidratados, *E. andrei* no fue capaz de aumentar su biomasa hasta niveles similares a los que alcanzaron las lombrices de esta especie alimentadas con estiércol vacuno (Figura 1.1). En contrapartida, hay que destacar que la reproducción de las lombrices fue más eficiente durante el vermicompostaje de los destríos de tomate que en el vermicompostaje de estiércol (Tabla 1.5). La adición a los destríos de tomate deshidratados de un 25% de paja permitió a las lombrices elevar su biomasa manteniendo la alta tasa de reproducción que ocurrió en el vermicompostaje de los destríos de tomate (Tabla 1.5).

A diferencia de los procesos de vermicompostaje tradicionales que fueron desarrollados a escala de laboratorio en los capítulos 1, 4 y 5 para biotransformar los restos de plantas y los destríos de tomate deshidratados, la acidez (pH: 3.9) y elevado contenido de agua (92%) de los destríos frescos de tomates recién desechados condicionó que el vermicompostaje de este material se llevara a cabo mediante un sistema de alimentación continua (Capítulos 2 y 3). Para el desarrollo de este proceso de vermicompostaje de alimentación continua se construyó un vermirreactor rectangular, en el cual se dispuso, en su parte inferior, una capa de estiércol ovino, en la que se inoculó una población inicial de aproximadamente 1000 lombrices de la especie *E. fetida*. El vermirreactor fue alimentado, durante 150 días, con destríos de tomates triturados. Posteriormente y una vez retiradas las lombrices, el vermicompost obtenido fue madurado durante dos meses. Con este sistema, las lombrices permanecieron protegidas del contacto directo del jugo de ácido de tomate, permitiendo a estas procesar paulatinamente los destríos de tomate que eran vertidos sobre la capa de estiércol. De esta manera se consiguió un duplicar la población y biomasa total de lombrices después de 90 días (Figura 2.1). Además de permitir biotransformar los destríos de tomate fresco desechados por los cultivos bajo plástico y en invernadero, este sistema de vermicompostaje de alimentación continua hace más económico el proceso de vermicompostaje, ya que el continuo aporte de destríos frescos hizo que no fuera necesario aportar agua para mantener la humedad del lecho de vermicompostaje a un nivel de adecuado para el desarrollo de las lombrices, algo que, como

se mencionó en el apartado I.2.7.6., es necesario en sistemas de vermicompostaje tradicionales.

Los parámetros químicos, bioquímicos y microbiológicos analizados en los capítulos 1, 2, 3, y 4 pusieron de manifiesto una significativa biotransformación de los restos de plantas y los destríos de tomate tras el proceso de vermicompostaje.

El vermicompostaje disminuyó significativamente los contenidos de carbono orgánico total (TOC) y carbono orgánico hidrosoluble (WSC) que inicialmente presentaban tanto los destríos secos de tomate como las mata de plantas mezcladas con lodos de papelera. Estos resultados indican que una parte significativa de los compuestos orgánicos que componen esos residuos fue mineralizada hasta CO_2 y/o transformada en biomasa de lombriz durante los procesos de vermicompostaje tradicional a escala de laboratorio expuestos en los capítulos 1 y 4. En el caso del vermicompostaje de destríos frescos de tomate mediante el sistema de alimentación continua a escala piloto descrito en los capítulos 2 y 3, los valores de TOC medidos mensualmente en el lecho del vermirreactor se mantuvieron por debajo de 300 g TOC kg^{-1} material (Tablas 2.2 y 3.1), lo cual indica que gran parte de los aproximadamente 1469 g de TOC que se descargaron mensualmente en el sistema a través del aporte semanal de 10 kg de destríos de tomate (367 g de TOC $semana^{-1}$) fueron mineralizado hasta CO_2 y/o transformado hasta biomasa de lombriz. Estudios pioneros en el campo del vermicompostaje revelaron como las lombrices pueden directamente descomponer los residuos orgánicos y asimilar el carbono contenido en las fracciones de materia orgánica más lábiles (Edwards, 1988). Además, como se ha indicado y referenciado en la introducción de esta Memoria (I.2.1), así como en los capítulos que la componen, la acción de las lombrices sobre el residuo acelera indirectamente su descomposición y mineralización, favoreciendo el desarrollo y la actividad de los microorganismos degradadores de materia orgánica en el residuo orgánico. Así, la actividad de las lombrices y microorganismos involucrados los procesos de vermicompostaje descritos en los capítulos 1, 2, 3 y 4 fue capaz de degradar la materia orgánica contenida en los diferentes residuos ensayados.

La biodegradación de esos residuos también quedó patente en las disminuciones significativas de los valores de relación C/N que inicialmente presentaban los destríos secos de tomate y las mezclas de restos de plantas y lodos de papelera, que tras los diferentes procesos de vermicompostaje se redujeron hasta llegar a niveles inferiores a 20 en los diferentes vermicomposts obtenidos (Tablas 1.2 y 4.2). Una significativa disminución de la relación C/N de materiales orgánicos ha sido tradicionalmente considerada como un signo de

su descomposición y estabilización a causa de procesos biológicos como compostaje o vermicompostaje (Senesi, 1989). Por esta razón, la legislación española establece que los materiales vermicompostados deben tener una relación C/N inferior a 20 (Gobierno de España, 2005). En el caso del vermicompostaje continuo de destríos de tomate frescos, el valor de la relación C/N se mantuvo por debajo de 12 desde el inicio del vermicompostaje y durante todo el proceso, a pesar de la gran cantidad de materia orgánica fresca que se incorporó periódicamente al sistema (Tablas 2.2 y 3.1). Este resultado indica que gran parte del residuo añadido a lo largo del proceso se biodegrada y estabilización continuamente. No obstante, Senesi (1989) ya advirtió en su clásica revisión sobre los materiales comportados, el valor de la relación C/N en sí mismo no es un buen indicador del grado de estabilidad de composts o vermicomposts, ya que estos materiales pueden contener una cantidad variable de compuestos orgánicos que son recalcitrantes a su biodegradación. Por este motivo, además de estudiar los cambios químicos que los residuos orgánicos experimentan durante el vermicompostados, es necesario valorar como el ese proceso afecta a la actividad biológica del residuo orgánico, a fin de confirmar los resultados químicos que sugieren la bioestabilización del residuo.

En los procesos de vermicompostaje desarrollados en los capítulos 1, 2, 3 y 4 se evaluaron también los cambios que sufren los valores de la actividad potencial de diferentes tipos de enzimas (oxido-reductasa e hidrolasas) presentes en los distintos residuos orgánicos como consecuencia de su vermicompostaje, a fin de confirmar la biodegradación y estabilización de los residuos. Los resultados de estos análisis mostraron que tras los diferentes procesos de vermicompostaje se produjo una significativa disminución general de los valores iniciales de las diferentes actividades enzimas en los destríos secos de tomate, solos o mezclados con paja o estiércol vacuno. Igualmente, el vermicompostaje de matas de planta de tomate mezcladas con lodos de papelera provocó una disminución significativa de los valores de las actividades enzimáticas analizadas. Benítez et al., 1999 estudiaron estas actividades enzimáticas como bioindicadores del proceso de vermicompostaje llegando a afirmar que el significativo descenso de la potencial actividad de diferentes hidrolasas (β-glucosidasa, fosofatasa acida, ureasa y proteasa) ocurrido en un proceso de vermicompostaje tradicional responde a que gran proporción de los compuestos orgánicos responsables de la síntesis de esas enzimas han sido degradados por la acción conjunta de las lombrices y microorganismos durante el proceso. Por otro lado, la disminución significativa de la actividad deshidrogenada del un residuo orgánico tras el vermicompostaje ha sido ampliamente usado como indicador de la estabilización biológica en multitud de estudios

diferentes sobre vermicompostaje de residuos orgánicos de diferente naturaleza (Bansal & Kapoor, 2000; Benítez et al., 2002; Garg et al., 2006; Kaushik & Garg, 2003; Nogales et al., 2005; Parthasarathi & Ranganathan, 1999; Sen & Chandra, 2009; Shanthi et al., 2010).

Aunque la reducción en la actividad potencial de enzimas hidrolasas y deshidrogenadas de los residuos orgánicos tras su vermicompostaje se considera un patrón común que refleja la degradación, mineralización y bioestabilización de esos materiales en procesos de vermicompostaje tradicionales (Nogales et al., 2008), en el caso de procesos de vermicompostaje de alimentación continua existe escasa información en la literatura científica sobre la evolución de estas actividades enzimáticas. Unicamente los trabajos realizados en Departamento de Ecología y Biología Animal de la Facultad de Ciencias perteneciente a Universidad de Vigo ha aportado interesante información sobre la evolución de diversas actividades enzimáticas durante un proceso de vermicompostaje continuo (Aira et al., 2006, 2007a, 2007b; Aira & Domínguez, 2008). No obstante, la información proporcionada en estos trabajos se limita a uso de un vermicompostador modular para el vermicompostaje de purines de cerdo. Por este motivo, los resultados descritos en el capítulo 2, donde se discute en profundidad la utilidad de monitorizar la evolución temporal de la actividad deshidrogenada y del valor total de actividades de las enzimas hidrolasas: β-glucosidasas, fosfatasa acidas, ureasas y proteasas durante el vermicompostaje continuo de destríos frescos de tomate y su posterior maduración representa información inédita en el ámbito de los procesos de vermicompostaje de alimentación continua. Incluso más novedoso, es el estudio recogido en el capítulo 3 sobre la actividad de enzimas que se encuentran asociadas a las sustancias húmicas que se producen durante este proceso de vermicompostaje continuo. Como resultados más destacados se observó que lo patrones temporales de las actividades totales de deshidrogenasa, β-glucosidasa, proteasa y ureasa se relacionaban con el crecimiento de las lombrices durante el proceso y, además, con la degradación de la materia orgánica que es continuamente incorporada al sistema. Por otra parte, los complejos humus-enzimas generados durante el periodo de vermicompostaje no pudieron resistir la desnaturalización, la desactivación y la degradación causada por la desecación al aire del material durante la fase de maduración. A pesar de esto, el vermicompost maduro obtenido a partir de los destríos de tomate tuvo un mayor contenido en complejos activos humus-enzima que los materiales vermicompostados.

Con una cierta independencia del tipo de sistema de vermicompostaje (tradicional y alimentando continuamente) que se empleó para biodegradar y bioestabilizar los diferentes tipos de residuos vegetales procedentes de cultivos de invernadero (restos de plantas y

destríos de tomates secos y frescos), los resultados recogidos en los capítulos 1, 2 y 4 muestran que esos proceso de vermicompostaje fueron capaces de degradar, mineralizar y biostabilizar esos residuos dando lugar a productos orgánicos con un importante contenido en elementos esenciales para la nutrición vegetal. En este sentido, los distintos vermicomposts procedentes de destríos secos de tomate (T), de destríos frescos de tomate (DT), y de restos de plantas de tomate mezclados con lodos de papelera (PS 2:1 y PS 1:1) mostraron valores de N entre 12 y 22 g kg^{-1} de P entre 2 y 10 g kg^{-1} y de K entre el 5 y 30 g kg^{-1} (Tablas 1.3, 2.3, 4.3). Esos valores fueron similares o incluso superiores a los que presentan otros vermicomposts obtenidos a partir de otros residuos orgánicos (Albanell et al., 2008; Benítez et al., 2002, Elvira et al., 2006, Garg et al., 2006, Nogales et al., 2005, Melgar et al., 2009, Sutrar, 2007) e incluso a aquellos que son producidos comercialmente por diferentes empresas. Además, los vermicomposts producidos a partir de los distintos residuos vegetales de invernadero como se describe en los capítulos 1, 2 y 4, registraron una concentración de metales pesados por debajo de los límites que la legislación española sobre productos fertilizantes requiere para que un compost o vermicompost sea considerado una enmienda orgánica de clase A o un substrato orgánico adecuado para cultivos vegetales (Real Decreto 824/2005 y Real Decreto 865/2010; Tabla I.4). En vista de los resultados recogidos en esos capítulos, se puede afirmar que los productos orgánicos resultantes del vermicompostaje de residuos vegetales de invernadero son materiales orgánicos con características que pueden permitir su valorización en como materiales útiles en agricultura y en restauración de suelos.

Además de las transformaciones químicas y bioquímicas que los procesos de vermicompostaje provocaron en los diferentes residuos vegetales de invernadero, la interacción entre lombrices y microorganismos durante los procesos de vermicompostaje descritos en los capítulos 1, 2, 3 y 4 desencadenó una significativa transformación de las comunidades microbianas que inicialmente presentaros estos residuos.

En los capítulos 1 y 2 y en el capítulo 4 se aplicaron la electroforesis en gel con gradiente desnaturalizante de fragmentos amplificados de los genes del ARNr por (DGGE) y en el análisis del contenido en ácidos grasos fosfolipídicos (PLFA), respectivamente, para los vermicompostaje llevados a cabo transformaron las comunidades iniciales de de los principales grupos de microorganismos involucrados en proceso de vermicompostaje: bacterias y hongos (Domínguez et al., 2010; Edwards & Fletcher, 1988). La aplicación de la DGGE de fragmentos de genes del ARNr baterianos en el análisis de la transformación de la comunidad bacteriana causada por el proceso de vermicompostaje expuesto en el capítulo 1

permite evidenciar como las distintas mezclas de tomate deshidratado con paja o estiércol presentaron comunidades microbianas que cambiaron significativamente como consecuencia del proceso de vermicompostaje (Fig. 1.4). De modo que las comunidades bacterianas en las diferentes mezclas de tomate con paja o estiércol deshidratado fueron más similares entré sí tras el proceso que antes de este proceso (Tabla 1.6). Esto sugiere un efecto homogenizador del proceso de vermicompostaje sobre las comunidades bacterianas de los residuos donde la presencia de tomate favoreció la existencia de comunidades bacterianas análogas. El análisis de las comunidades bacterianas desarrolladas durante el proceso de vermicompostaje de alimentación continua mediante DGGE mostró como la comunidad bacteriana inicial cambia como consecuencia de la actividad de las lombrices durante los 150 días de vermicompostaje para después transformarse en otra comunidad diferente cuando la fueron retirada y el material vermicompostado maduró (Figuras 2.4a). Esto indica la enorme influencia que tuvieron las distintas fases de este sobre la comunidad microbiana que se establece en el material orgánico. En este sistema de vermicompostaje continuo, el DGGE de los genes ARNr procedentes de los hongos que se desarrollaran en el proceso mostró un cambio de la comunidad de estos microorganismos que coincidió con la etapa donde la población y de biomasa de lombrices fue mayor. Como se discute en el capítulo 2, este hecho sugiere el enorme efecto que tienen las lombrices para condicionar la comunidad de hongos del material vermicompostado (Figuras 2.4b). Por último, en el capítulo 4, el análisis PLFA indicó que el vermicompostaje disminuyó significativamente la biomasa bacteriana y fúngica de las matas de planta mezcladas con lodos de papelera y modificó su abundancia en bacterias gram positivas, bacterias gram negativas, actinomicetos y hongos, de forma que los vermicompost resultantes de las diferentes mezclas de estos residuos presentaron uno porcentajes de estos microorganismos similares a los que se observan en un vermicompost comercial (Tabla 4.2, figura 4.3).

Un estudio más profundo de la estructura y composición de la comunidad bacteriana que alberga el vermicompost producido a partir de destríos frescos de tomate, vermicompost DT, se recoge en el capítulo 6. Este capítulo muestra las ventajas del uso combinado de la electroforesis en gel con gradiente desnaturalizante (DGGE) y del microchip de ADNr (COMPOCHIP) para investigar la comunidad bacteriana de este vermicompost y compararla con las comunidades microbianas que albergan otros tres vermicomposts obtenidos a partir del residuos orgánicos de origen diferente: estiércol vacuno comercial (CM), alperujo mezclado con biosolidos en proporción (OB) y orujo vinícola agotado mezclado con lías (WW), los cuales fueron procesados por con la misma especie de lombriz que el

vermicompost DT. Este estudio comparativo aporta una valiosa información en el campo de la microbiología del vermicompostaje, un aspecto del proceso poco conocido aun. Así, la comparación de las huellas indentificativas de los diferentes vermicomposts dejó patente que éstos albergaban comunidades bacterianas con un coeficiente de similitud medio cercano al 80% (Figura 6.1). El COMPOCHIP detectó la presencia de *Sphingobacterium*, *Streptomyces*, Alpha-Proteobacteria, Delta-Proteobacteria, y Firmicutes en todos los vermicomposts, aunque mostró diferencias entre los vermicomposts respecto la abundancia de los taxones bacterianos examinados (Figura 6.2 y 6.3). El contenido particular en esos taxones de los diferentes vermicompost ofrece una idea sobre la calidad microbiológica de estos vermicompost además de proporcionar una guía para buscar bacterias con capacidades provechosas en biotecnología usando estos vermicompost. Por ejemplo, en el caso particular del vermicompost de destríos de tomate, el análisis de su comunidad bacteriana mediante el COMPOCHIP reveló que este vermicompost posee un contenido en bacterias de la especie *Pseudomona aeruginosa* mayor que el de los otros vermicomposts (Figura 6.2). Puesto que es sobradamente conocido que algunas cepas de esta especie pueden actuar como patógenos oportunistas de humanos, el uso del COMPOCHIP para analizar vermicomposts permitió alertar sobre los posibles riesgos que entraña la manipulación del vermicompost en personas inmunodeprimidas. A pesar de este inconveniente, a la vista de varios estudios han puesto de manifiesto la habilidad de ciertas cepas de esta especie bacteriana para degradar diversos tipos de contaminantes y xenobióticos, como por ejemplo hidrocarburos y compuestos aromáticos derivados del petróleo (Das & Mukherjee, 2007; Ma et al., 2011; Zhang et al., 2011), polímeros sintéticos (Mukherjee et al., 2011) o pesticidas (Hussain et al., 2007; Malghani et al., 2009; Onbasili & Aslim, 2011), la presencia de *P. aeruginosa* en el vermicompost DT le otorga una potencial utilidad como material susceptible de ser investigado en subsiguientes estudios dirigidos al aislamiento de cepas de esta especie bacteriana con interés para procesos de biorremediación.

En base a los resultados obtenidos en el capítulo 6, el objetivo del capítulo 7 fue evaluar la tolerancia de la comunidad microbiana del vermicompost de destríos de tomate (DT) ante la adición de tres tipos diferentes de plaguicidas: el insecticida imidacloprid, el herbicida diuron, y el fungicida metalaxil, comparándola con aquella que presentan la comunidad microbiana de los otros vermicomposts ensayados (CM, OB y WW) en el capítulo 6. Para ello se empleó el sistema MicroResp^{TM} (apartado I.3. 2. 3.), una técnica que además permitió estimar la diversidad catabólica de cada uno de los vermicomposts. Además, en la investigación que se describe en el capítulo 7 también se examinaron la diversidad

bioquímica de los cuatro vermicomposts y la estructura de sus comunidades fúngicas mediante la determinación de cuatro actividades enzimáticas y el DGGE de genes ARNr 18S. De este modo, se observó que la respiración microbiana de los microorganismos presentes en el vermicompost DT no disminuye, aumentando incluso, cuando este vermicompost fue contaminado con dosis agrícolas de cada plaguicidas, lo cual si ocurrió en el caso del vermicompost comercial (CM) contaminado con imidacloprid o metalaxil (Fig. 7.4). Los resultados recogidos en ese capítulo sugieren que la capacidad de la comunidad microbiana de los vermicomposts para tolerar e incluso degradar plaguicidas parece estar relacionada con la diversidad funcional (bioquímica y catabólica) de los mismos. En este sentido, el vermicompostaje de destríos de tomate biotransformó esos residuos en un producto orgánico con un considerable potencial para ser utilizado en la construcción de sistemas de gestión de plaguicidas, como por ejemplo biofiltros o biobeds, los cuales son usados en prácticas agrícolas para evitar la contaminación ambiental que causan estos xenobióticos (Castillo et al., 2008; De Wilde et al., 2007)..

Como ha sido previamente mencionado en los capítulos 6 y 7, evaluar la comunidad microbiana que alberga un vermicompost es esencial no solo para conocer su potencial interés como material bioactivo con diferente usos sino también porque de esta manera se podría estimar el impacto que pueden tener los microorganismos contenidos esos materiales sobre la comunidad de un suelo cuando estos productos orgánicos son aplicados como enmiendas orgánicas con fines agrícolas y/o medioambientales. Desde este punto de vista, el estudio recogido en el capítulo 8 trató de proporcionar información sobre como afecta la adición a un suelo de un material vermicompostado en comparación con el efecto que causa el mismo residuo cuando este se estabiliza mediante otro proceso biológico diferente, como fue el caso del compostaje, el cual generó una comunidad microbiana diferente a la del vermicompost (Tabla 8.2). Bajo este planteamiento, un suelo agrícola fue enmendado con dos productos comercializados en la actualidad: un compost y/o un vermicompost, los cuales fueron producidos a partir de destríos de frutas y verduras desechadas por supermercados y comercios similares. Los diferentes análisis realizados en este estudio mostraron que tanto el compost como el vermicompost aumentaron la respiración basal del suelo agrícola de una manera similar (Figura 8.1), modificando el valor potencial de diferentes actividades enzimáticas (β-glucosidasa, ureasa, fosfatasa acida y arilsulfatasa), medidas en el suelo a lo largo de 28 días de incubación, de una manera bastante similar (Figuras 8.2 y 8.3). Esto pudo ser posiblemente debido a que ambas enmiendas presentaban una composición elemental muy parecida (Tabla 8.1), que parece ser el factor más determinante en los cambios que

causan en la bioquímica del suelo. Sin embargo, y después de cuatro semanas de incubación, se observó que las comunidades microbianas, evaluadas mediante el análisis de los ésteres metílicos de ácidos grasos (FAME), que se desarrollaron en las muestras del suelo sin enmendar y en las muestras del suelo enmendadas con un 3% de compost y/o vermicompost fueron diferentes (Figura 8.4). Además, las comunidades microbianas del suelo enmendado con compost cambiaron de forma distinta a como ocurrió cuando el mismo suelo fue enmendado con vermicompost (Figura 8.4). Esta diferenciación se relacionaría con la presencia de comunidades microbianas distintas en el compost y en el vermicompost (Tabla 8.2), lo cual confirma la importancia de investigar y conocer la comunidad microbiana de las enmiendas orgánicas que se aplican a los suelos. De esta manera el capítulo 5 pone de manifiesto la importancia de identificar grupos microbianos característicos de los compuestos vermicompostados.

Finalmente, con objeto de aportar información sobre la utilidad que pueden tener en procesos de biorremediación de suelos el vermicompost producidos a partir de destríos de tomate procedentes de invernadero, este vermicomposts se aplicó como enmienda orgánica para recuperar un suelo contaminado por metales pesados como se describe en el capítulo 9. Este vermicompost fue el escogido en función de la calidad de su materia orgánica y sus propiedades bioquímicas y microbiológicas descritas en los capítulos 2, 3, 6 y 7. Específicamente, en el estudio se ensayó este vermicompost frente a otro comercial en un sistema como enmienda del suelo contaminado por varios metales pesados en que además se sembraron plantas de trébol (*Trifolium repens*) con objeto de mejorar su estado de degradación y establecer una cubierta vegetal. Además, se testó la posibilidad de mejorar el efecto de ambos vermicompost mediante la adición de un inóculo de hongos micorrícos aislados de otro suelo contaminado por metales pesados. Los resultados obtenidos pusieron de manifiesto que la aplicación del vermicompost DT favoreció el desarrollo vegetal de plantas de trébol (Figura 9.1), ya que mejoró su nutrición (Tabla 9.2), pero impidió la acumulación de metales pesados en ella (Tabla 9.3). Además, la aplicación de vermicompost DT al suelo contaminado con metales pesados favoreció la estabilización de los mismos reduciendo la fracción de metales pesados que se presentaban en formas fácilmente extraíbles (Tabla 9.4.). Un efecto similar fue también observado en otros estudios donde se han enmendaros suelos contaminados por metales pesados con vermicomposts o composts de residuos oleícolas (Nogales y Benítez, 2006, 2007; Romero et al., 2005). Junto con esto cambios, la enmienda de este suelo degradado con vermicompost DT estimuló las actividades deshidrogenasa, β-glucosidasa y ureasa en el suelo (Figura 9.2) en una medida

mayor a la que se observo cuando el suelo fue enmendado con la misma cantidad de un vermicompost comercial. Por otro lado, la inoculación del suelo con hongos AM se tuvo escasos efectos sobre el crecimiento vegetal así como sobre la disponibilidad de metales y las actividades enzimáticas del suelo en comparación con la no inoculación. Los resultados de este último estudio sugiere que el vermicompost DT podría ser usado para favorecer el establecimiento de una cubierta vegetal sobre suelos contaminados por metales pesados a la vez que permitiría reducir la disponibilidad de estos contaminantes y aumentar la calidad bioquímica del suelo.

El aporte conjunto que suponen los resultados recogidos en los capítulos que componen esta tesis de investigación al campo del vermicompostaje permiten permitirán mejorar el uso de esta biotecnológica como una herramienta más eficaz para el tratamientos y reciclado de residuos orgánicos en materiales biológicos de un valorizados como enmiendas para suelos, proporcionando además un mayor conocimiento en el campo de la microbiología del proceso de vermicompostaje y sobre las comunidades microbianas asociadas a estos materiales orgánicos.

CONCLUSIONES

1. El elevado contenido en sales de los residuos vegetales (matas de plantas) generados por los cultivos en invernadero hace inviable su vermicompostaje. La mezcla de estos residuos con lodos de la fabricación de papel permite su reciclaje simultáneo mediante este proceso biotecnológico.

2. Una concentración de imidacloprid en matas de plantas procedentes de cultivos de invernadero superior a 2 mg kg^{-1} hace inviable su vermicompostaje.

3. El vermicompostaje de destríos frescos de tomate mediante un proceso de alimentación continua, que incorpora una fase de maduración posterior, es una opción económicamente viable para la gestión de estos residuos de invernadero ácidos y con un elevado contenido de agua.

4. Las actividades enzimáticas analizadas fueron bioindicadores útiles para valorar la biotransformación de los residuos vegetales de invernadero causada por su vermicompostaje así como para estimar la diversidad bioquímica de las comunidades microbianas presentes en los vermicomposts.

5. Los complejos húmus-enzimas generados durante la biotransformación de destríos de tomate mediante el vermicompostaje de alimentación continua no pudieron resistir la desnaturalización, desactivación y degradación causada por la fase final de maduración. Pese a esto, el vermicompost producido mostró mayor contenido de esos complejos que el sustrato orgánico inicial.

6. El vermicompostaje modificó significativamente las comunidades microbianas de los residuos vegetales de invernadero, disminuyendo su biomasa bacteriana y fúngica y cambiando también su estructura microbiana.

7. Los procesos de vermicompostaje diseñados fueron capaces de biodegradar y biotransformar los residuos vegetales de invernadero (matas de plantas y frutos de destríos) en materiales orgánicos con valor añadido para ser utilizados como enmiendas orgánicas de suelo en agricultura convencional, integrada y orgánica así como substratos orgánicos de cultivos vegetales de invernadero.

8. El uso conjunto del DGGE y el COMPOCHIP fue una estrategia rápida para comparar la comunidad bacteriana del vermicompost de destríos de tomate con la de otros vermicomposts, valorando la presencia de taxones bacterianos que permiten estimar la calidad microbiológica del vermicompost. Esta aproximación proporcionó una valiosa información para estudios dirigidos al aprovechamiento de las bacterias presentes en los vermicomposts.

9. El análisis de la respuesta respiratoria de la microbiota presente en el vermicompost de destríos de tomate a la adición de pesticidas mediante el sistema Microresp^{TM} permitió valorar su tolerancia a estos xenobióticos respecto a otras comunidades microbianas de vermicomposts.

10. La enmienda de un suelo agrícola con vermicompost de destríos de supermercado mejoró su actividad biológica y calidad bioquímica, modificando su estructura microbiana de forma diferente a como lo hizo un compost procedente del mismo residuo.

11. La enmienda de un suelo contaminado por metales pesados con vermicompost de destríos de tomate junto con la siembra de *Trifolium repens* fue una estrategia eficaz que redujo la disponibilidad de Fe, Cu, Zn, Cd y Pb del suelo, mejorando además su diversidad calidad bioquímica respecto al suelo sin enmendar.

CONCLUSIONS

1. High salt content in greenhouse vegetable wastes (plant debris) prevents the feasible vermicomposting of these wastes. Mixing these wastes with paper-mill sludge allows the simultaneous recycling of both wastes through this biotechnological process.

2. Greenhouse plant wastes containing imidacloprid concentrations over 2 mg kg^{-1} are unfeasible to be vermicomposted.

3. The vermicomposting of fresh tomato-fruit wastes by using a continuous-feeding process, which includes a final maturation phase, is an economically feasible option for biostabilizing such greenhouse wastes which are acid and have high water content.

4. The analysed enzyme activities were useful bioindicator for assessing the biotransformation of greenhouse vegetable wastes caused by vermicomposting as well as for estimating biochemical diversity of resident microbial communities in vermicomposts.

5. Humus-enzyme complexes originated by biotransformation of fresh tomato-fruit wastes through continuous-feeding vermicomposting were unable to Humic-enzyme complexes generated during the vermicomposting period were unable to resist denaturation, inactivation, and degradation caused by the final maturation phase. Despite this, the resulting vermicompost had higher content in humus-enzyme complexes than the initial organic-substrate.

6. Vermicomposting significantly modified the resident microbial communities in the greenhouse vegetable wastes, reducing their bacterial and fungal biomass and changing also their microbial structure.

7. The vermicomposting processes designed were able to biodegrade and biotransform greenhouse vegetable wastes (i.e. plant debris and damaged fruits) into valuable organic-materials to be used as organic soil amendments for conventional, integrated and organic agriculture or as organic growing media for greenhouse crops.

8. The joint use of DGGE and COMPOCHIP was a rapid strategy for comparing the resident bacterial communities in damaged-tomatoes vermicompost with that in other vermicomposts, assessing the presence of bacterial taxa which enables to estimate the vermicompost microbiological quality. This approach can also provide a worthwhile guidance for studies aimed at profiting from bacteria inhabiting vermicomposts.

9. The analysis of the respiratory response of the resident microbiota in damaged-tomatoes vermicompost to pesticide addition by using Microresp^{TM} system allowed to assess its tolerance to such xenobiotics with respect to other vermicompost microbial communities

10. Amending a cropland soil with vermicompost from grocery vegetable/fruit wastes improved its biological activity and biochemical quality modifying its microbial structure differently as compost produced from the same waste did.

11. Amending a heavy metal contaminated soil with damaged-tomatoes vermicomposts in combination with sowing *Trifolium repens* was an effective strategy which reduced availability of Fe, Cu, Zn, Cd y Pb in the soil, improving also its biochemical quality as compared with that in the soil without amending.

BIBLIOGRAFÍA

Adi, A.J., Noor, Z.M., 2009. Waste recycling: Utilization of coffee grounds and kitchen waste in vermicomposting. Bioresource Technology 100, 1027-1030.

Aguilar, F.J., González, P., 2002. Agricultural use of compost from municipal solid waste - Effects on soil physical properties. Journal of Solid Waste Technology and Management 28, 200-212.

Aira, M., Domínguez, J., 2008. Optimizing vermicomposting of animal wastes: Effects of rate of manure application on carbon loss and microbial stabilization. Journal of Environmental Management 88, 1525-1529.

Aira, M., Monroy, F., Domínguez, J., 2007b. *Eisenia fetida* (Oligochaeta, Lumbricidae) modifies the structure and physiological capabilities of microbial communities improving carbon mineralization during vermicomposting of pig manure. Microb. Ecol. 54, 662-671.

Aira, M., Monroy, F., Domínguez, J., 2006a. Changes in microbial biomass and microbial activity of pig slurry after the transit through the gut of the earthworm *Eudrilus eugeniae* (Kinberg, 1867). Biology and Fertility of Soils 42, 371-376.

Aira, M., Monroy, F., Domínguez, J., 2006b. *Eisenia fetida* (Oligochaeta, Lumbricidae) activates fungal growth, triggering cellulose decomposition during vermicomposting. Microbial Ecology 52, 738-747.

Aira, M., Monroy, F., Domínguez, J., 2007a. Earthworms strongly modify microbial biomass and activity triggering enzymatic activities during vermicomposting independently of the application rates of pig slurry. Science of the Total Environment 385, 252-261.

Albanell, E., Plaixats, J., Cabrero, T., 1988. Chemical changes during vermicomposting (*Eisenia fetida*) of sheep manure mixed with cotton industrial wastes. Biol. Fertl. Soils 6, 266-269.

Albiach, R., Canet, R., Pomares, F., Ingelmo, F., 2000. Microbial biomass content and enzymatic activities after the application of organic amendments to a horticultural soil. Bioresource Technology 75, 43-48.

Albiach, R., Canet, R., Pomares, F., Ingelmo, F., 2001. Organic matter components and aggregate stability after the application of different amendments to a horticultural soil. Bioresource Technology 76, 125-129.

Alef, K., Nannipieri, P., 1995. Methods in Soil Microbiology and Biochemistry. Academic Press, London,

Alkoaik, F., Ghaly, A.E., 2005. Effect of inoculum size on the composting of greenhouse tomato plant trimmings. Compost Science and Utilization 13, 262-273.

Alkoaik, F., Ghaly, A.E., 2006. Influence of dairy manure addition on the biological and thermal kinetics of composting of greenhouse tomato plant residues. Waste Management 26, 902-913.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143-169.

Anastasi, A., Varese, G.C., Voyron, S., Scannerini, S., Marchisio, V.F., 2004. Characterization of fungal biodiversity in compost and vermicompost. Compost Science and Utilization 12, 185-191.

Anastasi, A., Varese, G.C., Filipello Marchisio, V., 2005. Isolation and identification of fungal communities in compost and vermicompost. Mycologia 97, 33-44.

Antón, M.A., Muñoz, P., Castells, F., Montero, J.I., Soliva, M., 2005. Improving waste management in protected horticulture. Agronomy for Sustainable Development 25, 447-453.

Arancon, N.Q., Edwards, C.A., Atiyeh, R., Metzger, J.D., 2004. Effects of vermicomposts produced from food waste on the growth and yields of greenhouse peppers. Bioresource Technology 93, 139-144.

Arancon, N.Q., Edwards, C.A., Babenko, A., Cannon, J., Galvis, P., Metzger, J.D., 2008. Influences of vermicomposts, produced by earthworms and microorganisms from cattle manure, food waste and paper waste, on the germination, growth and flowering of petunias in the greenhouse. Applied Soil Ecology 39, 91-99.

Arancon, N.Q., Edwards, C.A., Bierman, P., Metzger, J.D., Lucht, C., 2005a. Effects of vermicomposts produced from cattle manure, food waste and paper waste on the growth and yield of peppers in the field. Pedobiologia 49, 297-306.

Arancon, N.Q., Edwards, C.A., Yardim, E.N., Oliver, T.J., Byrne, R.J., Keeney, G., 2007. Suppression of two-spotted spider mite (*Tetranychus urticae*), mealy bug (*Pseudococcus* sp) and aphid (*Myzus persicae*) populations and damage by vermicomposts. Crop Protection 26, 29-39.

Arancon, N.Q., Galvis, P.A., Edwards, C.A., 2005b. Suppression of insect pest populations and damage to plants by vermicomposts. Bioresource Technology 96, 1137-1142.

Atiyeh, R.M., Domínguez, J., Subler, S., Edwards, C.A., 2000. Changes in biochemical properties of cow manure during processing by earthworms (*Eisenia andrei*, Bouche) and the effects on seedling growth. Pedobiologia 44, 709-724.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 2002. Short protocols in molecular biology, Fifth ed. Wiley, New York.

Bansal, S., Kapoor, K.K., 2000. Vermicomposting of crop residues and cattle dung with *Eisenia foetida*. Bioresource Technology 73, 95-98.

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresource Technology 67, 297-303.

Benítez, E., Nogales, R., Masciandaro, G., Ceccanti, B., 2000. Isolation by isoelectric focusing of humic-urease complexes from earthworm (*Eisenia fetida*)-processed sewage sludges. Biology and Fertility of Soils 31, 489-493.

Benítez, E., Sainz, H., Melgar, R., Nogales, R., 2002. Vermicomposting of a lignocellulosic waste from olive oil industry: A pilot scale study. Waste Management and Research 20, 134-142.

Benítez, E., Sainz, H., Nogales, R., 2005. Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresource Technology 96, 785-790.

Bhattacharya, S.S., Chattopadhyay, G.N., 2004. Transformation of nitrogen during vermicomposting of fly ash. Waste Management and Research 22, 488-491.

Blaszak, M., Pelech, R., Graczyk, P., 2011. Screening of microorganisms for biodegradation of simazine pollution (obsolete pesticide Azotop 50 WP). Water, Air, &Amp; Soil Pollution 1-13.

Bouché, M.B., 1972. Lombrices de France: Ecologie et systematique. Annals of Zoologie 72, 1-671.

Buchanam, M.A., Rusell, E., Block, S.D., 1988. Chemical characterization and nitrogen mineralization potentials of vermicompost derived from differing organic wastes, in: Edwards, C.A., Neuhauser, E.F. (Eds.), Earthworms in waste and environmental management. SPB Academic Publishing BV, The Hague, pp. 231-240.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Applied and Environmental Microbiology 69, 3593-3599.

Cara, G. and Riviera, J., 1998. Residuos en la agricultura intensiva: El caso de Almería. In: Encuentro Medioambiental Almeriense: En busca de soluciones., Universidad de Almería, 128-132.

Castilla, N., 2004. Invernaderos de plástico: Tecnología y manejo. Ediciones Mundi-Prensa, Madrid.

Castillo, M.d.P., Torstensson, L., Stenström, J., 2008. Biobeds for environmental protection from pesticide use-a review. Journal of Agricultural and Food Chemistry 56, 6206-6219.

Chan, P.L.S., Griffiths, D.A., 1988. The vermicomposting of pre-treated pig manure. Biological Wastes 24, 57-69.

Chaoui, H.I., Zibilske, L.M., Ohno, T., 2003. Effects of earthworm casts and compost on soil microbial activity and plant nutrient availability. Soil Biology and Biochemistry 35, 295-302.

Chapman, S., Campbell, C., Artz, R., 2007. Assessing CLPPs using MicroResp^{TM}. Journal of Soils and Sediments 7, 406-410.

Chaudhuri, P., Pal, S., Gowtham Bhattachariee, T.K., Dey, S.K., 1993. Chemincal changes during vermicomposting (*Perionyx excavatus*) of kitchen waste trial ecosystems: Application to soils of the humid tropics. Biotropica 25, 130-150.

Chen, G., Zheng, Z., Yang, S., Fang, C., Zou, X., Luo, Y., 2010. Experimental co-digestion of corn stalk and vermicompost to improve biogas production. Waste Management 30, 1834-1840.

Contreras-Ramos, S.M., Álvarez-Bernal, D., Dendooven, L., 2008. Removal of polycyclic aromatic hydrocarbons from soil amended with biosolid or vermicompost in the presence of earthworms (*Eisenia fetida*). Soil Biology and Biochemistry 40, 1954-1959.

Cordovil, C.M., de Varennes, A., Pinto, R., Fernandes, R.C., 2011. Changes in mineral nitrogen, soil organic matter fractions and microbial community level physiological profiles after application of digested pig slurry and compost from municipal organic wastes to burned soils. Soil Biology and Biochemistry 43, 845-852.

Cuadrado, I., 2001. Estudio de la demanda de inputs auxiliares: Producción y manipulación en el sistema productivo agrícola almeriense. Fundación para la Investigación Agraria en la Provincia de Almería, Almeria.

Das, K., Mukherjee, A.K., 2007. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresource Technology 98, 1339-1345.

Datar, M.T., Rao, M., Reddy, S., 1997. Vermicomposting - A technological option for solid waste management. Journal of Solid Waste Technology and Management .

De Wilde, T., Spanoghe, P., Debaer, C., Ryckeboer, J., Springael, D., Jaeken, P., 2007. Overview of on-farm bioremediation systems to reduce the occurrence of point source contamination. Pest Management Science 63, 111-128.

Dees, P.M., Ghiorse, W.C., 2001. Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolates and extracted DNA. FEMS Microbiology Ecology 35, 207-216.

Degens, B.P., Harris, J.A., 1997. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biology and Biochemistry 29, 1309-1320.

Di Gennaro, P., Moreno, B., Annoni, E., García-Rodríguez, S., Bestetti, G., Benítez, E., 2009. Dynamic changes in bacterial community structure and in naphthalene dioxygenase expression in vermicompost-amended PAH-contaminated soils. Journal of Hazardous Materials 172, 1464-1469.

Domínguez, J., Velando, A., Aira, M., Monroy, F., 2003. Uniparental reproduction of *Eisenia fetida* and *E. Andrei* (Oligochaeta: Lumbricidae): Evidence of selfinsemination. Pedobiologia 47, 530-534.

Domínguez, J., 2004. State-of-the-Art and new perspectives on vermicomposting research, in: Edwards, C.A.(Ed.), Earthworm ecology. CRC Press LCC, Boca Raton, pp. 401-424.

Domínguez, J., Aira, M., Gómez-Brandón, M., 2010. Vermicomposting: earthworms enhance the work of microbes, in: Insam, H., Franke-Whittle, I., Goberna, M. (Eds.), Microbes at Work. Springer, Berlin, pp. 93-114.

Drake, H.L., Schramm, A., Horn, M.A., 2006. Earthworm gut microbial biomes: their Importance to soil microorganisms, denitrification, and the terrestrial production of the greenhouse gas N_2O, in: 65-87.

Dunbar, J., Ticknor, L.O., Kuske, C.R., 2001. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Applied and Environmental Microbiology 67, 190-197.

Eastman, B.R., 1999. Achieving pathogen stabilization using vermicomposting. BioCycle 40, 62-64.

Eastman, B.R., Kane, P.N., Edwards, C.A., Trytek, L., Gunadi, B., Stermer, A.L., Mobley, J.R., 2001. The effectiveness of vermiculture in human pathogen reduction for USEPA biosolids stabilization. Compost Science and Utilization 9, 38-49.

Edwards, C.A., 1988. Breakdown of animal, vegetable and industrial organic wastes by earthworms, in: Edwards, C.A., Neuhauser, E.F. (Eds.), Earthworms in waste and environmental management. SPB Academic Publishing BV, The Hague, pp. 21-31.

Edwards, C.A., Bohlen, P.J., 1996. Biology and ecology of earthworms., Third ed. Champman & Hall, London.

Edwards, C.A.,.Burrows, I., 1988. The potential of earthworm composts as plant growth media, in: Edwards, C.A., Neuhauser, E.F. (Eds.), Earthworms in waste and environmental management. SPB Academic Publishing BV, The Hague, pp. 221-229.

Edwards, C.A., Fletcher, K.E., 1988. Interactions between earthworms and microorganisms in organic-matter breakdown. Agriculture, Ecosystems & Environment 24, 235-247.

Elvira, C., Domínguez, J., Mato, S., 1997. The growth and reproduction of *Lumbricus rubellus* and *Dendrobaena rubida* in cow manure mixed cultures with *Eisenia andrei*. Applied Soil Ecology 5, 97-103.

Elvira, C., Goicoechea, M., Sampedro, L., Mato, S., Nogales, R., 1996. Bioconversion of solid paper-pulp mill sludge by earthworms. Bioresource Technology 57, 173-177.

Elvira, C., Mato, S., Nogales, R., 1995. Changes in heavy metal extractability and organic matter fractions after vermicomposting of sludges from a paper mill industry and wastewater treatment plant. Fresenius Environmental Bulletin 4, 503-507.

E.P.A. 1994. Reregistration Eligibility Decision (RED). Metalaxyl. 320 pp. United States Environmental Protection Agency. Washington, USA.

Engelstad, F., 1991. Impact of earthworms on decomposition of garden refuse. Biology and Fertility of Soils 12, 137-140.

Escobar, A., 1998. Aspectos ambientales de la agricultura en invernaderos, in: Pérez, J., Cuadrado, I. (Eds.), Tecnología de invernaderos. Consejería de Agricultura y Pesca, FIAPA, Caja Rural de Almería, Almería, pp. 487-512.

FAO. 2006. Guidelines for soil description. 4th edition. Rome

Fehr, M., Calçado, M.D.R., Romão, D.C., 2002. The basis of a policy for minimizing and recycling food waste. Environmental Science & Policy 5, 247-253.

Fleming, T.P., Richards, K.S., 1982. Localization of adsorbed heavy metals on the earthworm body surface and theri retrieval by chelation. Pedobiologia 23, 415-418.

Food and Agriculture Organization, 2002. El cultivo protegido en clima mediterráneoRoma.

Fracchia, L., Dohrmann, A.B., Martinotti, M.G., Tebbe, C.C., 2006. Bacterial diversity in a finished compost and vermicompost: Differences revealed by cultivation-independent analyses of PCR-amplified 16S rRNA genes. Applied Microbiology and Biotechnology 71, 942-952.

Frank, R., Klauck, C., Stonefield, K.I., 1983. Metal transfer in vermicomposting of sewage sludge and plant wastes. Bulletin of Environmental Contamination and Toxicology 31, 673-679.

Franke-Whittle, I.H., Klammer, S.H., Insam, H., 2005. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. Journal of Microbiological Methods 62, 37-56.

Franke-Whittle, I., Knapp, B., Fuchs, J., Kaufmann, R., Insam, H., 2009a. Application of COMPOCHIP microarray to investigate the bacterial communities of different composts. Microbial Ecology 57, 510-521.

Franke-Whittle, I.H., Goberna, M., Pfister, V., Insam, H., 2009b. Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities. Journal of Microbiological Methods 79, 279-288.

Fu, Y., Shao, L., Liu, H., Tong, L., Liu, H., 2011. Ethylene removal evaluation and bacterial community analysis of vermicompost as biofilter material. Journal of Hazardous Materials 192, 658-666.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan. 28, 123-134.

Garg, V.K., Chand, S., Chhillar, A., Yadav, A., 2005. Growth and reproduction of *Eisenia foetida* in various animal wastes during vermicomposting. Applied Ecology and Environmental Research 3, 51-59.

Garg, V.K., Gupta, R., Yadav, A., 2008. Potential of vermicomposting technology in solid waste management, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current Developments in Solid-state Fermentation. Springer New York468-511.

Garg, V.K., Kaushik, P., 2005. Vermistabilization of textile mill sludge spiked with poultry droppings by an epigeic earthworm Eisenia foetida. Bioresource Technology 96, 1063-1071.

Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied and Environmental Microbiology 57, 2351-2359.

Garrido-Frenich, A., Arrebola, F., González-Rodríguez, M., Vidal, J., Díez, N., 2003. Rapid pesticide analysis, in post-harvest plants used as animal feed, by low-pressure gas chromatography–tandem mass spectrometry. Analytical and Bioanalytical Chemistry 377, 1038-1046.

Giacomazzi, S., Cochet, N., 2004. Environmental impact of diuron transformation: a review. Chemosphere 56, 1021-1032.

Gobierno de España, 2005. Real Decreto 824/2005 sobre productos fertilizantes, in: BOE. Gobierno de España, Madrid, pp. 25602-25669.

Gómez-Brandón, M., Aira, M., Lores, M., Domínguez, J., 2011a. Changes in microbial community structure and function during vermicomposting of pig slurry. Bioresource Technology 102, 4171-4178.

Gómez-Brandón, M., Lazcano, C., Lores, M., Domínguez, J., 2010a. Detritivorous earthworms modify microbial community structure and accelerate plant residue decomposition. Applied Soil Ecology 44, 237-244.

Gómez-Brandón, M., Lazcano, C., Lores, M., Domínguez, J., 2011b. Short-term stabilization of grape marc through earthworms. Journal of Hazardous Materials 187, 291-295.

Gómez-Brandón, M., Lores, M., Domínguez, J., 2010b. A new combination of extraction and derivatization methods that reduces the complexity and preparation time in determining phospholipid fatty acids in solid environmental samples. Bioresource Technology 101, 1348-1354.

Gopal, M., Gupta, A., Sunil, E., Thomas, G., 2009. Amplification of plant beneficial microbial communities during conversion of coconut leaf substrate to vermicompost by *Eudrilus* sp. Current Microbiology 59, 15-20.

Gopalakrishnan, S., Pande, S., Sharma, M., Humayun, P., Kiran, B.K., Sandeep, D., Vidya, M.S., Deepthi, K., Rupela, O., 2011. Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium* wilt of chickpea. Crop Protection 30, 1070-1078.

Gupta, R., Garg, V.K., 2009. Vermiremediation and nutrient recovery of non-recyclable paper waste employing *Eisenia fetida*. Journal of Hazardous Materials 162, 430-439.

Gutiérrez-Miceli, F.A., Santiago-Borraz, J., Montes Molina, J.A., Nafate, C.C., bud-Archila, M., Oliva Llaven, M.A., Rincón-Rosales, R., Dendooven, L., 2007. Vermicompost as a soil supplement to improve growth, yield and fruit quality of tomato (*Lycopersicum esculentum*). Bioresource Technology 98, 2781-2786.

Hait, S., Tare, V., 2011. Vermistabilization of primary sewage sludge. Bioresource Technology 102, 2812-2820.

Hamilton, D.W., Murie, M.E., Khan, A., Ndegwa, P.M., 2008. Vermicomposting of poultry litter: Process optimization. In: American Society of Agricultural and Biological Engineers Annual International Meeting 2008, 11, 6672-6679.

Haruta, S., Kondo, M., Nakamura, K., Aiba, H., Ueno, S., Ishii, M., Igarashi, Y., 2002. Microbial community changes during organic solid waste treatment analyzed by double gradient-denaturing gradient gel electrophoresis and fluorescence in situ hybridization. Applied Microbiology and Biotechnology 60, 224-231.

Head, I.M., Saunders, J.R., Pickup, R.W., 1998. Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial Ecology 35, 1-21.

Hedman, B., Näslund, M., Marklund, S., 2006. Emission of PCDD/F, PCB, and HCB from combustion of firewood and pellets in residential stoves and boilers. Environmental Science & Technology 40, 4968-4975.

Hernández, D., Mansenét, V., Puiggrós Jové, J.M. 1999. Use of Confidor 200 SL in vegetable cultivation in Spain. Pflanzenschutz-Nachrichten Bayer 52, 364-375.

Hiraishi, A., 1999. Isoprenoid quinones as biomarkers of microbial populations in the environment. Journal of Bioscience and Bioengineering 88, 449-460.

Hiraishi, A., 2002. Vermicomposting of household biowaste using *Eisenia fetida*: Evaluation of waste reduction efficiency and coexistent microbial community structure. Seibutsu-Kogaku Kaishi 80, 283-290.

Hobson, A.M., Frederickson, J., Dise, N.B., 2005. CH_4 and N_2O from mechanically turned windrow and vermicomposting systems following in-vessel pre-treatment. Waste Management 25, 345-352.

Hu, H.Y., Fujie, K., Urano, K., 1994. Effect of temperature on the reaction rate of bacteria inhabiting the aerobic microbial film for wastewater treatment. Journal of Fermentation and Bioengineering 78, 100-104.

Huang, D.L., Zeng, G.M., Feng, C.L., Hu, S., Lai, C., Zhao, M.H., Su, F.F., Tang, L., Liu, H.L., 2010. Changes of microbial population structure related to lignin degradation during lignocellulosic waste composting. Bioresource Technology 101, 4062-4067.

Hussain, S., Arshad, M., Saleem, M., Khalid, A., 2007. Biodegradation of α- and β-endosulfan by soil bacteria. Biodegradation 18, 731-740.

Jiang, W., Qu, D., Mu, D., Wang, L., 2003. Protected cultivation of horticultural crops in china, in: Janick, J.(Ed.), Horticultural Reviews. John Wiley & Sons, Inc.115-162.

Kale, R.D., Bano, K., Krisgnamoorthy, R.V., 1982. Potential of *Perionix excavatus* for utilizing organic wastes. Pedobiologia 23, 419-425.

Kale, R.D., Mallesh, B.C., Kubra, B., Bagyaraj, D.J., 1992. Influence of vermicompost application on the available macronutrients and selected microbial populations in a paddy field. Soil Biology and Biochemistry 24, 1317-1320.

Kaushik, P., Garg, V.K., 2003. Vermicomposting of mixed solid textile mill sludge and cow dung with the epigeic earthworm *Eisenia foetida*. Bioresource Technology 90, 311-316.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T., 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods 58, 169-188.

Knapp, B.A., Podmirseg, S.M., Seeber, J., Meyer, E., Insam, H., 2009. Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. Soil Biology and Biochemistry 41, 2299-2307.

Krause, L., Diaz, N.N., Edwards, R.A., Gartemann, K.H., Krömeke, H., Neuweger, H., Pühler, A., Runte, K.J., Schlüter, A., Stoye, J., Szczepanowski, R., Tauch, A., Goesmann, A., 2008. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. Journal of Biotechnology 136, 91-101.

Lazcano, C., Gómez-Brandón, M., Domínguez, J., 2008. Comparison of the effectiveness of composting and vermicomposting for the biological stabilization of cattle manure. Chemosphere 72, 1013-1019.

Lee, D.H., Zo, Y.G., Kim, S.J., 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Applied and Environmental Microbiology 62, 3112-3120.

Li, X., Xing, M., Yang, J., Huang, Z., 2011. Compositional and functional features of humic acid-like fractions from vermicomposting of sewage sludge and cow dung. Journal of Hazardous Materials 185, 740-748.

Loisel, P., Harmand, J., Zemb, O., Latrille, E., Lobry, C., Delgenes, J.P., Godon, J.J., 2006. Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environmental Microbiology 8, 720-731.

Lores, M., Gómez-Brandón, M., Pérez-Díaz, D., Domínguez, J., 2006. Using FAME profiles for the characterization of animal wastes and vermicomposts. Soil Biology and Biochemistry 38, 2993-2996.

Ma, C., Wang, Y., Zhuang, L., Huang, D., Zhou, S., Li, F., 2011. Anaerobic degradation of phenanthrene by a newly isolated humus-reducing bacterium, *Pseudomonas aeruginosa strain* PAH-1, Journal of Soils and Sediments 11, 923-929

Malato, S., Blanco, J., Cáceres, J., Fernández-Alba, A. R., Agüera, A., Rodríguez, A., 2002. Photocatalytic treatment of water-soluble pesticides by photo-Fenton and TiO2 using solar energy. Catalysis Today 76, 209-220.

Malghani, S., Chatterjee, N., Hu, X., Zejiao, L., 2009. Isolation and characterization of a profenofos degrading bacterium. Journal of Environmental Sciences 21, 1591-1597.

Manivannan, S., Balamurugan, M., Parthasarathi, K., Gunasekaran, G., Ranganathan, L.S., 2009. Effect of vermicompost on soil fertility and crop productivity-beans (*Phaseolus vulgaris*). Journal of Environmental Biology 30, 275-281.

Manna, M.C., Singh, M., Kundu, S., Tripathi, A.K., Takkar, P.N., 1997. Growth and reproduction of the vermicomposting earthworm *Perionyx excavatus* as influenced by food materials. Biology and Fertility of Soils 24, 129-132.

Manzano-Agugliaro, F., 2007. Gasificación de residuos de invernadero para la obtención de energía eléctrica en el sur de España: Ubicación mediante SIG. Interciencia 32, 131-136.

Marschner, P., 2007. Soil microbial community structure and function assessed by FAME, PLFA and DGGE-Advantages and Limitations, in: Varma, A., Oelmüller, R. (Eds.), Advanced Techniques in Soil Microbiology. Springer, Berlin, pp. 181-200.

Marsh, L., Subler, S., Mishra, S., Marini, M., 2005. Suitability of aquaculture effluent solids mixed with cardboard as a feedstock for vermicomposting. Bioresource Technology 96, 413-418.

Martín-Gil, J., Navas-Gracia, L.M., Gómez-Sobrino, E., Correa-Guimaraes, A., Hernández-Navarro, S., Sánchez-Báscones, M., del Carmen Ramos-Sánchez, M., 2008. Composting and vermicomposting experiences in the treatment and bioconversion of asphaltens from the *Prestige* oil spill. Bioresource Technology 99, 1821-1829.

Martínez, F., Calero, B.J., Nogales, R., Rovesti, L., 2003. Lombricultura. Manual práctico. Unidad de producciones gráficas MINREX, La Habana.

Melgar, R., Benítez, E., Nogales, R., 2009. Bioconversion of wastes from olive oil industries by vermicomposting process using the epigeic earthworm *Eisenia andrei*. Journal of Environmental Science and Health Part B 44, 488-495.

Ministerio de medio ambiente y medio rural y marino, 2010. Encuesta sobre Superficies y Rendimientos de Cultivos. Gobierno de España, Madrid.

Molin, S., Givskov, M., 1999. Application of molecular tools for in situ monitoring of bacterial growth activity. Environmental Microbiology 1, 383-391.

Mondini, C., Fornasier, F., Sinicco, T., 2004. Enzymatic activity as a parameter for the characterization of the composting process. Soil Biology and Biochemistry 36, 1587-1594.

Monroy, F., Aira, M., Domínguez, J., 2009. Reduction of total coliform numbers during vermicomposting is caused by short-term direct effects of earthworms on microorganisms and depends on the dose of application of pig slurry. Science of the Total Environment 407, 5411-5416.

Moon, K.E., Lee, S.Y., Lee, S.H., Ryu, H.W., Cho, K.S., 2010. Earthworm cast as a promising filter bed material and its methanotrophic contribution to methane removal. Journal of Hazardous Materials 176, 131-138.

Moore-Kucera, J., Dick, R.P., 2008. PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. Microbial Ecology 55, 500-511.

Moreno, B., Vivas, A., Nogales, R., Benítez, E., 2009a. Solvent tolerance acquired by Brevibacillus brevis during an olive-waste vermicomposting process. Ecotoxicology and Environmental Safety 72, 2109-2114.

Moreno, B., Vivas, A., Nogales, R., Macci, C., Masciandaro, G., Benítez, E., 2009b. Restoring biochemical activity and bacterial diversity in a trichloroethylene-contaminated soil: The reclamation effect of vermicomposted olive wastes. Environmental Science and Pollution Research 16, 253-264.

Moreno, B., Nogales, R., Macci, C., Masciandaro, G., Benitez, E., 2011. Microbial eco-physiological profiles to estimate the biological restoration of a trichloroethylene-contaminated soil. Ecological Indicators 11, 1563-1571.

Mukherjee, K., Tribedi, P., Chowdhury, A., Ray, T., Joardar, A., Giri, S., Sil, A.K., 2011. Isolation of a *Pseudomonas aeruginosa* strain from soil that can degrade polyurethane diol. Biodegradation 22, 377-388.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695-700.

Narihiro, T., Yamanaka, Y., Hiraishi, A., 2003. High culturability of bacteria in commercially available personal composters for fed-batch treatment of household biowaste. Microbes and Environments 18, 94-99.

Ndegwa, P.M., Thompson, S.A., 2000. Effects of C-to-N ratio on vermicomposting of biosolids. Bioresource Technology 75, 7-12.

Nogales, R., Benítez, E., Elvira, C., Gallardo-Lara, F., 1995. Uso agrícola de compost y vermicompot de basuras urbanas (I): Procesos, madurez y calidad de los productos. Residuos 26, 53-57.

Nogales, R., Cifuentes, C., Benítez, E., 2005. Vermicomposting of winery wastes: A laboratory study. J. Environ. Sci. Health. 40, 659-673.

Nogales, R., Domínguez, J., Mato, S., 2008. Vermicompostaje, in: Moreno, J., Moral, R. (Eds.), Compostaje. Ediciones Mundi Prensa, Madrid, pp. 187-208.

Nogales, R., Elvira, C., Benítez, E., Thompson, R., Gómez, M., 1999. Feasibility of vermicomposting dairy biosolids using a modified system to avoid earthworm mortality. Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes 34, 151-169.

Nogales, R., Thompson, R., Calmet, A., Benítez, E., Gómez, M., Elvira, C., 1998. Feasibility of vermicomposting residues from olive oil production obtained using two stage centrifugation. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 33, 1491-1506.

Onbasili, D., Aslim, B., 2011. Biodegradation of 2,4-Dichlorophenoxy acetic acid (2,4-D) herbicide by *Pseudomonas* spp. strains. Fresenius Environmental Bulletin 20, 1438-1446.

Orozco, F.H., Cegarra, J., Trujillo, L.M., Roig, A., 1996. Vermicomposting of coffee pulp using the earthworm *Eisenia fetida*: Effects on C and N contents and the availability of nutrients. Biology and Fertility of Soils 22, 162-166.

Pardossi, A., Tognoni, F., Incrocci, L., 2004. Mediterranean Greenhouse Technology. Chron. Hort. 44, 28-34.

Parra, S., Aguilar, F.J., Calatrava, J., 2008. Decision modelling for environmental protection: The contingent valuation method applied to greenhouse waste management. Biosystems Engineering 99, 469-477.

Parra, S., Pérez, J.J., Calatrava, J., 2001. Vegetal waste from protected horticulture in southeastern Spain: characterisation of environmental externalities. In: Proceedings of

the fifth International Symposium on Protected Cultivation in Mild Winter Climates: Current Trends for Sustainable Technologies., Cartagena, Spain, Acta Horticulturae, 559, 787-792.

Parthasarathi, K., 2007. Life cycle of *Lampito mauritii* (Kinberg) in comparison with *Eudrilus eugeniae* (Kinberg) cultured on different substrates. Journal of Environmental Biology 28, 803-812.

Parthasarathi, K., Ranganathan, L.S., 1999. Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. European Journal of Soil Biology 35, 107-113.

Parthasarathi, K., Ranganathan, L.S., 1998. Pressmud Vermicast are 'hot spots' of fungi and bacteria. Ecology, Environment and Conservation 4, 81-86.

Parthasarathi, K., Ranganathan, L.S., 2000. Aging effect on enzyme activities in pressmud vermicasts of *Lampito mauritii* (Kinberg) and *Eudrilus eugeniae* (Kinberg). Biol. Fertl. Soils 30, 347-350.

Pascual, J.A., Moreno, J.L., Hernández, T., García, C., 2002. Persistence of immobilised and total urease and phosphatase activities in a soil amended with organic wastes. Bioresource Technology 82, 73-78.

Pizl, V., Novakova, A., 2003. Interactions between microfungi and *Eisenia andrei* (Oligochaeta) during cattle manure vermicomposting. Pedobiologia 47, 895-899.

Pramanik, P., Ghosh, G.K., Ghosal, P.K., Banik, P., 2007. Changes in organic - C, N, P and K and enzyme activities in vermicompost of biodegradable organic wastes under liming and microbial inoculants. Bioresource Technology 98, 2485-2494.

Rajesh Banu, J., Esakkiraj, S., Nagendran, R., Logakanthi, S., 2005. Biomanagement of petrochemical sludge using an exotic earthworm *Eudrilus eugineae*. Journal of Environmental Biology 26, 43-47.

Ranjard, L., Poly, F., Nazaret, S., 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Research in Microbiology 151, 167-177.

Reddy, M.V., Ohkura, K., 2004. Vermicomposting of rice-straw and its effects on sorghum growth. Tropical Ecology 45, 327-331.

Riggle, D., Holmes, H., 1994. New horizons for commercial vermiculture. BioCycle 35, 58-62.

Ritchie, N.J., Schutter, M.E., Dick, R.P., Myrold, D.D., 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. Applied and Environmental Microbiology 66, 1668-1675.

Rodríguez-Canché, L.G., Cardoso Vigueros, L., Maldonado-Montiel, T., Martínez-Sanmiguel, M., 2010. Pathogen reduction in septic tank sludge through vermicomposting using *Eisenia fetida*. Bioresource Technology 101, 3548-3553.

Romero, E., Plaza, C., Senesi, N., Nogales, R., Polo, A., 2007. Humic acid-like fractions in raw and vermicomposted winery and distillery wastes. Geoderma 139, 397-406.

Saha, S., Mina, B.L., Gopinath, K.A., Kundu, S., Gupta, H.S., 2008. Relative changes in phosphatase activities as influenced by source and application rate of organic composts in field crops. Bioresource Technology 99, 1750-1757.

Sangwan, P., Kaushik, C.P., Garg, V.K., 2008. Vermiconversion of industrial sludge for recycling the nutrients. Bioresource Technology 99, 8699-8704.

Schloss, P.D., Hay, A.G., Wilson, D.B., Walker, L.P., 2003. Tracking temporal changes of bacterial community fingerprints during the initial stages of composting. FEMS Microbiology Ecology 46, 1-9.

Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K.J., Szczepanowski, R., Tauch, A., Tilker, A., Viehöver, P., Goesmann, A., 2008. The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. Journal of Biotechnology 136, 77-90.

Schnürer, J., Rosswall, T., 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Applied and Environmental Microbiology 43, 1256-1261.

Schutter, M.E., Dick, R.P., 2000. Comparison of Fatty Acid Methyl Ester (FAME) Methods for Characterizing Microbial Communities. Soil Science Society of America Journal 64, 1659-1668.

Schwieger, F., Tebbe, C.C., 1998. A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Applied and Environmental Microbiology 64, 4870-4876.

Schwinn, F., Staub, T., Urech, P.A., 1977. A new type of fungicide against diseasescaused by Oomycetes. Meded Fac Landbouwwet Rijksuniv Gent 42, 1181–1188

Sekiguchi, H., Tomioka, N., Nakahara, T., Uchiyama, H., 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnology Letters 23, 1205-1208.

Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresource Technology 100, 804-811.

Sen, B., Hamelin, J., Bru-Adan, V., Godon, J.J., Chandra, T.S., 2008. Structural divergence of bacterial communities from functionally similar laboratory-scale vermicomposts assessed by PCR-CE-SSCP. Journal of Applied Microbiology 105, 2123-2132.

Senesi, N., 1989. Composted materials as organic fertilizers. Science of the Total Environment 81-82, 521-542.

Sharma, S., Aneja, M.K., Mayer, J., Munch, J.C., Schloter, M., 2005. Diversity of transcripts of nitrite reductase genes (nirK and nirS) in rhizospheres of grain legumes. Applied and Environmental Microbiology 71, 2001-2007.

Sigfito Agroenvases, S.L., 2009. Sistema integrado de gestión de envases fitosanitarios. http://www.sigfito.es,

Singh, U.S., Tripathi, R.K., 1982. Physicochemical and biological properties of metalaxyl. Indian Journal Mycology Plant Patholology 12, 287-294

Singleton, D.R., Hendrix, P.F., Coleman, D.C., Whitman, W.B., 2003. Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). Soil Biology and Biochemistry 35, 1547-1555.

Sinha, J., Biswas, C.K., Ghosh, A., Saha, A., 2010a. Efficacy of vermicompost against fertilizers on *Cicer* and *Pisum* and on population diversity of N_2 fixing bacteria. Journal of Environmental Biology 31, 287-292.

Sinha, R.K., Agarwal, S., Chauhan, K., Chandran, V., Soni, B.K., 2010b. Vermiculture technology: Reviving the dreams of Sir Charles Darwin for scientific use of earthworms in sustainable development programs. Technology and Investment 1, 155-172.

Soil Survey Staff, 2010. Keys to soil taxonomy. Eleventh edition. 338 p.United States Department of Agriculture Agriculture Handbook. Natural Resources Conservation Service. Washungton, USA

Song, X.Z., Zhao, C.X., Wang, X.L., Li, J., 2009. Study of nitrate leaching and nitrogen fate under intensive vegetable production pattern in northern China. Comptes Rendus Biologies 332, 385-392.

Srivastava, R., Kumar, D., Gupta, S.K., 2005. Bioremediation of municipal sludge by vermitechnology and toxicity assessment by *Allium cepa*. Bioresource Technology 96, 1867-1871.

Stanghellini, C., Kempkes, F.L.K., Knies, P., 2003. Enhancing environmental quality in agriculture systems. In: Proceedings of the International Symposium on Managing Greenhouse Crops in Saline Environment, Pisa, Italy, Acta Horticulturae, 609, 277-283.

Suárez-Estrella, F., Vargas-García, M.C., López, M.J., Moreno, J., 2004. Survival of *Fusarium oxysporum* f.sp. *melonis* on plant waste. Crop Protection 23, 127-133.

Sukul, P., Spiteller, M. J., 2000. Metalaxyl: persistence, degradation, metabolism, an analytical methods. Revue Environmental Contamination of Toxicology 164, 1-26.

Sukul, P., Spiteller, M. J., 2001. Persistence, Fate, and Metabolism of [14C]Metalaxyin Typical Indian Soils Agric. Food Chemistry 49, 2352-2358.

Suthar, S., 2007. Influence of different food sources on growth and reproduction performance of composting epigeics: *Eudrilus eugeniae*, *Perionyx excavatus* and *Perionyx sansibaricus*. Applied Ecology and Environmental Research 5, 79-92.

Suthar, S., 2008a. Bioconversion of post harvest crop residues and cattle shed manure into value-added products using earthworm *Eudrilus eugeniae* Kinberg. Ecological Engineering 32, 206-214.

Suthar, S., 2008b. Bioremediation of aerobically treated distillery sludge mixed with cow dung by using an epigeic earthworm *Eisenia fetida*. Environmentalist 28, 76-84.

Suthar, S., Singh, S., 2008b. Vermicomposting of domestic waste by using two epigeic earthworms (*Perionyx excavatus* and *Perionyx sansibaricus*). International Journal of Environmental Science and Technology 5, 99-106.

Suthar, S., Singh, S., 2009. Bioconcentrations of metals (Fe, Cu, Zn, Pb) in earthworms (eisenia fetida), inoculated in municipal sewage sludge: Do earthworms pose a possible risk of terrestrial food chain contamination? Environmental Toxicology 24, 25-32.

Suthar, S., Singh, S., 2008a. Feasibility of vermicomposting in biostabilization of sludge from a distillery industry. Science of the Total Environment 394, 237-243.

Suzuki, M., Rappe, M.S., Giovannoni, S.J., 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Applied and Environmental Microbiology 64, 4522-4529.

Szczech, M., Smolinska, U., 2001. Comparison of suppressiveness of vermicomposts produced from animal manures and sewage sludge against *Phytophthora nicotianae* Breda de Haan var. *nicotianae*. Journal of Phytopathology 149, 77-82.

Szczech, M.M., 1999. Suppressiveness of vermicompost against Fusarium wilt of tomato. Journal of Phytopathology 147, 155-161.

Tang, J.C., Kanamori, T., Inoue, Y., Yasuta, T., Yoshida, S., Katayama, A., 2004. Changes in the microbial community structure during thermophilic composting of manure as detected by the quinone profile method. Process Biochemistry 39, 1999-2006.

Tang, J.C., Maie, N., Tada, Y., Katayama, A., 2006. Characterization of the maturing process of cattle manure compost. Process Biochemistry 41, 380-389.

Tiquia, S.M., 2005. Microbial community dynamics in manure composts based on 16S and 18S rDNA T-RFLP profiles. Environmental Technology 26, 1101-1114.

Tolón, A., Lastra, X., 2010. La agricultura intensiva del poniente almeriense: Diagnóstico e instrumentos de gestión ambiental. Revista Electrónica De Medio Ambiente 8, 18-40.

Tomati, U., Grappelli, A., Galli, E., 1988. The hormone-like effect of earthworm casts on plant growth. Biol. Fertl. Soils 5, 288-294.

Tomlin, C. D. S., 2003. The Pesticide Manual. 12nd. British Crop Protection Council. UK. 1300 pp.

Torsvik, V., Goksoyr, J., Daae, F.L., 1990. High diversity in DNA of soil bacteria. Applied and Environmental Microbiology 56, 782-787.

Tourova, T.P., 2003. Copy number of ribosomal operons in prokaryotes and its effect on phylogenetic analyses. Microbiology 72, 389-402.

Tripathi, G., Bhardwaj, P., 2004. Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg). Bioresource Technology 92, 275-283.

Urech, P. A., Schwinn, F., Staub, T., 1977. CGA 48988, a novel fungicide for the control of late blight, downy mildew and related soil borne diseases, En Proceedings of the British Crop Protection Conference, Brighton, Vol 2, pp. 623. Boots, Nottingham,.

Urrestarazu, M., Mazuela, P.C., Boukhalfa, A., Arán, A., Salas, M.D.C., 2005. Oxygen content and its diurnal variation in a new recirculanting water soilless culture for horticultural crops. HortScience 40, 1729-1730.

Vasanthi, D., Kumaraswamy, K., 1999. Efficacy of vermicomposts to improve soil fertility and rice yield. Journal of the Indian Society of Soil Science 47, 268-272.

Vaz-Moreira, I., Silva, M.E., Manaia, C.M., Nunes, O.C., 2008. Diversity of bacterial isolates from commercial and homemade composts. Microbial Ecology 55, 714-722.

Verkhovtseva, N.V., Osipov, G.A., Bolysheva, T.N., Kasatinov, V.A., Kuzmina, N.V., Antsiferova, E.J., Alexeeva, A.S., 2002. Comparative investigation of vermicompost microbial communities, in: Insam, H., Riddech, N., Klammer, S.H. (Eds.), Microbiology of composting. Springer, Berlin, pp. 99-110.

Vivas, A., Moreno, B., Del Val, C., Macci, C., Masciandaro, G., Benítez, E., 2008. Metabolic and bacterial diversity in soils historically contaminated by heavy metals and hydrocarbons. Journal of Environmental Monitoring 10, 1287-1296.

Vivas, A., Moreno, B., García-Rodríguez, S., Benítez, E., 2009. Assessing the impact of composting and vermicomposting on bacterial community size and structure, and microbial functional diversity of an olive-mill waste. Bioresource Technology 100, 1319-1326.

von Mersi, W., Schinner, F., 1991. An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol. Fert. Soils 11, 216-220.

Warman, P.R., AngLopez, M.J., 2010. Vermicompost derived from different feedstocks as a plant growth medium. Bioresource Technology 101, 4479-4483.

WHO, 2004. The WHO Recommended Classification of Pesticides by Hazard. World Health Organization. Geneva. 56 pp.

Wong, M.L., Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. BioTechniques 39, 75-85.

Yakushev, A., Byzov, B., 2009. Hydrolase activity as an indicator of the state of microbial communities in vermicomposts. Moscow University Soil Science Bulletin 64, 93-98.

Yang, S., Li, J., Zheng, Z., Zhang, Y., Zhang, B., 2009. Vermicompost as an accelerator of methane production. International Journal of Environmental Engineering 1, 52-63.

Yardim, E.N., Arancon, N.Q., Edwards, C.A., Oliver, T.J., Byrne, R.J., 2006. Suppression of tomato hornworm (*Manduca quinquemaculata*) and cucumber beetles (*Acalymma vittatum* and *Diabotrica undecimpunctata*) populations and damage by vermicomposts. Pedobiologia 50, 23-29.

Yasir, M., Aslam, Z., Kim, S.W., Lee, S.W., Jeon, C.O., Chung, Y.R., 2009. Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity. Bioresource Technology 100, 4396-4403.

Yasir, M., Aslam, Z., Song, G.C., Bibi, F., Jeon, C.O., Chung, Y.R., 2010. *Chitinophaga vermicomposti* sp. nov., with antifungal activity, isolated from vermicompost. International Journal of Systematic and Evolutionary Microbiology 60, 268-.

Yasmin, S., D'Souza, D., 2010. Effects of pesticides on the growth and reproduction of earthworm: a review. Applied and Environmental Soil Science 2010, 1-9.

Zaller, J.G., 2007. Vermicompost as a substitute for peat in potting media: Effects on germination, biomass allocation, yields and fruit quality of three tomato varieties. Scientia Horticulturae 112, 191-199.

Zambare, V.P., Nilegaonkar, S.S., Kanekar, P.P., 2011a. Use of agro-residues for protease production and application in degelatinization of waste photographic films. Research Journal of Biotechnology 6, 62-65.

Zambare, V.P., Nilegaonkar, S.S., Kanekar, P.P., 2011b. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. New Biotechnology 28, 173-181.

Zang, Y., Zhong, Y., Luo, Y., y Kong, Z. M., 2000. Genotoxicity of two novel pesticides for the earthworm. Eisenia fetida. Environmental Pollution 108, 271-278.

Zdruli, P., Jones, R.J.A., Montanarella, L., 2004. Organic matter in the soils of southern Europe, in: European Soil Bureau Research Report, EUR 21083

EN. Office for Official Publications of the European Communities, Luxembourg, pp. 1-17.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A review. Biology and Fertility of Soils 29, 111-129.

Zhang, Z., Hou, Z., Yang, C., Ma, C., Tao, F., Xu, P., 2011. Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. Bioresource Technology 102, 4111-4116.

ANEXO

ARTÍCULOS CIENTÍFICOS PUBLICADOS

Bioresource Technology 101 (2010) 9654–9660

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Feasibility of vermicomposting for vegetable greenhouse waste recycling

Manuel J. Fernández-Gómez *, Esperanza Romero, Rogelio Nogales

Department of Environmental Protection, Estación Experimental del Zaidín (EEZ), CSIC, Profesor Albareda 1, 18008 Granada, Spain

ARTICLE INFO

Article history:
Received 28 April 2010
Received in revised form 26 July 2010
Accepted 27 July 2010
Available online 1 August 2010

Keywords:
Eisenia andrei
Enzyme activity
Phytotoxicity test
Genetic fingerprinting
PCR-DGGE

ABSTRACT

This study was conducted in order to evaluate the feasibility of *Eisenia andrei* for vermicomposting heterogeneous-plant (HP), tomato-plant (P), and damaged tomato-fruit (T) greenhouse vegetable wastes. Earthworm growth and reproduction were monitored over a 12-week period, and variations in chemical parameters, enzyme activity, phytotoxicity test, and genetic fingerprinting of bacterial communities were evaluated. While high rates of salinity prevented earthworm survival in HP and P (>10 dS m^{-1}), T was vermicomposted recording an adequate earthworm growth and cocoon production. The latter waste was successfully stabilized, as indicated by the significant decrease in its TOC content (~13–26%) and C:N ratio (~16–36%) and its high germination indices (~39–72%). The similar enzyme activities levels and bacterial community fingerprintings recorded in diverse vermicomposts obtained from T waste indicate that this type of waste favoured the existence of analogous bacterial communities responsible for the high degree of stabilization and maturity detected.

1. Introduction

The use of greenhouses is expanding worldwide, currently covering over 1.5 million hectares (Espi et al., 2006), as these crop systems improve food production and protect crops from adverse meteorological conditions and pests. The Mediterranean basin has the largest area occupied by greenhouses in Europe approximately 200,000 (Pardossi et al., 2004). Mediterranean greenhouse systems enable high-value vegetables to be produced from autumn through spring (Castilla and Hernández, 2005). However, these intensive agricultural systems generate large amounts of waste, creating an unsustainable environment that adversely affects ecological integrity and human health due to they cause agricultural pest, riverbeds pollution and uncontrolled burning, among other things (Parra et al., 2008). Vegetable waste, which can be classified into plant residues and rejected and/or damaged fruit waste, accounts for most of these residues. Plant wastes consist of stems, twigs and roots, which are discarded after the harvesting. Fruit waste consists of unmarketable fruit due to bruising, inappropriate size, rotting, or insect attack, as well as marketable fruits that are discarded in order to control market prices, accounting for around 25% of total greenhouse vegetable waste. Plant and fruit wastes are often placed in special containers and air-dried in order to reduce volumes.

The enormous quantities of biomass from greenhouse vegetable waste need to be recycled into nutrient-enriched organic products, which could be used for agricultural and land restoration purposes, as the low organic content (⩽2%) of 75% of southern European topsoil (Zdruli et al., 2004) is a major problem that could be solved by adding organic amendments to the degraded soils. Low-cost and environmentally appropriate technologies, such as composting or vermicomposting, should therefore be favoured over other disposal methods. Nevertheless, due to the agricultural management procedures used in these intensive agricultural systems based on chemical fertilizers and high-yield crops, greenhouse vegetable wastes are characterized by specific features that hinder the use of these biological technologies. Unlike crop waste from conventional farming, greenhouse vegetable wastes are hardly ever bioconverted due to its low C/N, high salinity and ammonia content and may also contain large amounts of pollutants such as pesticides. Compared with conventional crop wastes, very little study has been carried out on the biostabilization of greenhouse vegetable waste.

Vermicomposting biotechnologies involve the bio-oxidation and stabilization of organic matter through the joint action of earthworms and microorganisms under aerobic and mesophilic conditions. Vermicomposting has greater mass-reduction capacity than composting over a shorter processing time and generates products with higher humus content and significantly lower phytotoxicity (Lorimor et al., 2001). Vermicompost is also more marketable than compost due to its more attractive appearance and higher nutrient content and microbial activity (Nogales et al., 2008). It is well known that vermicomposting is effective for managing of crop wastes from conventional farming systems (Bansal and Kapoor, 2000; Suthar, 2008) through various epigeic earthworms such as *Eisenia fetida*, *Eisenia andrei*, *Perionyx excavatus*, and *Eudrilus eugeniae*. However, an extensive survey of the literature has led us to conclude that no study of greenhouse vegetable

* Corresponding author. Tel.: +34 958 181600/125; fax: +34 958 129600.
E-mail address: manuelj.fernandez@eez.csic.es (M.J. Fernández-Gómez).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.07.109

waste vermicomposting has been carried out. The *E. fetida* and *E. andrei* earthworm species, due to their high tolerance in relation to environmental variables such as pH, moisture, and temperature (Nogales et al., 2008), could represent the best option in developing a vermicomposting system for recycling organic waste in temperate climates.

The study of enzyme activity has proved helpful for assessing the stability of vermicomposted end products (Benítez et al., 1999). A decrease in dehydrogenase and overall hydrolytic activity has thus been associated with the stabilization of organic matter during the vermicomposting process. Molecular tools based on PCR amplification of the 16S rRNA gene, such as clone libraries, fluorescence in situ hybridization (FISH), restriction fragment length polymorphism (T-RFLP), Q-PCR, and microarrays, have been widely used to study bacterial communities investigating different aspects i.e. identification and/or in-situ detection of certain species, species richness, bacterial population size and genetic community structure. Among all DNA-based methods, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA is a useful tool for characterizing the genetic structure of bacterial communities in composts, as this technique can be used without subsequent sequencing and provides the genetic fingerprinting characteristics of each bacterial community. Recently, Sen and Chandra (2009) have reported that this fingerprinting method is essential for improving composting and vermicomposting technology. They showed that DGGE is capable of evaluating the relationship between changes in physico-chemical and microbial community structures occurring during the vermicomposting process due to the presence of earthworms.

In view of the above, the principal objective of this study was to assess the feasibility of using *E. andrei* to vermicompost the enormous quantities of heterogeneous-plant, tomato-plant, and damaged tomato-fruit vegetable waste produced by Mediterranean greenhouse crops. Chemical parameters, enzyme activity, and germination indices were determined in order to evaluate chemical and biochemical changes caused by vermicomposting. DGGE of PCR-amplified 16S rDNA was carried out to genetically fingerprint bacterial communities in greenhouse vegetable waste and the resulting vermicompost in order to show the impact of earthworms on the bacterial genetic structure of mature vermicompost.

2. Methods

2.1. Earthworms and organic-waste collection

Non-clitellated earthworms (*E. andrei*) were selected from a culture bank at the Estación Experimental del Zaidín (CSIC), Granada, Spain.

Heterogeneous-plant (HP), tomato-plant (P), and damaged tomato-fruit (T) wastes, three of the most abundant greenhouse vegetable residues in Andalusia (Southern Spain), where the study was carried out, were tested. HP waste is made up of a mixture of stems, twigs, and roots from different types of greenhouse crops; P waste, consisting of stems, twigs, and roots, comes from greenhouse tomato crops; T waste consists of unsold damaged tomatoes which are unloaded into ponds after harvesting. All these waste were collected from an organic waste-treatment plant located in El Egido, Almería (Spain) where they were being accumulated and air dried. The chemical characteristics of these greenhouse wastes, analyzed as described below, are shown in Table 1.

2.2. Experimental design

The wastes were over-dried at 25 °C and chopped. For the purposes of optimum vermicomposting, they were mixed with cow dung (D) or straw (S) to boost their low C:N ratios to >20. The greenhouse wastes selected were therefore assayed on their own (HP, P, T) and also mixed with either cow dung (HP/D, P/D, T/D) or straw (HP/S, P/S, T/S) in 2:1 and 4:1 ratios, meaning that a total of 15 waste mixtures were tested for vermicomposting. Cow dung, regarded as an excellent material for *E. andrei* development (Nogales et al., 2008), was assayed on its own as control.

Table 1
Chemical composition of the organic waste used.

Organic wastes[a]	TOC ($g\ kg^{-1}$)	TKN ($g\ kg^{-1}$)	C:N ratio	pH	EC ($dS\ m^{-1}$)
HP	151 ± 2.4	22 ± 0.9	7 ± 0.2	8.5 ± 0.02	17.0 ± 0.10
P	315 ± 4.1	35 ± 0.8	9 ± 0.1	7.6 ± 0.02	12.0 ± 0.01
T	460 ± 1.5	23 ± 1.2	20 ± 0.9	8.3 ± 0.02	5.0 ± 0.03
S	571 ± 3.2	1.9 ± 0.1	294 ± 9.0	7.3 ± 0.01	2.4 ± 0.01
D	401 ± 2.0	15 ± 0.5	27 ± 1.0	9.3 ± 0.02	9.2 ± 0.02

TOC, total organic carbon; TKN, total Kjeldahl nitrogen; EC, electrical conductivity.
[a] HP, heterogenic-plant wastes; P, tomato-plant waste; T, tomato-fruit waste; D, cow dung; S, wheat straw.

Fifty grams (dw) samples of each material were placed in triplicate in 500 ml glass pots and aerated for a week to eliminate substances that are toxic to the earthworms, as reported by Elvira et al. (1996). Moisture content was adjusted to 80–85% and five non-clitellated earthworms weighing between 0.17 and 0.31 g were inoculated. Samples were kept in darkness at 24 °C for 12 weeks maintaining the constant moisture conditions by periodical watering.

Mortality rates, earthworm biomass, and cocoon population were evaluated weekly by hand. At the end of the process, the earthworms were removed. Samples from both the initial materials and resulting vermicompost were homogenized and divided into two subsamples, one of which was dried and finely ground for chemical analysis and germination testing, while the other was stored at −20 °C for enzyme activity and DNA analysis.

2.3. Chemical analysis

The pH and electrical conductivity (EC) were measured in a 1:10 sample:water (w/v) ratio. Total organic carbon (TOC) and total Kjeldhal nitrogen (TKN) were determined using the dichromate oxidation and Kjeldahl methods, respectively (M.A.P.A., 1986). Water-soluble carbon (WSC) was extracted at 60 °C for 1 h with distilled water (1:10 sample:water w/v) and determined using the dichromate oxidation method. Total P was measured using the nitrovanadomolybdate method, total K and Na using photometry, and total Ca, Mg and micronutrients (Fe, Mn, Cu, and Zn) using atomic-absorption spectrometry after a digestion of the samples with HNO_3:$HClO_4$ (Williams, 1984).

2.4. Enzyme activity analysis

Total enzyme activities were determined in triplicate, with each reaction tube containing 0.2 g sample. Dehydrogenase activity was determined according to the von Mersi and Schinner (1991) method modified by García et al. (1997). The urease, acid-phosphatase, and β-glucosidase hydrolytic enzymes were determinated according to the methods reported by Nannipieri et al. (1980) and Tabatabai (1982).

2.5. Germination index

To assess the maturity and phytotoxicity levels of the initial materials and final vermicomposts, the germination index (GI) was calculated according to the technique describe by Zucconi

et al. (1981), which involve incubating cress seeds (*Lepidium sativum*) with 1:10 sample:water (w/v) extracts in darkness at 25 °C for 24 h.

2.6. DNA extraction and bacterial community structure analysis

Total DNA was extracted from 250 mg of the sample using the MoBio UltraClean Soil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol, although the second step (inhibitor removal solution) was repeated.

The V3 hypervariable region of 16S rRNA gene was amplified using the 338F (Lane, 1991) and 518R-GC clamp (Muyzer et al., 1993) primers. PCR was performed in a personal Mastercycler® (Eppendorf AG, Hamburg, Germany) using 2 μl DNA with a total volume of 25 μl containing 0.2 μM primers, 1× reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20], 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} BSA, and 0.025 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany). Non-template controls were included in parallel. PCR began with denaturing at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were verified by electrophoresis on 1.5% agarose gels, and their concentrations were measured.

DGGE was carried out by loading 100 ng of PCR products into 8% (w:v) polyacrylamide gels containing a gradient of denaturants ranging from 40% to 65% (100% denaturant consisting of 40% [v/v] formamide and 7 M urea) (Muyzer et al., 1993). Gels were run in an INGENYphorU System (Ingeny International BV, The Netherlands) at 60 °C for 16 h at 100 V. The gels were stained with silver nitrate using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Germany) and photographed for subsequent image analysis, as describe below.

2.7. Data analysis

One-way ANOVA was used to analyze the significant difference between treatments with mean separation based on Tukey's test. A paired-sample *t*-test was also performed to assess the difference between parameters measured in the initial material and the final vermicompost for each treatment. Statistical analyses were conducted at >95% confidence level ($P < 0.05$) using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

A comparison of DGGE banding patterns was made with the GelCompar II software (Applied Maths, Kortrijk, Belgium). After conversion of the scanned gels, the bands were normalized and a reference position defined to align the patterns for accurate comparison after associating the bands with the standard pattern. Pattern similarity values were calculated by comparing the densitometric curves using the Pearson correlation similarity coefficient. The Dendrograms were calculated through the use of the unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm. A position tolerance of 1% was set for band matching.

3. Results and discussion

3.1. Chemical parameters

All earthworms died in the initial materials containing heterogeneous-plant or tomato-plant wastes after 24 h. This can be attributed to the high EC of these wastes (Table 1), as the earthworms were able to develop in substrates containing mixtures of tomato-fruit waste (T; T/S 2:1; T/S 4:1; T/D 2:1 and T/D 4:1) and cow dung (D), which had lower salinity levels. Gunadi and Edwards (2003) reported that *E. fetida* died in vegetable wastes (i.e. lettuce, pea, celery, etc.) due to their high EC (12.8 ± 1.7 dS m^{-1}). They also reported that these worms survived for only three weeks in a vermibed composed of supermarket fruit wastes (i.e. cucumber, pear, apple, etc.).

In the viable substrates, the vermicomposting significantly altered the chemical properties of the initial materials (Table 2). The pH levels significantly increased after the vermicomposting process, except in the case of the control treatment (D). These higher pH values could be explained by the disappearance of organic acids in tomato-fruit waste and/or to the mineralization of proteins generating ammonium. In all treatments, TOC content diminished by between 13% and 26%, with treatment T recording the largest TOC reduction. Although earlier studies have shown that earthworms mineralize cattle dung more easily than other organic wastes (Suthar, 2009), the tomato-fruit waste may give rise to a specific microbial community responsible for the high C mineralization rate. This hypothesis was partly confirmed by the significant reduction in WSC values in all vermicomposting treatments except in the case of cow dung (D). In treatment D, this organic fraction, which represents the most easily metabolisable organic matter, could be degraded prior to the addition of earthworms during the previous cow dung maturation stage.

The C:N ratio decreased by between 16% and 23% for treatments T, T/D 2:1, T/D 4:1, and D, and by over 35% for T/S 2:1 and T/S 4:1. This ratio has been widely used as an index of maturity and stability, since a decline from the initial C:N value to a final value <20 usually indicates an high degree of organic matter stabilization (Senesi, 1989), as evidenced by the C:N ratio of under 20 required by Spanish legislation for fertilizer product (Government of Spain, 2005). All the vermicomposts produced met this requirement except in the case of the vermicompost from cow dung. This could be due to the lower initial TKN content in this cow dung (Table 3) compared with the other substrates.

Table 2
Chemical analyses of initial materials (I) and final vermicomposts (F).

	pH			EC (dS m^{-1})			TOC (g kg^{-1})			WSC (g kg^{-1})			C:N ratio		
Treatments[a]	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test
D	9.3 a	9.4	0.68	9.2 a	8.7 a	0.20	401 a	319 a	0.00*	29.1 a	31.8 a	0.11	26.9 c	22.7 a	0.02*
T	8.3 bc	9.7	0.00*	5.0 d	5.3 c	0.22	460 c	338 ab	0.01*	82.3 e	20.3 de	0.00*	20.2 a	16.6 b	0.03*
T/S (2:1)	7.9 c	9.4	0.01*	5.0 d	5.9 bc	0.00*	458 c	357 b	0.01*	72.8 d	28.2 b	0.00*	24.4 b	15.6 b	0.03*
T/S (4:1)	8.4 bc	9.2	0.04*	5.4 cd	5.5 c	0.26	433 bc	340 ab	0.01*	68.2 c	22.3 cd	0.00*	24.1 ab	15.6 b	0.01*
T/D (2:1)	8.8 ab	9.7	0.03*	5.6 c	6.7 b	0.03*	384 a	335 ab	0.03*	41.6 b	23.2 c	0.00*	21.4 ab	16.5 b	0.01*
T/D (4:1)	8.3 bc	9.6	0.01*	6.2 b	5.5 c	0.02*	430 b	337 ab	0.02*	66.4 c	18.9 e	0.00*	21.4 ab	17.4 b	0.01*

TOC, total organic carbon; WSC, water soluble carbon; EC, electrical conductivity. Means in the same column followed by same letters are not significantly different from each other. *t*-Test, *P* values of paired-sample *t*-test.

* Significant difference between the initial material and final vermicomposts.

[a] Refer to text, for explanation of treatment abbreviations.

Table 3
Total macronutrients and sodium content (g kg^{-1}) of initial materials (I) and final vermicomposts (F).

Treatments[a]	TKN			P			K			Ca			Mg			Na		
	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test
D	15.0 a	14.1 a	0.30	5.1 bcd	6.9 a	0.06	28.4 a	34.1 a	0.01*	15.8 a	20.6 b	0.02	8.5 a	10.8 a	0.04*	2304 a	2829 a	0.03*
T	22.8 b	20.4 bc	0.11	4.9 bc	9.7 c	0.00*	23.6 b	28.8 b	0.02*	7.7 c	16.7 cd	0.00*	3.3 c	6.1 c	0.00*	798 c	1522 cd	0.00*
T/S (2:1)	18.9 a	23.0 c	0.17	3.9 a	8.1 ab	0.01*	21.8 b	30.3 b	0.00*	6.5 c	14.7 de	0.01*	2.4 c	5.1 d	0.01*	556 d	1371 d	0.00*
T/S (4:1)	18.0 a	21.8 bc	0.02*	4.4 ab	7.9 ab	0.00*	21.7 b	28.6 b	0.00*	7.0 c	14.2 e	0.00*	2.8 c	5.3 cd	0.00*	663 cd	1198 d	0.00*
T/D (2:1)	18.0 a	20.3 bc	0.06	5.8 d	8.4 b	0.00*	24.7 b	33.5 a	0.01*	11.6 b	18.6 bc	0.00*	5.4 b	8.5 b	0.00*	1363 b	1864 c	0.04*
T/D (4:1)	20.1 ab	19.4 b	0.61	5.6 cd	8.2 b	0.01*	24.2 b	34.8 a	0.01*	11.6 b	23.1 a	0.01*	5.2 b	10.6 a	0.00*	1164 b	2344 b	0.00*

TKN, total Kjeldhal nitrogen. Means in the same column followed by same letters are not significantly different from each other ($P < 0.05$). *t*-Test, *P* values of paired-sample *t*-test.
* Significant difference between the initial material and final vermicompost.
[a] Refer to text, for explanation of treatment abbreviations.

Regarding the changes in macronutrients caused by vermicomposting (Table 3), TKN concentration significantly changed only in treatment T/S 4:1. Earlier studies have reported that vermicomposting may enrich the N-content of vermicompost due to N additions by earthworms in the form of mucus, enzymes or nitrogenous excretory substances (Tripathi and Bhardwaj, 2004) and as a consequence of nitrogen transformation mediated by the vermicompost's microbiota through organic matter mineralization and microbial nitrogen-fixing (Bhattacharya and Chattopadhyay, 2004). On the other hand, reductions in N concentrations have been reported in vermicomposting due to in-vivo denitrification within the worm's digestive tract (Hobson et al., 2005). Part of the N-content in the initial substrate is also transformed into earthworm protein. In this study, N concentration in the end product could be explained by the N-content in the initial substrate, earthworm growth, and decomposition efficiency during vermicomposting. Total concentrations of K, Ca, Mg, Na (Table 3), and micronutrients (Table 4) significantly increased in all substrates after the vermicomposting processes. Higher percentage increases were recorded in vermicompost containing tomato-fruit waste compare with the vermicompost from cow dung. This could be explained by cow dung's lower mineralization rates, as indicated by the higher WSC content and C:N ratio recorded for this vermicompost as compared to other types (Table 2). The nutrient concentration increased in the vermicomposts obtained, as the earthworms reduced waste mass by enhancing organic matter mineralization. This increase in total nutrients concentration caused by *E. andrei* was previously described by Elvira et al. (1996) and recently corroborated by other authors (Garg et al., 2006; Suthar, 2007). By contrast, Garg and Kaushik (2005) reported a decrease in total potassium, total calcium, and heavy metal concentrations (Fe, Zn, Pb and Cd) after vermicomposting and concluded that this could be due to leaching of these cations caused by excess water drainage. This factor can be ruled out in our study, as the substrates were placed in pots with sealed bottoms in order to prevent leaching. This vermicomposting system avoided nutrient loss and the vermicompost obtained was rich in plant nutrients.

3.2. Earthworm growth and reproduction

Despite differences in the chemical characteristics of each viable mixture used for developing *E. andrei*, earthworm growth showed similar patterns, although some significant variations were observed (Fig. 1 and Table 5). Maximum individual biomass was recorded after 4 weeks of vermicomposting in treatments T, T/S 2:1, T/S 4:1, and D, while T/D 2:1 and 4:1, peaked a week earlier. As expected, the highest values for individual biomass and net biomass were recorded by control treatment D, whereas T showed the lowest values. On the other hand, cocoon production (Table 5) started one week earlier in the substrate containing tomato-fruit waste than in control treatment D. Total cocoon production in

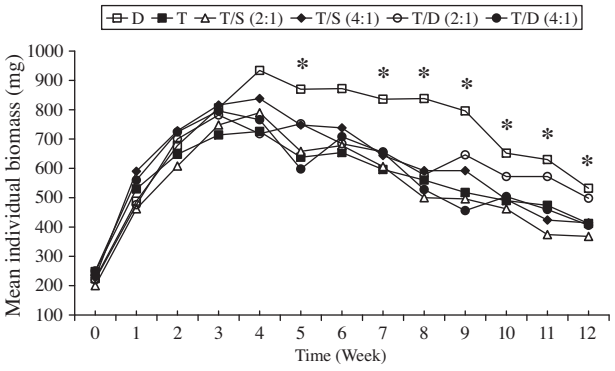


Fig. 1. Earthworm growth during the vermicomposting process of greenhouse vegetable wastes. The asterisk (*) denotes a significant difference between treatments at a given time.

Table 4
Total micronutrients content (mg kg^{-1}) of initial materials (I) and final vermicomposts (F).

Treatments[a]	Fe			Mn			Cu			Zn		
	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test	I	F	*t*-Test
D	1861 a	3050 a	0.00*	82 a	126 a	0.01*	19 a	29 a	0.01*	59 a	80 a	0.02*
T	1887 a	6183 d	0.00*	45 c	182 c	0.00*	24 b	68 c	0.00*	48 ab	136 d	0.00*
T/S (2:1)	1432 b	4683 b	0.00*	40 c	150 b	0.00*	20 a	58 b	0.00*	50 ab	111 b	0.00*
T/S (4:1)	1572 b	5590 d	0.00*	42 c	150 b	0.00*	23 ab	61 b	0.00*	46 b	126 c	0.00*
T/D (2:1)	1922 a	5199 c	0.00*	69 b	155 b	0.00*	26 b	57 b	0.00*	59 a	114 b	0.00*
T/D (4:1)	1990 a	4989 bc	0.00*	64 b	172 c	0.00*	26 b	79 d	0.00*	59 a	110 b	0.01*

Means in the same column followed by same letters are not significantly different from each other. *t*-test: *P* values of paired-sample *t*-test.
* significant difference between the initial material and final vermicompost.
[a] Refer to text, for explanation of treatment abbreviations.

Table 5
Growth and reproduction parameters of *E. andrei* in the feasible tomato-fruit material and control cow dung.

	Treatments[a]					
	D	T	T/S (2:1)	T/S (4:1)	T/D (2:1)	T/D (4:1)
Mean initial biomass [A] (mg worm^{-1})	224	248	200	228	220	250
Mean final biomass (mg worm^{-1})	532 a	412 abc	368 c	414 c	498 ab	406 bc
Week of maximum individual biomass	4	4	4	4	3	3
Maximum individual biomass [B] (mg worm^{-1})	934 a	726 b	790 ab	838 ab	782 ab	796 ab
Maximum growth rate (mg worm^{-1} week^{-1})	178 a	120 b	148 ab	153 ab	187 a	182 a
Net biomass gained [B]–[A] (mg worm^{-1})	710 a	478 b	590 ab	610 a	562 ab	546 ab
Starting cocoon production (week)	4	3	3	3	3	3
Total cocoon production (number)	59 d	222 ab	229 ab	265 a	138 c	168 bc

Means in the same row followed by same letters are not significantly different from each other.
[a] Refer to text, for explanation of treatment abbreviations.

treatment D was more than half that for treatments containing tomato-fruit waste. The highest level of cocoon production was recorded in treatment T/S 4:1. This difference in cocoon production rates is related to the quality of the waste material used (Nogales et al., 2008). Tomato-fruit waste, which can be improved through small additions of straw, would therefore be an appropriate greenhouse waste for effective earthworm development.

3.3. Enzyme activity

Dehydrogenases, which are intracellular enzymes involved in oxidative phosphorylation, can be used to assess overall microbial activity (García et al., 1997). Dehydrogenase activity values recorded in final tomato-fruit waste vermicomposts were significantly lower than those for the control treatment D (Fig. 2a). This could be related to the higher WSC content in the vermicompost from cow dung (D) as compared to other vermicomposts (Table 2). The lower dehydrogenase activity recorded in vermicomposts from tomato-fruit waste could mean that the organic matter present in this material was metabolised faster than the organic matter from cow dung. Benítez et al. (1999) have demonstrated a close correlation between WSC and dehydrogenase activity in vermicomposting, showing that the dehydrogenase activity provides a clear indication of the dynamics of organic-matter degradation which is useful in characterizing the status of vermicomposts.

As with dehydrogenase activity, hydrolytic enzyme levels analyzed in this study were lower in end products than in the initial materials (Fig. 2b). β-glucosidases, which catalyze the hydrolysis of cellobiose and other disaccharides, play a major role in the decomposition of organic C compounds. The β-glucosidase activity in tomato-fruit waste (T) recorded the highest level of initial activity, possibly due to the fact that it also recorded the highest WSC content (T) was the highest, and it could be related to its highest WSC content (Table 2). Significant decreases showed by this enzyme activity after vermicomposting indicated that this process was capable of degrading the large labile organic C fraction in

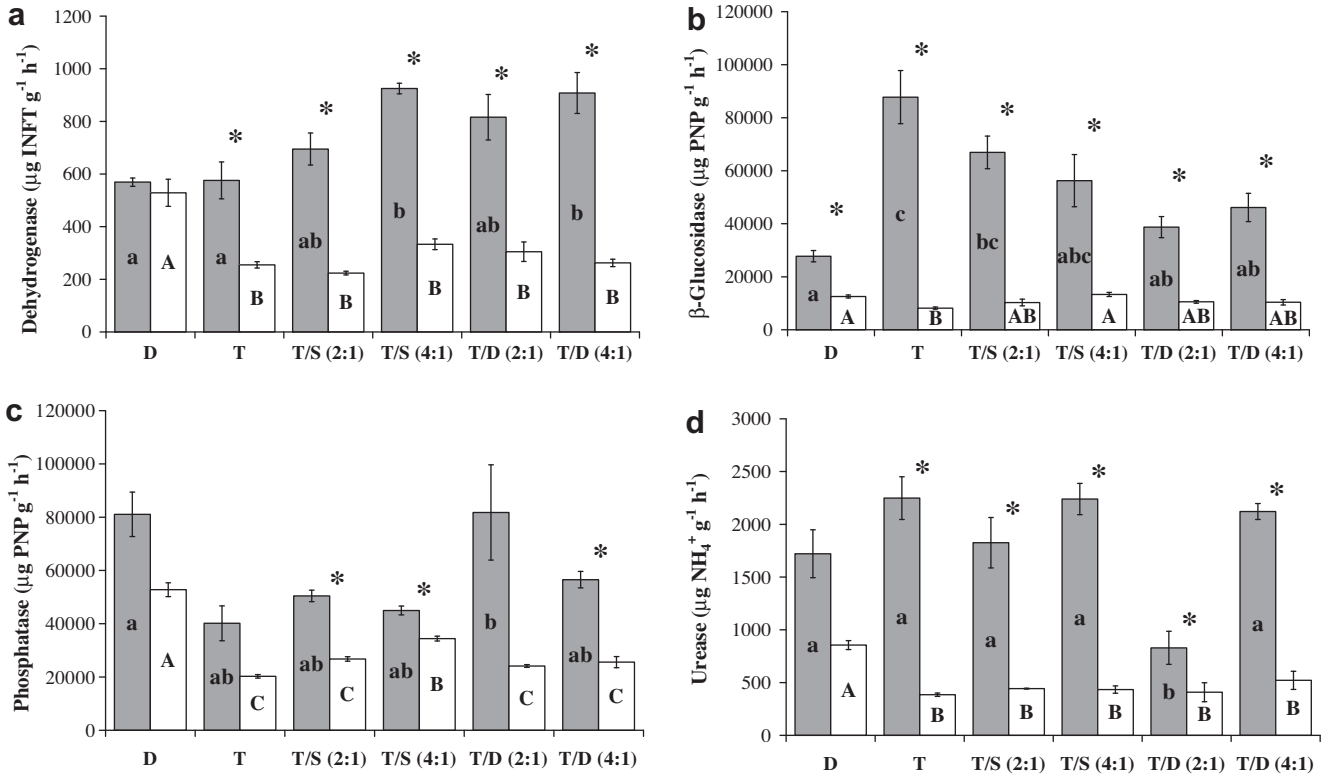


Fig. 2. Enzyme activities in initial materials (■) and final vermicomposts (□). Error bars represent the mean standard error. Lowercase or capital letters indicate significant differences among initial materials or final vermicomposts, respectively. The asterisk (*) indicates a significant difference between the initial material and final vermicompost from each treatment.

greenhouse waste T. Acid-phosphatases, which catalyse the hydrolysis of organic phosphomonoester to an inorganic phosphate form, behaved in a similar way to β-glucosidases, although phosphatase activity fell significantly only in treatments T/D 4:1, T/S 2:1, and T/S 4:1 (Fig. 2c). This could be due to residual amounts of organic-phosphate compounds, which may act as enzyme-synthesis-inducing substrates. Urease activity decreased significantly after vermicomposting in all treatments except in the case of the control treatment D (Fig. 2d). This was possibly due to the higher level of recalcitrant ureic compound in cow dung. Both phosphatase and urease activity recorded in the end product from cow dung was significantly higher than in the other vermicomposts. According to Pramanik et al. (2007), these activities detected in vermicompost vary depending on the type of organic wastes used. These researchers found higher levels of phosphatase and urease activity in vermicompost from cow dung than from other organic wastes.

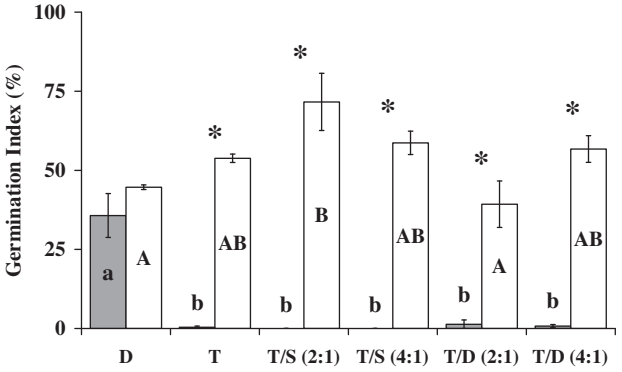


Fig. 3. Germination indices in initial materials (■) and final vermicomposts (□). Error bars represent the mean standard error. Lowercase or capital letters indicate significant differences among the initial materials or final vermicomposts, respectively. The asterisk (∗) indicates a significant difference between initial material and final vermicompost from each treatment.

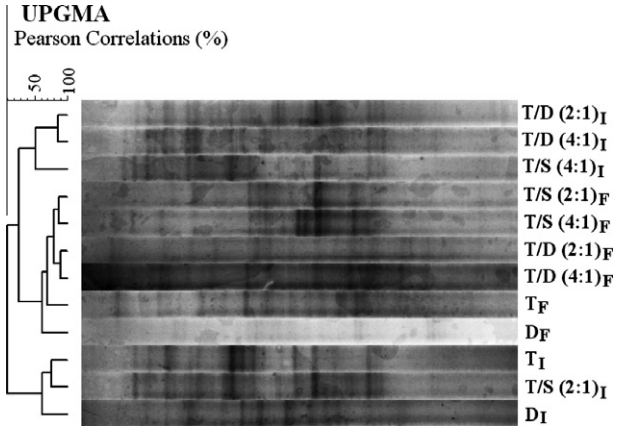


Fig. 4. Dendrogram from cluster analysis of bacterial community structure from initial material (I) and final vermicompost (F) samples based on DGGE patterns of amplified partial 16S rRNA genes.

3.4. Germination index

Germination index (GI) values were close to zero in the initial materials containing tomato-fruit wastes (Fig. 3). After vermicomposting, GI values significantly increased by over 50% in all treatments except in the case of the treatments D and T/D 2:1. According to Zucconi et al. (1981), composts which have a GI value higher than 50% are considered no-phytotoxic and stable for agricultural application. The lower GI values could be explained by the higher EC values recorded in treatments D and T/D 2:1. According to Iannotti et al. (1994), cress seed germination was greatly inhibited mainly due to high salinity levels in compost. Benítez et al. (2002) also reported low GI percentages in vermicompost from cattle manure as a consequence of its high EC level (6.6 dS m^{-1}). These findings indicate that vermicomposting is an effective system for eliminating the phytotoxic effects of unprocessed tomato-fruit waste from greenhouse crops.

3.5. Bacterial community

The bacterial community was genetically fingerprinted in each initial substrate and resulting vermicompost. An initial analysis of DGGE banding patterns from replicate samplings revealed a similarity coefficient >95% (data not shown). To compare all the samples on the one gel, a single sample was therefore run on the final gel. UPGMA analysis of this DGGE showed that all vermicomposts clustered into a clear group, whereas the initial materials showed no grouping (Fig. 4). Within this cluster, the vermicomposts from cow dung (D_F) and from tomato-fruit waste (T_F) showed the lowest similarity value (Table 6). On the other hand, the bacterial community fingerprinting showed by the different vermicomposts con-

Table 6
Similarity value among DGGE patterns from initial materials (I) and final vermicomposts (F).

Treatment[a]	I						F					
	D	T	T/S (2:1)	T/S (4:1)	T/D (2:1)	T/D (4:1)	D	T	T/S (2:1)	T/S (4:1)	T/D (2:1)	T/D (4:1)
I												
D	100											
T	68.1	100										
T/S (2:1)	58.6	76.7	100									
T/S (4:1)	17.3	38.9	59.9	100								
T/D (2:1)	15.3	4.9	51.2	44.3	100							
T/D (4:1)	7.6	0	44.1	61.1	87.5	100						
F												
D	0	0	0	39.3	13.7	32.9	100					
T	0	0	0	0	31.9	29.5	30.8	100				
T/S (2:1)	0	0	0	18.9	39.3	49.7	72.2	68.9	100			
T/S (4:1)	1.7	0	0	2.6	43.9	44.7	52.4	76.6	88.9	100		
T/D (2:1)	0	0	0	16.6	38.9	31.3	81.5	69.4	85.9	74.6	100	
T/D (4:1)	0	0	0	0.9	17.4	31.3	75.9	71.2	84.2	75.2	90.5	100

All values are % Pearson correlation similarity coefficient.
[a] Refer to text, for explanation of treatment abbreviations.

taining tomato-fruit waste were highly similar, with an average similarity coefficient >71%.

It is clear from our findings that *E. andrei* greatly transformed the different bacterial communities of each substrate producing vermicomposts characterized by analogous bacterial communities. The impact of earthworms on bacterial community have recently been described by Sen and Chandra (2009), who reported that analysis of DGGE fingerprinting showed divergent bacterial community development in compost and vermicompost obtained from the same initial material. They also reported that the dissimilar genetic fingerprintings observed in compost and vermicompost were due to differences in the functional responses of microbial communities in terms of enzyme activity and community-level physiological profiles. In line with this finding, the lower similarity value observed in the fingerprinting of vermicompost from cow dung could explain the chemical and biochemical differences previously reported between this vermicompost and those containing tomato-fruit waste. The high similarity values recorded on the basis of fingerprintings from vermicompost containing tomato-fruit waste may also indicate that this type of waste favoured the existence of specialized bacterial communities, thus explaining its higher rates of organic matter mineralization and stabilization. The DGGE technique is therefore useful for assessing the degree of maturity of end products. It also generates a characteristic fingerprinting that shows the major changes in bacterial structure caused by the vermicomposting of tomato-fruit waste using *E. andrei*. This is consistent with previous research based on other fingerprinting methods. Using FAME analysis, Lores et al. (2006) reported that the specific fingerprintings of microbial communities in vermicompost depend on the type of substrate and earthworm species used.

4. Conclusions

These findings represent a sound basis for applying the vermicomposting process to the recycling of greenhouse vegetable wastes, where salt content was the main factor restricting earthworm development. Our study shows that vermicomposting is a feasible means of recycling damaged tomato-fruit waste, where, although *E. andrei* recorded lower growth than in the cow dung control treatment, cocoon production was higher. Chemical and enzymatic analyses and germination indices showed that vermicomposting was effective in biostabilizing this waste. We also show that DGGE technique generates genetic fingerprintings that accurately reflect the degree of stabilization in vermicompost.

Acknowledgements

This study was financed by "Junta de Andalucía" project P05-AGR-00408. Manuel J. Fernández Gómez thanks the Science and Innovation Ministry for their FPU doctoral grant (AP2006-03452). The authors also thank Mª Angeles Delgado for technical support.

References

Bansal, S., Kapoor, K.K., 2000. Vermicomposting of crop residues and cattle dung with *Eisenia foetida*. Bioresour. Technol. 73, 95–98.

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol. 67, 297–303.

Benítez, E., Sainz, H., Melgar, R., Nogales, R., 2002. Vermicomposting of a lignocellulosic waste from olive oil industry: a pilot scale study. Waste Manage. Res. 20, 134–142.

Bhattacharya, S.S., Chattopadhyay, G.N., 2004. Transformation of nitrogen during vermicomposting of fly ash. Waste Manage. Res. 22, 488–491.

Castilla, N., Hernández, J., 2005. The plastic greenhouse industry of Spain. Chron. Hort. 45, 15–20.

Elvira, C., Goicoechea, M., Sampedro, L., Mato, S., Nogales, R., 1996. Bioconversion of solid paper-pulp mill sludge by earthworms. Bioresour. Technol. 57, 173–177.

Espi, E., Salmeron, A., Fontecha, A., García, Y., Real, A.I., 2006. Plastic films for agricultural applications. J. Plastic Film Sheet. 22, 85–102.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan. 28, 123–134.

Garg, P., Gupta, A., Satya, S., 2006. Vermicomposting of different types of waste using *Eisenia foetida*: a comparative study. Bioresour. Technol. 97, 391–395.

Garg, V.K., Kaushik, P., 2005. Vermistabilization of textile mill sludge spiked with poultry droppings by an epigeic earthworm *Eisenia foetida*. Bioresour. Technol. 96, 1063–1071.

Government of Spain, 2005. Real Decreto 824/2005 sobre productos fertilizantes, in: BOE. Government of Spain, Madrid, pp. 25602–25669.

Gunadi, B., Edwards, C.A., 2003. The effects of multiple applications of different organic wastes on the growth, fecundity and survival of *Eisenia fetida* (Savigny) (Lumbricidae). Pedobiologia 47, 321–329.

Hobson, A.M., Frederickson, J., Dise, N.B., 2005. CH_4 and N_2O from mechanically turned windrow and vermicomposting systems following in-vessel pretreatment. Waste Manag. 25, 345–352.

Iannotti, D.A., Grebus, M.E., Toth, B.L., Madden, L.V., Hoitink, H.A.J., 1994. Oxygen respirometry to assess stability and maturity of composted municipal solid waste. J. Environ. Qual. 23, 1177–1183.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New York, pp. 115–175.

Lores, M., Gómez-Brandón, M., Pérez-Díaz, D., Domínguez, J., 2006. Using FAME profiles for the characterization of animal wastes and vermicomposts. Soil Biol. Biochem. 38, 2993–2996.

Lorimor, J., Fulhage, C., Zhang, R., Funk, T., Sheffield, R., Sheppard, C., Newton, G.L., 2001. Manure management strategies/technologies. In: Rice, J.M., Caldwell, D.F., Humenik, F.J. (Eds.), White Paper on Animal Agriculture and the Environment for National Center for Manure and Animal Waste Management. ASABE, Michigan, p. 52.

M.A.P.A., 1986. Official methods of analysis, Plants, Organic Products, Fertilizers, Soils, Waters and Organic Fertilizers.Vol. III. Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación, Madrid.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011–1016.

Nogales, R., Domínguez, J., Mato, S., 2008. Vermicompostaje. In: Moreno, J., Moral, R. (Eds.), Compostaje. Ediciones Mundi Prensa, Madrid, pp. 187–208.

Pardossi, A., Tognoni, F., Incrocci, L., 2004. Mediterranean greenhouse technology. Chron. Hort. 44, 28–34.

Parra, S., Aguilar, F.J., Calatrava, J., 2008. Decision modelling for environmental protection: the contingent valuation method applied to greenhouse waste management. Biosyst. Eng. 99, 469–477.

Pramanik, P., Ghosh, G.K., Ghosal, P.K., Banik, P., 2007. Changes in organic – C, N, P and K and enzyme activities in vermicompost of biodegradable organic wastes under liming and microbial inoculants. Bioresour. Technol. 98, 2485–2494.

Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresour. Technol. 100, 804–811.

Senesi, N., 1989. Composted materials as organic fertilizers. Sci. Total Environ. 81–82, 521–542.

Suthar, S., 2007. Vermicomposting potential of *Perionyx sansibaricus* (Perrier) in different waste materials. Bioresour. Technol. 98, 1231–1237.

Suthar, S., 2008. Bioconversion of post harvest crop residues and cattle shed manure into value-added products using earthworm *Eudrilus eugeniae* Kinberg. Ecol. Eng. 32, 206–214.

Suthar, S., 2009. Vermicomposting of vegetable-market solid waste using *Eisenia fetida*: impact of bulking material on earthworm growth and decomposition rate. Ecol. Eng. 35, 914–920.

Tabatabai, M.A., 1982. Methods of soil analysis, part 2, chemical and microbiological properties. Soil Enzymes 907, 943.

Tripathi, G., Bhardwaj, P., 2004. Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg). Bioresour. Technol. 92, 275–283.

von Mersi, W., Schinner, F., 1991. An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol. Fertl. Soils 11, 216–220.

Williams, S., 1984. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Zdruli, P., Jones, R.J.A., Montanarella, L., 2004. Organic matterin the soils of southern Europe, in: European Soil Bureau Research Report, E U R 21083 EN. Office for Official Publications of the European Communities, Luxembourg. pp. 1–17.

Zucconi, F., Forte, M., Monaco, A., De Bertoldi, M., 1981. Biological evaluation of compost maturity. BioCycle 22, 27–29.

Anexo

Waste Management 30 (2010) 2461–2468

Contents lists available at ScienceDirect

Waste Management

journal homepage: www.elsevier.com/locate/wasman

Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops

Manuel J. Fernández-Gómez [a,*], Rogelio Nogales [a], Heribert Insam [b], Esperanza Romero [a], Marta Goberna [b]

[a] Department of Environmental Protection, Estación Experimental del Zaidín (EEZ), CSIC, Profesor Albareda 1, 18008 Granada, Spain
[b] Institute of Microbiology, University of Innsbruck, Technikerstraβe 25, A-6020 Innsbruck, Austria

ARTICLE INFO

Article history:
Received 11 March 2010
Accepted 2 July 2010
Available online 2 August 2010

ABSTRACT

Huge quantities of discarded fruits generated from greenhouse crops represent a worldwide environmental problem. The aim of this work was to assess the efficiency of vermicomposting as a recycling management option for biotransforming tomato-fruit wastes from greenhouses into an organic nutrient-rich product available for agricultural purposes. A pilot vermireactor was constructed. It was provided with a manure layer, where an initial population of *Eisenia fetida* was introduced and fed continuously at a high organic loading rate (13.6 kg TOC m^{-3} wk^{-1}) for 150 days. Vermicompost chemical and enzymatic parameters as well as the bacterial and fungal community structure were determined for 210 days (vermicomposting plus a maturation period). Earthworm biomass increased after 90 days, and then declined due to increasing pH, electrical conductivity and ammonium concentration. The temporal patterns of dehydrogenase, β-glucosidase, protease and urease were related to earthworm growth and the stabilization of organic matter. Bacterial DGGE profiles differed between the period of degradation of labile substrates and the maturation step. Fungal communities at the stage of maximum earthworm biomass differed most, suggesting a gut passage effect. The end product was chemically stable and enriched in nutrients, demonstrating that tomato-fruit wastes can be successfully vermicomposted into a valuable soil amendment. We suggest continuous-feeding vermicomposting as an environmentally sound management option for greenhouse wastes.

1. Introduction

In Spain, 66,000 ha are dedicated to greenhouse crops, 65% of which are located in Andalusia (Spanish Ministry of Agriculture, Fisheries and Food, 2007). These profitable systems have a negative impact on the environment where they are located because of the enormous quantities of wastes produced, which have become one of most important problems associated to this agricultural practice. These waste were traditionally eliminated by abandon them in dry ravines or empty areas originating uncontrolled burning, blocking of riverbeds, poisoning of cattle and sheep and a negative visual impact on the landscape. In addition, landfills containing these wastes are a potential breeding ground for diseases and pests and a source of greenhouse gas (methane) (Parra et al., 2008). Therefore, the greenhouse wastes should be recycled and reused in order to achieve environmentally suitable agricultural management. A particular kind of vegetable waste are the fruit wastes, which consist of fruits unsuitable for marketing due to bruising, inappropriate size, rotting, or insect attack, as well as marketable fruits that are discarded so as to raise their market prices. Only in the region of Motril (Granada, Southern Spain), where this study was conducted, 15,000 Tm of tomato-fruit wastes were discarded in 2007, representing 24% of all greenhouse wastes. Tomatoes comprised 80% of all fruit wastes from greenhouses and were generated continuously over the year.

This huge quantity of wastes could be converted into a nutrient-enriched bio-fertilizer and used for agricultural purposes or land restoration. Vermicomposting, a process involving the biostabilization of organic wastes by the joint action of earthworms and microorganisms, has proven to be a low-cost and rapid technique for the efficient management of vegetable wastes (Bansal and Kapoor, 2000; Suthar, 2008), using a variety of epigeic earthworms, e.g. *Eisenia fetida*, *Perionyx excavatus*, and *Eudrilus eugeniae*. Among these species, *E. fetida* might be the best choice for developing a vermicomposting process in temperate climates due to its tolerance of a broad range of environmental conditions, i.e. pH, moisture, and temperature (Nogales et al., 2008). Recent studies have shown the efficiency of *E. fetida* to vermicompost supermarket vegetable and fruit wastes using traditional (non-continuous) vermicomposting processes (Gunadi and Edwards, 2003; Suthar, 2009). To date, the vermicomposting of fruit wastes using a continuous-feeding system has not been studied.

* Corresponding author. Tel.: +34 958 181600x225; fax: +34 958 129600.
E-mail address: manuelj.fernandez@eez.csic.es (M.J. Fernández-Gómez).

0956-053X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.wasman.2010.07.005

Enzyme activities have been used as indicators of the time course of organic matter in vermicomposting systems from a small to large scale (Benítez et al., 1999, 2002; Nogales et al., 2005). Overall decreases in dehydrogenase and total hydrolytic activities have been related to the decline in microbial activity and available substrates during the decomposition of organic matter. Despite that these enzymes have been widely used in composting and non-continuous vermicomposting, they have still not been evaluated as tools for monitoring a continuous vermicomposting system. On the other hand, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of genes coding for SSU rRNA has recently been used to determine the genetic structure of bacterial communities during vermicomposting at the laboratory scale with bin systems (Sen and Chandra, 2009). However, temporal changes in enzyme activities and microbial community structure have not been researched jointly throughout a continuously fed vermicomposting process.

In view the above, the objective of this study was to test the efficiency of *E. fetida* to vermicompost tomato-fruit wastes from greenhouse crops using a pilot-scale continuous-feeding vermicomposting system. A rectangular vermireactor was designed and tomato-fruit wastes were added over a 150-day vermicomposting process. The time course of the chemical parameters, enzyme activities, and genetic structure of the fungal and bacterial communities proliferating in the vermibed (i.e. the vermireactor substrate) were tracked in order to evaluate the role of earthworms and monitor the stabilization of organic wastes during this biotransformation process. Finally, the nutrient content was analysed in the end product in order to assess the quality of this vermicompost as an agricultural resource.

2. Materials and methods

2.1. Experimental set-up

A rectangular metal pilot-scale (0.6 m × 0.9 m × 0.2 m) vermireactor was designed. A 0.1 cm mesh was placed at the bottom of the vermireactor. A 5 cm layer containing 15 kg dry weight of mature sheep manure (36% moisture, pH 8.6, 1.8 dS m^{-1} electrical conductivity (EC), 138 g kg^{-1} total organic carbon (TOC), 9.6 g kg^{-1} total Kjeldhal nitrogen (TKN), and C:N ratio 14; see also Table 3) was placed on the mesh to provide an initial habitat for the earthworms. Sheep manure was selected since it is optimal for worm growth and it is intensively produced close to the greenhouses in Southern Spain. A total of 500 g of clitellated and non-clitellated earthworms (*E. fetida*) were inoculated in the manure layer. These were obtained from the earthworm culture bank at the "Estación Experimental del Zaidín" (CSIC) in the city of Granada (Southern Spain). Fifteen days after the earthworms were added, the vermireactor was fed with 10 kg of the liquid-paste of tomato-fruit wastes (92% moisture, pH 3.9, 1.4 dS m^{-1} EC, 459 g kg^{-1} TOC, 23 g kg^{-1} TKN and C/N ratio 20), which were taken from the organic-waste-treatment plant in Motril (Granada province, Spain). This operation was repeated weekly for 5 months, during which the organic loading rate was kept at 13.6 kg TOC m^{-3} wk^{-1}. After this period, the earthworms were removed by hand, and the vermicompost was matured in a pile for 2 months. The vermicomposting process was conducted under a controlled temperature (25 °C) with no water was applied beyond that contained in the liquid-paste of tomato-fruit wastes.

Throughout the process, five 70 cm^2 cylindrical cores were used to take regularly distributed samples from the vermireactor. The number and weight of earthworms in each core were recorded every 15 days, and the worms were replaced in the vermireactor. The content of each core was collected monthly during vermicomposting and at the end of the maturation period. Each sample was divided into two subsamples, of which one was stored at −20 °C for enzyme activity and molecular biological analyses, while the other was air dried and finely ground for chemical analyses.

2.2. Chemical analyses

The pH and electrical conductivity (EC) of the vermibed were measured with a glass electrode using a 1:10 sample:water (w:v) ratio. Total organic C (TOC) and total Kjeldahl N (TKN) were determined using the dichromate oxidation and Kjeldahl method, respectively (M.A.P.A., 1986). Water-soluble carbon (WSC) was extracted at 60 °C for 1 h with distilled water (1:10 sample:water w/v) and then determined with potassium dichromate and sulphuric acid at 160 °C for 30 min. Subsequently, the amount of Cr^{3+} produced by the reduction of Cr^{6+} was quantified spectrophotometrically at 590 nm (Sims and Haby, 1971). The ammonium (NH_4^+) concentration was determined after extraction with 2 M KCl using a modified salicylate–nitroprusside colorimetric method (Kandeler and Gerber, 1988). Total phosphorus was measured using the ammonium vanadomolybdate method; total K and Na were measured by flame-photometry; and total Ca and Mg and micronutrients (Fe, Mn, Cu, Zn) were determined by atomic absorption spectrometry after digesting the samples with HNO_3:$HClO_4$ mixture (Williams, 1984).

2.3. Enzyme activities

Total enzyme activities were determined in triplicate, each reaction tube containing 0.2 g sample. Dehydrogenase activity was measured after the extraction of iodonitrotetrazolium formazan (INTF), produced by the reduction of 2-p-iodophenyl-3 p-nitrophenyl-5 tetrazolium chloride, with a mixture of 1.5:1 acetone:tetrachloroethylene. INTF was measured in a spectrophotometer at 490 nm (García et al., 1997). The enzyme activities β-glucosidase and acid phosphatase were quantified by estimating the amount of p-nitrophenol (PNP) produced from 4-nitrophenyl-β-D glucanopyranoside (PNG) and 4-nitrophenyl phosphate (PNPP) following Tabatabai (1982) and Tabatabai and Bremner (1969), respectively. Urease and protease activities were determined using urea and N-a-benzoyl-L-argininamide, respectively, as substrates (Nannipieri et al., 1980; Bonmatí et al., 1998). The NH_4^+ released was measured using an ammonium-selective electrode (ORION Research Inc., Beverly, MA, USA, mod. 95-12).

2.4. DNA extraction and PCR–DGGE analyses

Total DNA was extracted in triplicate from 0.25 g of samples taken on days 0 (M0), 30 (M1), 90 (M3), and 150 (M5) of vermicomposting and from the matured vermicompost (V) by means of the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. Briefly, this DNA extraction kit involves mechanical and chemical lysis of the cells, precipitation of non-DNA substances and capture of total DNA on a silica membrane for its washing and subsequent elution. DNA solutions were checked for quality by electrophoresis in 1% agarose gels stained with ethidium bromide.

The PCR was performed with 1–2 μl DNA in a total volume of 25 μl containing 0.2 μM of each primer (Table 1), 1× reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20] (GeneCraft, Münster, Germany), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin, 0.025 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany) and sterile water. Non-template controls, containing all the components except DNA templates, were included in parallel. Amplification was performed in a PCR Express cycler (ThermoHybaid) using

Table 1
PCR primer sets, PCR, and DGGE conditions used in this study.

Primer set	Annealing positions	Sequence 5′–3′	Specificity	Annealing (°C)	Amplicon length (bp)[c]	Denaturing gradient (%)	References
F984[a]	968–984[b]	AACGCGAAGAACCTTAC	Bacteria	61	433	40–65	Heuer et al. (1997)
–R1378	1378–1401[b]	CGGTGTGTACAAGGCCCGGGAACG					
R1[a]	18 rDNA	AICCATTCAATCGGTAIT (I = Inosin)	Fungi	50	390	30–60	Vainio and Hantula (2000)
–F390	18 rDNA	CGATAACGAACGAGACCT					

[a] Primers with a GC clamp at the 5′ end according references.
[b] Numbering according to the *rrs* gene of *Escherichia coli*.
[c] Calculated from the *rrs* gene of *E. coli* or from the product length described in Vainio and Hantula (2000).

an initial denaturation at 94 °C for 8 min, followed by 30 amplification cycles, each consisting of 1 min at 94 °C, 1 min annealing at the specific temperature in Table 1, and 1 min at 72 °C, followed by a final extension step for 10 min at 72 °C. Proper sizes of amplification products were verified by electrophoresis in 1.5% agarose gels stained with ethidium bromide and inspected under a UV–transilluminator. The PCR product concentration was determined with the PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA, USA). Fluorescence was measured with an Anthos Zenyth 3100 multimode detector (Anthos Labtec, Austria) and the Software for Anthos Multimode Detectors (Version 2.0.0.13).

The denaturing gradient gel electrophoresis (DGGE) was conducted by loading 100 ng of PCR products into 8% (w/v) polyacrylamide gel in 1× TAE (20 mM Tris–Cl, 10 mM acetate, 0.5 mM Na_2EDTA) containing a gradient of denaturants (100% denaturants consisting of 40% [v/v] formamide and 7 M urea) as indicated in Table 1. A 100 bp DNA ladder (Genecraft®, Germany) served as marker. Gels were run in an INGENYphorU System (Ingeny International BV, The Netherlands) at 60 °C for 16 h at 100 V. Gels were stained with silver nitrate (Sanguinetti et al., 1994) using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Germany) and photographed for subsequent image analysis.

2.5. Data analysis

The time course of all parameters determined during vermicomposting was statistically tested using a repeated-measures analysis of variance (ANOVA). The Least Significant Difference test (LSD) was used for *post hoc* mean separation. A paired-sample *t*-test was performed to assess the differences of chemical parameters between initial sheep manure and the final mature vermicomposts. All statistical analyses were conducted using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

A comparison of DGGE patterns was made with the GelCompar II software (Applied Maths, Kortrijk, Belgium). After conversion of the scanned gels, the bands were normalized and a reference position defined to align the patterns for proper comparison after associating the bands with the standard. Similarity values among banding patterns were calculated based on the comparison of the corresponding densitometric curves using the Pearson correlation similarity coefficient. Dendrograms were calculated with the unweighted-pair group method using arithmetic averages (UPGMA) clustering algorithm. A position tolerance of 1% was set for band matching.

3. Results and discussion

3.1. Earthworm development and chemical changes during vermicomposting

Weekly applications of tomato-fruit wastes did not increase the total earthworm biomass during the first 75 days of vermicomposting (Fig. 1a). Nevertheless, a pronounced increase was observed

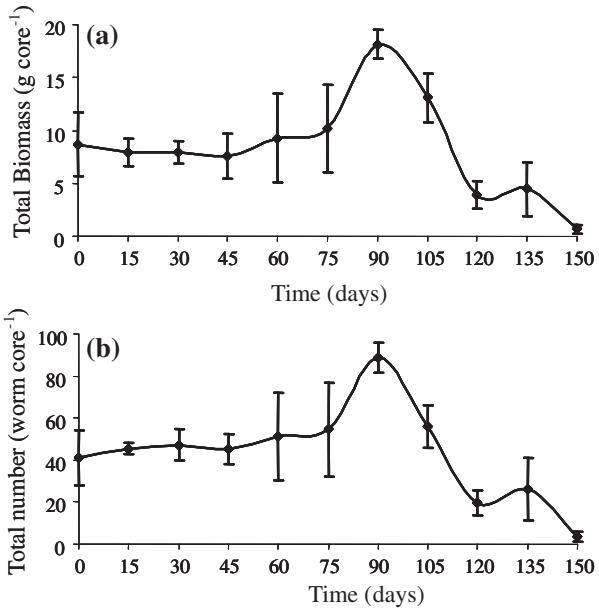


Fig. 1. Evolution of biomass (a) and total number of earthworms (b) during the continuous-feeding vermicomposting process. Each symbol indicates the mean ± standard error ($n = 5$).

from day 75 to day 90, peaking at 18.6 g earthworm per core (ca. 1400 g earthworms in the whole vermireactor), which was twofold the initial value. This is consistent with the life-cycle of *E. fetida*, which population doubles between 54 and 91 days after cocoon deposition under optimal feeding conditions (Nogales et al., 2008). Importantly, the moisture content in the liquid-paste of tomato-fruit wastes was high enough so as to allow normal earthworm development without further watering. Edwards (1988) reported that *E. fetida* can survive at moisture contents between 50% and 90%, in agreement with our results.

From day 90 to 120, although more tomato-fruit was added, the total earthworm biomass decreased abruptly and continued to decline slightly until the end of vermicomposting (day 150). This trend of earthworm biomass mirrored the reduction in the number of earthworms (Fig. 1b), and not the individual worm weights, which remained basically constant throughout the vermicomposting (data not shown). The increased earthworm mortality during this period could be explained by the rise in the pH value, electrical conductivity (EC) and NH_4^+ levels in the vermibed, which have been previously shown to be lethal to earthworms (Edwards, 1988). The vermibed pH increased from 8.3 to 9.5 during the 150 days period of earthworm activity and up to 10.4 after maturation (Table 2). This can be attributed to the increase in NH_4^+ concentration, exceeding 3000 mg kg^{-1} on day 90 and remaining high until the end of the process. This could be the result of the high mineralization of proteins from the tomato-fruit waste and sheep manure. In

Table 2
Temporal changes in chemical parameters during the continuous-feeding vermicomposting process and after maturation period.

Time (days)	pH	EC (dS m^{-1})	Moisture (%)	TOC (g kg^{-1})	TKN (g kg^{-1})	C/N	WSC (g kg^{-1})	NH_4^+ (mg kg^{-1})
Continuous-feeding vermicomposting process								
0	8.3^{a}	1.68^{a}	56^{b}	139^{a}	12.1^{a}	11.4	17.8	348^{a}
30	9.1^{b}	2.09^{ab}	58^{b}	170^{a}	14.4^{ab}	11.9	21.3	362^{a}
60	9.5^{b}	2.55b	57^{b}	188^{ab}	16.6^{bc}	11.3	24.2	1380^{bc}
90	9.1^{b}	3.01^{c}	69^{a}	208^{b}	17.1^{c}	12.1	23.3	3303^{e}
120	8.6^{a}	3.85^{d}	71^{a}	267^{c}	23.0^{d}	11.6	26.7	1968^{bc}
150	9.5^{b}	4.48^{f}	57^{b}	279^{c}	23.9^{d}	11.7	25.2	2561de
Maturation period								
210	10.4^{c}	4.40^{f}	29^{c}	148^{a}	12.9^{a}	11.4	16.9	2264^{cd}

All values are means of five replicates.
EC: electrical conductivity; TOC: total organic carbon; WSC: water-soluble carbon.
Data with the same letter are not significantly different ($p < 0.05$).

addition, it must be considered that under a basic pH, ammonium (NH_4^+) turns into the more toxic ammonia (NH_3). EC values also increased continuously up to a maximum of 4.5 dS m^{-1} after 150 days (Table 2) indicating the release of salts during the decomposition of organic substances. Gunadi and Edwards (2003) reported that *E. fetida* died in vegetable waste (e.g. lettuce, pea, celery, etc.) with a high EC value (12.8 ± 1.7 dS m^{-1}) and NH_4^+ concentrations (1878.1 ± 67.8 mg kg^{-1}), and these worms survived only 3 weeks in supermarket fruit wastes (e.g. cucumber, pear, apple, etc.) at pH 4.1, EC 4.5 dS m^{-1} and 3.5 mg NH_4^+ kg^{-1} recorded in that vermibed. Likewise, Mitchell (1997) found *E. fetida* unable to survive in cattle manure at pH 9.5 and 5.0 dS m^{-1} EC.

During the 150 days vermicomposting, the values of TOC, TKN and WSC significantly increased by 100%, 96% and 35%, respectively, compared to their initial values (Table 2). This was not surprising, since fresh organic matter was continuously incorporated by the addition of tomato-fruit waste at a high loading rate. These results contrast with the general decline in TOC value observed in non-continuous-feeding vermicomposting systems (Benítez et al., 1999, 2002; Nogales et al., 2005). Hence, a maturing period was considered necessary to complete the mineralization of the carbonaceous and organic-N compounds contained in the vermibed. Indeed, TOC, TKN, and WSC all decreased after the maturation period, down to levels similar to the initial values. Despite the changes during the vermicomposting process, the C:N ratio (TOC:TKN) remained constant at values from 11 to 12. The C:N ratio has been traditionally used as index for estimating compost maturity and stability. Senesi (1989) reported that although a decline from the initial C:N value, which should be not >30 in the substrate, to a final value <20, generally indicates an advanced degree of organic matter stabilization in the product and is considered desirable for a mature composts, for particular substrates this index is not considered a reliable indicator, since raw materials used may have a highly variable lignin:cellulose ratio, or they may contain other recalcitrant component, varying their biodegradability. In spite of this fact, the Spanish legislation for fertilizers (Government of Spain, 2005) states that the C:N value of a compost to be used as an organic amendment in agriculture should always be <20.

3.2. Enzyme activity during vermicomposting and vermicompost maturation

Dehydrogenases (DH-ase) are intracellular enzymes involved in the oxidative phosphorylation process, so that the activity of these enzymes has been widely used to assess the overall microbial activity in soils as well as in other biotransformation processes (García et al., 1997; Castaldi et al., 2008). The low DH-ase recorded at the start of the experiment (Fig. 2a) indicated reduced microbial activity in the mature manure used as the starter material. This enzyme activity sharply increased at day 30, as a result of microbial stimulation by the initial application of tomato-fruit waste and the supply of microbes thriving in the residue. Parthasarathi and Ranganathan (1999) demonstrated that fresh earthworm castings released into the vermicompost substrate have higher microbial activity and viable cell numbers. Still, a more appreciable increase was recorded between days 90 and 120. This could be a consequence of the significant increase in TOC and TKN content due to an excessive waste-loading rate, which also raised the moisture level reducing the oxygenation conditions in the vermibed between days 90 and 120 (Table 2). On the other hand, the mentioned increase coincided with the sudden decrease in total earthworm biomass, suggesting that the burst in microbial activity could be related to the decomposition of the worm bodies. Immediately after day 120, DH-ase activity fell to values similar to those recorded at the start of the experiment and remained stable during the maturation phase. According to Benítez et al. (1999), during a non-continuously fed vermicomposting systems, a peak in the DH-ase activity pattern would allow the end of the hydrolytic phase to be distinguished, corresponding with the maximum earthworm biomass, from the start of the maturation phase. Our results show that DH-ase is useful for monitoring continuously fed vermicomposting processes, its rise pointing to imminent system instability and thus indicating the need of reducing the organic loading rate.

The β-glucosidase activity, which catalyses the hydrolysis of cellobiose and other disaccharides, resembled dehydrogenase in behaviour, although its maximum was detected on day 90, a month before the peak registered by dehydrogenase (Fig. 2b). The application of tomato-fruit waste, which has a high carbohydrate and soluble sugar content (averaging 4% of the total dry weight), activated these enzymes. However, β-glucosidase was presumably also fostered by the high earthworm biomass. This conjecture is supported by the positive correlation between the two parameters (Pearson's correlation coefficient = 0.456, $p < 0.05$). This result agrees with those reported by Parthasarathi and Ranganathan (2000) who detected higher carbohydrase activities in castings from earthworms grown in organic wastes. Thus, the strong decline of this enzyme between days 90 and 120 could be related to the sharp reduction in the earthworm population. Therefore, β-glucosidase activity, which is easy and inexpensive to analyse, appears to be useful to monitor the earthworm population while avoiding the laborious earthworm biomass determination.

Acid phosphatases are enzymes of agricultural value because they catalyse the hydrolysis of organic phosphomonoester to an inorganic phosphate form, which can be taken up by plants. The initial activity was high (Fig. 2c) and decreased progressively until the end of the experiment, presumably due to the rise in pH to over 9.0 (Table 2), which is considerably higher than the optimum for

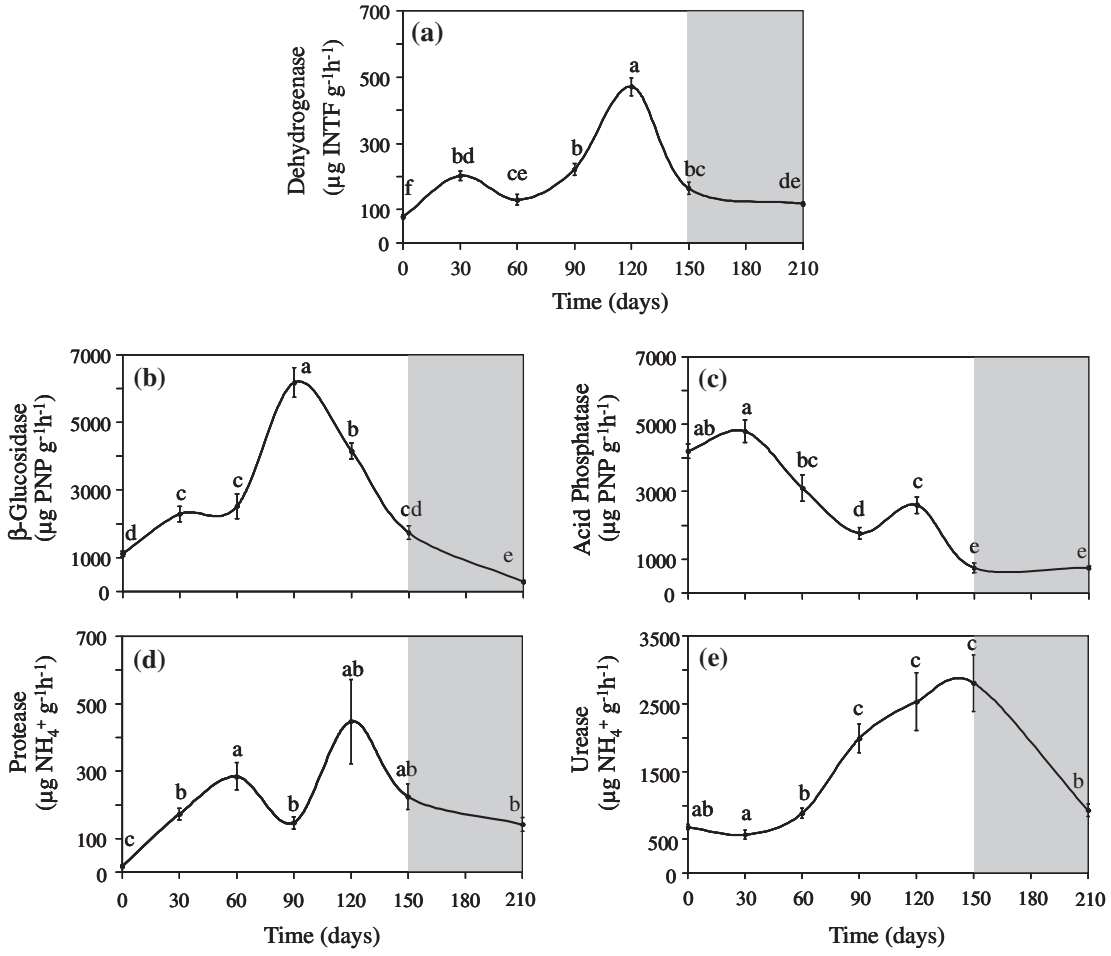


Fig. 2. Enzyme activities during the continuous-feeding vermicomposting process (□) and after maturation period (■). Data with the same letter are not significantly different ($p < 0.05$). Each symbol indicates the mean ± standard error ($n = 5$).

this enzyme (between 4 and 6.5) (Speir and Ross, 1978). Therefore, acid phosphatases do not seem to be good process indicators in this continuous-feeding vermicomposting system.

Protease activity increased from days 0 to 60 (Fig. 2d), indicating the depolymerization of N-containing compounds into dissolved organic nitrogen. The subsequent decline of protease activity at day 90 could be due to the transformation of available N-containing compounds in worm tissues at that moment of maximum earthworm biomass. A further increase of protease activity was recorded from day 90 to 120, coinciding with increasing microbial activity and decreasing earthworm biomass. The death of earthworms could have released peptides which stimulated protease synthesis by the microbiota. Thus, it should be noted that protease activity patterns would potentially reflect earthworm mortality.

Urease activity remained more or less stable during the first 60 days, then increased until day 90, and remained high until day 150 (Fig. 2e). This enzyme acts on carbon nitrogen (C–N) bonds other than the peptide linkage and is involved in the hydrolysis of urea to carbon dioxide and ammonium. In this sense, the dynamics of urease activity could be used as an indicator of high ammonium production in the vermibed, as it suggests the positive correlation between the two parameters (Pearson's correlation coefficient = 0.600, $p < 0.01$). Eventually, this enzyme activity decreased after maturation as a consequence of the mineralization of urea-type substrates.

3.3. Changes in elemental composition in the mature vermicompost

Vermicomposting with *E. fetida* converted the sheep manure together with tomato-fruit wastes into a nutrient-rich product which can be used for agricultural purposes. As compared to the initial manure, the mature vermicompost had significantly higher TKN content, as well as other plant nutrients, i.e. K, Cu, and Zn (Table 3). This increase may respond to the high mineralization of nitrogen from decaying earthworm tissues jointly to the release of other

Table 3
Elemental composition of initial manure layer and mature vermicompost.

Parameters	Initial sheep manure	Mature vermicompost	*t*-Test[a]
TKN (g kg^{-1})	9.6	12.9	0.001*
P (g kg^{-1})	2.60	2.04	0.020^{ns}
K (g kg^{-1})	8.9	22.7	0.006*
Ca (g kg^{-1})	24.6	18.6	0.020^{ns}
Mg (g kg^{-1})	9.54	7.20	0.033^{ns}
Na (g kg^{-1})	2469	1385	0.001*
Fe (mg kg^{-1})	15,166	8588	0.001*
Mn (mg kg^{-1})	315	256	0.222^{ns}
Cu (mg kg^{-1})	13.1	27.4	0.001*
Zn (mg kg^{-1})	68.0	99.3	0.001*

^{ns}Non-significant difference.
All values represent mean of three replicates.
[a] Paired-sample *t*-test.
* Significant at $p < 0.01$.

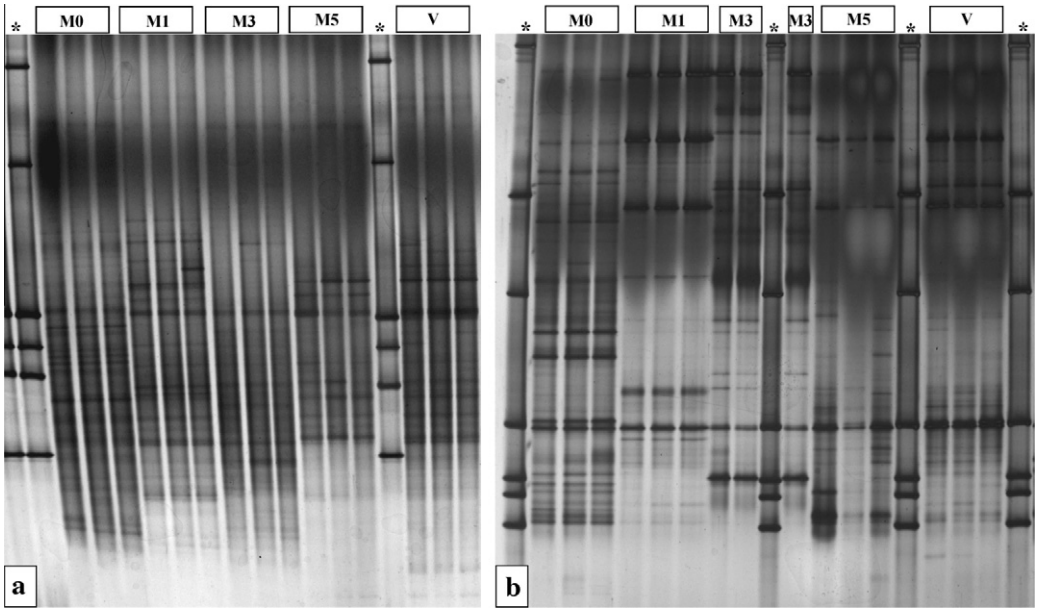


Fig. 3. Photographed of DGGE gels from triplicate samples taken during the continuous-feeding vermicomposting on days 0 (M0), 30 (M1), 90 (M3), 150 (M5) and mature vermicompost (V). (a) DGGE from bacterial 16S rDNA gene fragments PCR products. (b) DGGE from fungal 18S rRNA gene fragments. Ladder lanes are labelled with ∗.

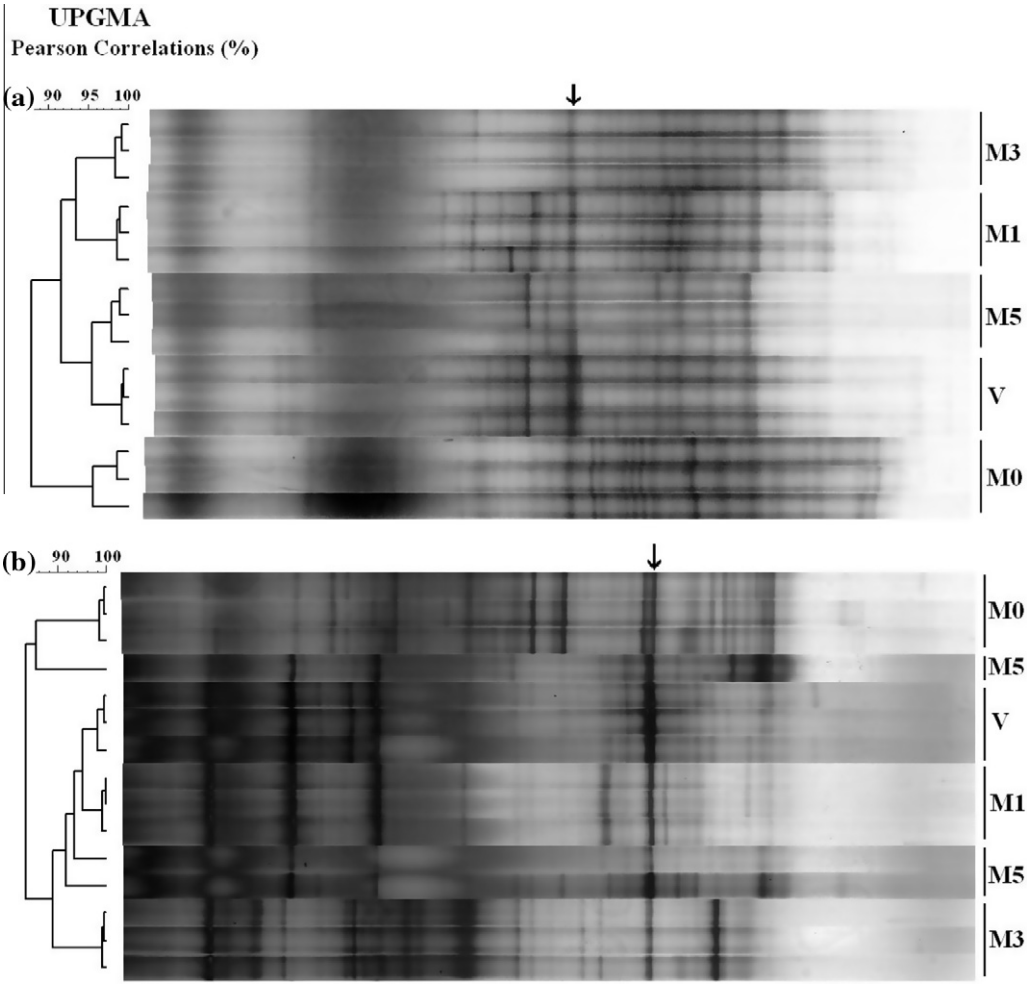


Fig. 4. Dendrogram depicting the relatedness of bacterial (a) and fungal communities (b) from triplicate samples taken during the continuous-feeding vermicomposting on days 0 (M0), 30 (M1), 90 (M3), 150 (M5) and mature vermicompost (V).

nutrients by the oxidation of organic matter (Lee, 1985). In agreement with these results, Tripathi and Bhardwaj (2004) reported increases in N and K, 150 days after *E. fetida* inoculation in a non-continuous vermicomposting system. Conversely, total Fe and Na measured in the vermicompost showed lower concentrations than that in the sheep manure, whereas P, Ca, Mg, and Mn did not show any significant change. Except in the case of Na, these results contrast with those commonly observed in non-continuous vermicomposting processes, in which these elements tend to increase as a result of organic matter mineralization (Benítez et al., 2002; Nogales et al., 2005).

3.4. Microbial community structure during vermicomposting and vermicompost maturation

The bacterial and fungal DGGE profiles were generated from a total of 15 samples (Fig. 3), i.e. three replicate vermicompost samples taken on days 0 (M0), 30 (M1), 90 (M3), 150 (M5), and the mature vermicompost (V). DGGE profiles of triplicate samples were found to be highly reproducible since, with one exception, the replicates from the same period of vermicomposting were more similar to each other than to other samples (Fig. 4). A complex banding pattern with more than 40 detectable bands appeared in all samples, indicating a high bacterial diversity in these complex microbial systems.

An UPGMA analysis of these DGGE profiles showed that the bacterial community profiles clustered into two main groups (Fig. 4a): samples from day 0 (M0) were discriminated from another cluster grouping all others (M1, M3, M5 and V). This implies that the bacterial community present in the initial samples was strongly affected by earthworm digestion. Therefore, the earthworm's activity could modify the microbial diversity of the vermibed by selectively feeding on and/or stimulating specific taxa. Recently, Monroy et al. (2009) demonstrated that the passage of the pig slurry through the gut of *E. fetida* reduced the total coliform numbers by 98%, but did not reduce the total abundance of bacteria providing a sound evidence of this selective effect. Within the main cluster including M1, M3, M5, and V, a temporal variation was also evident as M1 and M3 clustered separately from M5 and V. This suggests a change in the bacterial community concurrent with the two steps detected in the dynamics of vermicomposting. The first step was probably characterized by a bacterial community associated with the degradation of labile organic substrates. In the second step, marked by a decrease in microbial activity, the bacterial DGGE profiles presumably reflected a community involved in the stabilization of organic matter.

As for bacteria, UPGMA analysis clustered fungal profiles into two groups (Fig. 4b): one consisted of samples M0, and the other M1, M3, V and two out of three M5 samples. Within this cluster, a subcluster composed of samples taken on day 90 (M3) was evident. This points out a difference in the fungal community structure as a consequence of the high earthworm biomass recorded in this period (Fig. 1a), indicating that gut passage selected certain fungal species. According to Schönholzer et al. (1999) fungi represent a considerable food source for earthworms and are digested during gut passage. Likewise, Tiunov and Scheu (2000) found distinct effects on the abundance of certain phyla by gut passage through earthworms (*Lumbricus terrestris*).

All DGGE profiles from both bacterial and fungal communities shared a dominant band in the position indicated by an arrow in Fig. 4. The intensity of this band increased in the final vermicompost (V). This might represent microbial taxa associated with this type of vermicomposting. Toyota and Kimura (2000) demonstrated that *E. fetida* presents an indigenous gut-associated microflora which could contribute to the microbial community in mature vermicomposts. On the other hand, the gut microbiota of *Lumbricus rubellus* was found to be substantially affected by its diet (Knapp et al., 2009). Lores et al. (2006) reported that the specific microbial community fingerprint of a vermicompost is dependent on the animal manure and earthworm species used in the process.

4. Conclusions

The continuous-feeding system assayed was an effective method to revalue the liquid-paste of tomato-fruit waste under high organic loading rate conditions (13.6 kg TOC m^{-3} wk^{-1}). Thanks to the high moisture content of this waste, an extra water addition was not required to maintain optimal moisture for earthworm development, which is fundamental for the economy of the process under semi-arid conditions. Among all parameters studied, the enzyme activities were useful indicators of the process and should be used in subsequent trials to further optimise the waste-loading rate in order to allow even longer earthworm persistence and shorten the vermicomposting process (e.g. by reducing the amount of tomato-fruit wastes added and/or the frequency of feeding; by reducing the time of the vermicomposting process from 150 to 90 days and/or by starting the maturation step before). The study of the bacterial and fungal communities showed strong and specific changes in the vermibed's microbiota as a consequence of the degradation and stabilization of organic matter. The mature vermicompost constituted a value-added product which may be used as an organic amendment for agricultural soils. Therefore, the continuous-feeding vermicomposting has proven an adequate option for recycling tomato-fruit waste from greenhouses in an environmentally acceptable way.

Acknowledgements

This study was founded by the Junta de Andalucia (P05-AGR-00408). Manuel J. Fernández-Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant and his temporal stay in the University of Innsbruck (AP2006-03452). M. Goberna thanks support by the Marie Curie Actions (MEIF-CT-2006-041034). We thank the organic waste treatment plant in Motril (Granada, Southern Spain) for providing the tomato-fruit wastes. The authors also thank Celia Cifuentes for her technical support and David Nesbitt for assisting in the translation of the manuscript into English.

References

Bansal, S., Kapoor, K.K., 2000. Vermicomposting of crop residues and cattle dung with *Eisenia foetida*. Bioresour. Technol. 73, 95–98.

Benítez, E., Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol. 67, 297–303.

Benítez, E., Sainz, H., Melgar, R., Nogales, R., 2002. Vermicomposting of a lignocellulosic waste from olive oil industry: a pilot scale study. Waste Manage. Res. 20, 134–142.

Bonmatí, M., Ceccanti, B., Nannipieri, P., 1998. Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol. Biochem. 30, 2113–2125.

Castaldi, P., Garau, G., Melis, P., 2008. Maturity assessment of compost from municipal solid waste through the study of enzyme activities and water-soluble fractions. Waste Manage. 28, 534–540.

Edwards, C.A., 1988. Breakdown of animal, vegetable and industrial organic wastes by earthworms. In: Edwards, C.A., Neuhauser, E.F. (Eds.), Earthworms in Waste and Environmental Management. SPB Academic Publishing BV, The Hague, pp. 21–31.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plant 28, 123–134.

Government of Spain, 2005. Real Decreto 824/2005 Sobre Productos Fertilizantes. BOE. Government of Spain, Madrid, pp. 25602–25669.

Gunadi, B., Edwards, C.A., 2003. The effects of multiple applications of different organic wastes on the growth, fecundity and survival of *Eisenia fetida* (Savigny) (Lumbricidae). Pedobiologia 47, 321–329.

Heuer, H., Krsek, M., Baker, P., Smalla, K., Wellington, E.M., 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S

rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63, 3233–3241.
Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertl. Soils 6, 68–72.
Knapp, B.A., Podmirseg, S.M., Seeber, J., Meyer, E., Insam, H., 2009. Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. Soil Biol. Biochem. 41, 2299–2307.
Lee, K.E., 1985. Earthworms. Their Ecology and Relationships with Soils and Land Use, first ed. Academic Press, Sydney.
Lores, M., Gómez-Brandón, M., Pérez-Díaz, D., Domínguez, J., 2006. Using FAME profiles for the characterization of animal wastes and vermicomposts. Soil Biol. Biochem. 38, 2993–2996.
M.A.P.A., 1986. Official methods of analysis. Plants, Organic Products, Fertilizers, Soils, Waters and Organic Fertilizers, vol. III. Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación, Madrid.
Mitchell, A., 1997. Production of *Eisenia fetida* and vermicompost from feed-lot cattle manure. Soil Biol. Biochem. 29, 763–766.
Monroy, F., Aira, M., Domínguez, J., 2009. Reduction of total coliform numbers during vermicomposting is caused by short-term direct effects of earthworms on microorganisms and depends on the dose of application of pig slurry. Sci. Total Environ. 407, 5411–5416.
Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011–1016.
Nogales, R., Cifuentes, C., Benítez, E., 2005. Vermicomposting of winery wastes: a laboratory study. J. Environ. Sci. Health. 40, 659–673.
Nogales, R., Domínguez, J., Mato, S., 2008. Vermicompostaje. In: Moreno, J., Moral, R. (Eds.), Compostaje. Ediciones Mundi Prensa, Madrid, pp. 187–208.
Parra, S., Aguilar, F.J., Calatrava, J., 2008. Decision modelling for environmental protection: the contingent valuation method applied to greenhouse waste management. Biosyst. Eng. 99, 469–477.
Parthasarathi, K., Ranganathan, L.S., 1999. Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. Eur. J. Soil Biol. 35, 107–113.
Parthasarathi, K., Ranganathan, L.S., 2000. Aging effect on enzyme activities in pressmud vermicasts of *Lampito mauritii* (Kinberg) and *Eudrilus eugeniae* (Kinberg). Biol. Fertl. Soils 30, 347–350.
Sanguinetti, C.J., Neto, E.D., Simpson, A.J.G., 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17, 914–921.
Schönholzer, F., Hahn, D., Zeyer, J., 1999. Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. FEMS Microbiol. Ecol. 28, 235–248.
Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresour. Technol. 100, 804–811.
Senesi, N., 1989. Composted materials as organic fertilizers. Sci. Total Environ. 81–82, 521–542.
Sims, J.R., Haby, V.A., 1971. Simplified colorimetric determination of soil organic matter. Soil Sci. 112, 137–141.
Spanish Ministry of Agriculture, Fisheries and Food, 2007. Encuesta sobre Superficies y Rendimientos de Cultivos. Resultados, Madrid.
Speir, T.W., Ross, D.J., 1978. Soil phosphatase and sulphatase. In: Burns, R.G. (Ed.), Soil Enzymes. Academic Press, London, pp. 197–250.
Suthar, S., 2008. Bioconversion of post harvest crop residues and cattle shed manure into value-added products using earthworm *Eudrilus eugeniae* Kinberg. Ecol. Eng. 32, 206–214.
Suthar, S., 2009. Vermicomposting of vegetable-market solid waste using *Eisenia fetida*: impact of bulking material on earthworm growth and decomposition rate. Ecol. Eng. 35, 914–920.
Tabatabai, M.A., 1982. Soil enzymes. In: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. ASA and SSSA, Madison, pp. 907–943.
Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301–307.
Tiunov, A.V., Scheu, S., 2000. Microfungal communities in soil, litter and casts of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. Appl. Soil Ecol. 14, 17–26.
Toyota, K., Kimura, M., 2000. Microbial community indigenous to the earthworm *Eisenia foetida*. Biol. Fertl. Soils 31, 187–190.
Tripathi, G., Bhardwaj, P., 2004. Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg). Bioresour. Technol. 92, 275–283.
Vainio, E., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104, 927–936.
Williams, S., 1984. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed. Association of Official Analytical Chemists, Arlington.

Manuel Jesús Fernández-Gómez Ph.D. student, Manuel Fernández-Gómez obtained his Bachelor in Biology (2006) from the University of Granada (Spain). Since 2007, he has started his doctoral studies thanks to a Spanish Government Fellowship collaborating in the projects recycling greenhouse wastes as amendments for soil restoration and bioremediation processes. In 2008, he stayed in Innsbruck (Austria) for 5 months to work with Dr. Insam investigating microbial communities involved in vermicomposting and composting process. Likewise in 2009, he worked for 4 months as Scholar Visitor at the Ohio State University (United States) developing soil microbial ecology studies join to Dr. Richard P. Dick.

Rogelio Nogales Vargas-Machuca Ph.D. in Biology. Scientific researcher in the Estación Experimental del Zaidín, Granada (Spanish Council for Scientific Research, CSIC). Head of the Research Group "Soil-Plant Relationships". His research fields of expertise include recycling "vermicomposting" of organic wastes and in reuse of organic amendments for soils and agricultural crops protection. He has participated in more than 35 national and international funded R&D projects. He has authored more than 60 scientific articles and reviews, three books, more than 70 book's chapters and has directed 6 doctoral theses related with biotransformation and revalue of organic wastes.

Heribert Insam Ph.D. in Microbiology 1985, worked in Braunschweig, Germany and Calgary, Canada and is now Associate Professor at the Institute of Microbiology, University of Innsbruck (Austria). Head of the K-Regio Center BioTreaT (Biological Treatment and Recycling Technologies) and head of the Research Group Microbial Ecology. His fields of expertise are soil and environmental microbiology, recycling of organic wastes (composting and anaerobic digestion, biogas) and molecular ecology. He has participated in more than 30 funded R&D projects. He has authored more than 100 scientific articles and reviews, is editor of four books, expert evaluator of EU and Editor-in-chief of the journal Applied Soil Ecology.

Esperanza Romero Taboada Ph.D. in Pharmacy in 1990. Scientific researcher at the Estación Experimental del Zaidín, that is a research Center of the Spanish Council for Scientific Research (CSIC) Agency in Granada-Spain. She is specialist in the reutilization of organic wastes as soil amendments to control the behaviour of contaminants in the environment and to protect the quality of soil and water resources. She has participated in 11 international and 20 national R&D projects. Has 42 articles in journal included in the Journal Citation Reports and has more than 25 articles in other journals, as chapter of books, monographs, etc.

Marta Goberna Estellés Ph.D. in Biology (2004). Hired researcher at the Centro de Edafología y Biología Aplicada del Segura (Murcia, Spanish Council for Scientific Research, CSIC). She has worked in Valencia (2000–2006), Innsbruck (2006–2009, Marie Curie IntraEuropean Fellowship) and Murcia (since 2009, Marie Curie Reintegration Grant). Her fields of expertise are molecular microbial ecology of soils and organic wastes, and the use of microbial indicators for assessing soil degradation and restoration. MG is co-author of 12 articles in top international journals, seven contributions to book chapters or other journals, and co-editor of a book. Member of the editorial board of Applied Soil Ecology.

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 1/11

Hydrolases Activities of Extracted Humic Substances During Vermicomposting of Damaged Tomatoes Wastes Using a Continuous-Supplying System

Manuel J. Fernández-Gómez, Esperanza Romero, Celia Cifuentes and Rogelio Nogales

Abstract The behaviour of extracelullar hydrolytic enzyme activities in a continuous-supply vermicomposting system has never been studied previously. The aim of this study was to assess under such system the extracellular enzyme activities glucosidase, urease, acid phosphatase, and protease, which were extracted following the pyrophosphate-extraction method at pH 7.1. Vermicomposting was carried out using a medium-sized rectangular vermireactor continually supplied with damaged tomatoes (10 kg week^{-1}) during a 5-month period on a layer of mature sheep manure. The earthworms were then removed, and the vermicomposted organic material was matured over a 2-month period. Total earthworm biomass reached its greatest value after 3 months, coinciding with peak levels for pyrophosphate-extractable carbon content and extracellular urease, acid phosphatase and protease activities. Extracellular β-glucosidase activity peaked in the 4th month, when microbial activity was also at its greatest level. At the end of the vermicomposting period, phosphatase and protease activity decreased to levels similar to those recorded at the beginning of the vermicomposting process. By contrast, β-glucosidase and urease activity values were grater than those recorded at the start of the vermicomposting process. Humic-enzyme complexes generated during the vermicomposting period were unable to resist denaturation, inactivation, and degradation caused by the air-drying during the maturation phase. Although the mature vermicompost obtained showed higher content in humus-enzyme complexes than the initial mature sheep manure, the assayed continuous-supply vermicomposting system was unefficient for enhancing the formation of stabilised humus-enzymes complexes.

M. J. Fernández-Gómez · E. Romero · C. Cifuentes · R. Nogales (✉)
Department of Enviromental Protection, Estación Experimental del Zaidín, CSIC, c/Profesor Albareda 1, 18008 Granada, Spain
e-mail: rnogales@eez.csic.es

C. Trasar et al. (eds.), *Soil Enzymology in the Recycling of Organic Wastes and Environmental Restoration*, Environmental Science and Engineering,
DOI: 10.1007/978-3-642-21162-1_22, © Springer-Verlag Berlin Heidelberg 2011

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 2/11

1 Introduction

In Spain, approximately 66,100 ha are dedicated to greenhouse horticultural crops, most of which is located in Andalusia (Southern Spain). This intensive agricultural system generates large amounts of different types of waste every year, including vegetable wastes, which have become the greatest problem associated with this type of agriculture by exacerbating environmental problems associated with this crop. Damaged fruit waste consists of unmarketable fruits due to bruising, inappropriate size, rotting, and insect attack as well as marketable fruits that are discarded to raise market prices. In the Motril region alone (Granada, Southern Spain), about 15,000 Tm of fruit waste were rejected in 2007, representing 24% of all greenhouse waste generation. Particularly, tomato fruit accounts for 80% of all damaged greenhouse fruit waste generates in this region.

Vermicomposting is an effective low-cost method of transforming organic waste into a stable end-product called vermicompost, which is characterized by relatively high levels of humus-like substances, active microorganisms and enzymes. This biological process can be carried out on a small, medium, or large scale by using a windrow, bed, or bin system and flow-through reactors. The type of system adopted—non-continuous batch-supply mode or continuous-flow supply mode—depends on the nature of the waste input recycled (Edwards 1995). Previous works have shown that non-continuous vermicomposting systems are capable of producing organic soil amendments and biofertilizers from agricultural, agroindustrial, and urban waste (Elvira et al. 1998; Nogales et al. 2005; Melgar et al. 2009). However, to date, a continuous-supply system has never been used to transform fruit wastes into stabilized organic soil amendment.

Formation of complexes between humic substances and extracellular enzymes is a mechanism to stabilize and protect enzymes in soil, avoiding its denaturation, inactivation and degradation (Burns 1982; Nannipieri et al. 1996). In non-continuous vermicomposting processes, the activity of some extracellular hydrolytic enzymes extracted with pyrophosphate increased or remained constant, thus suggesting that the humus enzyme complexes resisted microbial and earthworm attack (Benítez et al. 2000, 2005). This is particularly relevant from an ecological point of view, as stabilised and active humus-enzyme complexes in soil environments can reactivate soil C, N, and P-cycles (Pascual et al. 2002). In addition, the active humus-enzyme complexes in the vermicomposts have been found to be particularly useful for soil biochemical remediation when they are used as soil amendments (Benítez et al. 2004; Romero el al. 2005)

As the behavior of extracellular hydrolytic enzyme activity in continuous-supply vermicomposting systems has never been studied, the objective of this study was to analyze the evolution of extracellular enzyme activities during a continuous vermicomposting in order to monitor the process. The relationship between extracellular enzyme activities and humic-like substances was also studied as a possible tool for characterizing the degree of stability of the vermicompost obtained as well as its potential biochemical contributions to soil quality

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 3/11

and fertility. To do this, β-glucosidase, acid phosphatase, protease, and urease extracellular enzyme activities were determined in the pyrophosphate extract during a continuous-supply vermicomposting process using damaged tomato-fruit.

2 Materials and Methods

A medium-sized rectangular metallic vermireactor (0.6 × 0.9 × 0.2 m) was designed, and 0.1 cm mesh was placed at the bottom of the vermireactor. A 5 cm layer containing 15 kg dry weight of sheep manure, whose chemical characteristics are described in Table 1, was placed on the mesh to provide an appropriate habitat for earthworms. A total of 500 g of clitellated and non-clitellated earthworms (*Eisenia fetida*) were inoculated in this layer. The worms came from a culture stock in the Estación Experimental del Zaidín (CSIC) in Granada (Spain). Fifteen days after earthworm inoculation, the vermireactor was filled with liquid-paste from damaged tomatoes (moisture: 92%, pH: 3.9, EC: 1.8 dS m^{-1}, TOC: 459 g kg^{-1}, TKN: 23 g kg^{-1} and C/N: 20) at a loading rate of 10 kg $week^{-1}$. The damaged tomatoes were obtained from greenhouse crops in the Motril region (Granada, Southern Spain). Vermicomposting was carried out under controlled temperature conditions (25°C), and no water was added other than that already present in the semiliquid tomato paste. After 5 months, the earthworms were removed by hand, and the vermicomposted organic substrate was left in a pile for a 2-month maturation period without water addition. During the vermicomposting process, the organic substrate was sampled monthly by means of five cylindrical cores (600 cm^3), which were evenly placed in the vermireactor. In each core, earthworm biomass was recorded, and the worms were replaced in the vermireactor. A fraction of the organic substrate ($\sim$100 g) contained in each core was taken, homogenized and stored in plastic vials at −20°C for analysis. In addition, five cores were also taken from the initial layer of sheep manure and the vermicompost obtained after the maturation period for analysis. Chemical and enzyme activities analysis of organic substrate contained in each core was carried out in triplicate.

The pH, electrical conductivity (EC), total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) were determined according to validated methods (MAPA 1986). The ammonium–N concentration (NH_4^+–N) was determined after extraction with 2 M KCl using a modified salicylate-nitroprusside colorimetric method (Kandeler and Gerber 1988). Water-soluble carbon (WSC) was extracted with distilled water (1:10 w/v) by mechanical shaking at 60°C for 1 h. WSC was analyzed in the supernatant after centrifugation 8,000 × g.

The pyrophosphate extractable carbon (PEC) was extracted with $Na_2P_4O_7$ (0.1 M, pH 7.1) in a 1:10 (w:v) ratio by mechanical shaking at 37°C for 24 h. The suspension was centrifuged at 8,000 × g; the supernatant was filtered through a 0.45 µm Millipore membrane, which was then dialysed for 7 days against distilled water, which was changed daily, in order to generate the pyrophosphate

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 4/11

Table 1 Chemical analyses in the sheep manure (S), at initial (V0) and after the vermicomposting period (V5), and in mature vermicompost (MV)

	pH	EC dS m^{-1}	NH_4^+–N mg kg^{-1}	TOC g kg^{-1}	TKN g kg^{-1}	WSC g kg^{-1}	C/N	Moisture %
S	8.6 ± 0.3^c	1.8 ± 0.1^b	438 ± 32^c	138 ± 6^b	9.6 ± 0.2^c	12 ± 0.5^c	14 ± 0.1^a	7 ± 0.2^c
V0	8.3 ± 0.5^c	1.7 ± 0.1^b	271 ± 42^b	140 ± 3^b	12 ± 1.2^b	18 ± 1.5^b	11 ± 0.6^b	55 ± 1^a
V5	9.5 ± 0.5^b	4.5 ± 0.1^a	$1{,}992 \pm 285^a$	280 ± 12^a	24 ± 0.9^a	25 ± 3^a	12 ± 0.5^b	57 ± 3^a
MV	10.4 ± 0.3^a	4.4 ± 0.03^a	$1{,}761 \pm 71^a$	148 ± 6^b	13 ± 0.4^b	17 ± 0.2^b	11 ± 0.1^b	25 ± 0.8^b

In each parameter, different letters indicate significant differences ($P < 0.05$)

extractable carbon (PEC) solution. Humic acid-like compounds (HAL) were extracted from 10 ml of a PEC solution by means of acidification with 97% H_2SO_4 (pH 1.0) and then centrifuged. The precipitate was dissolved in 10 ml of 0.5 M NaOH to obtain the HAL solution. To determine the C content of the WSC, PEC, and HAL solutions, 1 ml of each solution was digested with 1 ml of 1 N $K_2Cr_2O_7$ and 2 ml of 97% H_2SO_4 at 160°C for 30 min. Spectrophotometry ($\lambda = 590$ nm) was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} (Sims and Haby 1971).

Dehydrogenase activity was determined incubating 0.5 of solid organic sample at 25°C for 20 h with 0.2 mL of 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium chloride (INT). Controls without INT were also incubated. The iodonitrotetrazolium formazan (INTF) produced by the reduction of INT was extracted with acetone:tetrachloroethene (1.5:1) and measured in a spectrophotometer at 490 nm (García et al. 1997). To determine the absolute extracellular β-glucosidase and the absolute extracellular acid phosphatase activity, 0.5 ml of PEC solution was incubated at 37°C for 2 h with 0.5 ml of each enzyme-substrate solution and 2 ml of 0.1 M maleate buffer at pH 6.5. An enzyme-substrate solution with 0.05 M 4-nitrophenyl-β-D-glucanopyranoside (*p*-NG) or 0.115 M *p*-nitrophenyl phosphate (*p*-NPP) was used as enzyme-substrate for β-glucosidase or acid phosphatase activity, respectively. Controls were run as an enzyme test, but the enzyme-substrate was added at the end of incubation, before the determination of enzyme-product. The *p*-nitrophenol (*p*-NP) produced in the enzyme reaction was extracted and determined spectrophotometrically at 398 nm as described by Tabatabai and Bremner (1969). Absolute extracellular urease and protease activities were determined using 0.5 ml of PEC solution, 2 ml of 6.4% urea or 0.5 ml N-α-benzoyl-L-argininamide respectively as substrates, and 2 ml of 0.1 M phosphate buffer at pH 7.0. Controls were run as an enzyme test, but distilled waster was added instead of the enzyme-substrate. Enzyme tests and controls were incubated at 37°C for 2 h and straight afterwards the reaction was stopped cooling down at 2°C for 15 min as described by Nannipieri et al. (1980). The NH_4^+ released into the solution from the hydrolytic reaction was measured using an ammonium-selective electrode (ORION, mod. 95–12). The specific extracellular enzyme activity was calculated from the absolute extracellular enzyme activity values divided by the C content of the PEC solution.

The evolution of all parameters determined during the whole process was statistically tested using repeated analysis of variance (ANOVA) measures. The least significant difference test (LSD) was used for post-hoc mean separation. A paired-sample *t*-test was performed to assess the differences in chemical parameters between initial sheep manure and mature vermicompost. All statistical analyses were conducted using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

3 Results and Discussion

The weekly application of damaged tomatoes did not significantly increase total earthworm biomass during the first two months of vermicomposting (Fig. 1). Nevertheless, a pronounced increase was observed in the 3rd month, when the percentage of total earthworm biomass was 3.3-fold higher than that at the beginning of the process. However, earthworm biomass decreased sharply in the 4th month, in coincidence with the peak in the microbial activity, as indicated by dehidrogenase activity (Fig. 1), which is used to assess overall microbial activity during vermicomposting (Benítez et al. 1999). From the 4th month until the end of the vermicomposting process, total earthworm biomass decreased slightly, though not significantly. Conversely, a significant fall in dehydrogenase activity could suggest a microbial turnover in the vermireactor microbiota. The reduction in earthworm biomass could be due to the following factors (shown in Table 1): (i) an increase in ammonium concentration in the substrate as a consequence of the mineralization of proteins from the tomato-fruit waste and manure layer, (ii) an increase in pH value due to the decomposition of abundant organic anions in tomatoes, as reported for other plant residues (Xu et al. 2006), and (iii) an increase in electrical conductivity (EC) recorded in the vermireacot substrate due to the weekly salt intake by the tomatoes waste added to the vermireactor. These increases in the organic substrates are well known to negatively affect normal earthworm development during the vermicomposting process (Nogales et al. 2008). The increases in ammonium, pH and EC could be explained by: (i) excessive fresh organic matter in the vermireactor at the 3rd month as consequence of continuous additions of tomato-fruit waste and (ii) the high level of earthworm activity in the 3rd month which greatly stimulated microbial activity. These two factors triggered microbial growth and activity in the vermireactor substrate, leading to imminent system instability. To optimize the benefits of this continuous-supply system in subsequent trials, the organic loading rate should therefore be reduced, the earthworm population halved in the 3rd month, and another vermireactor constructed with an additional manure layer.

The pyrophosphate extractable carbon (PEC) and the humic-acid like substances (HAL) contents (Fig. 2) peaked in the 3rd month as a result of large amounts of organic matter from additions of tomato-fruit waste. The increase in both PEC and HAL may indicate that all the fresh substrate added was efficiently

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 6/11

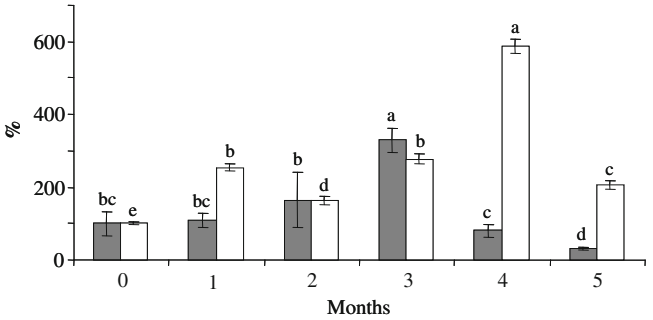


Fig. 1 Percentage changes in total earthworm biomass (■) and dehydrogenase activity (□) during the vermicomposting period. *Bars* represent standards errors. In each parameter, columns with different letters indicate significant differences ($P < 0.05$)

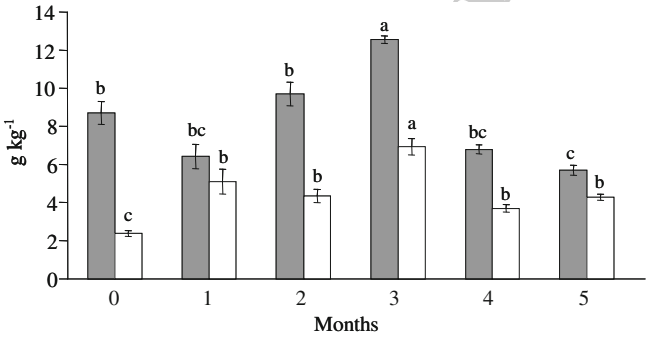


Fig. 2 PEC (■) and HAL (□) during the vermicomposting process. *Bars* represent standards errors. In each parameter, columns with different letters indicate significant differences ($P < 0.05$)

transformed by earthworms. However, from the 4th month until the end of the vermicomposting process, while more tomato-fruit waste was added, PEC and HAL content fell sharply. This could be attributed to a fall in earthworm biomass together with an increase in microbial activity. The PEC solution contains both humic-like and more recalcitrant compounds (Benítez et al. 2000) which were possibly not degraded by earthworms. Both types of compound could have subsequently been degraded by the proliferation of microorganisms in the 4th month. From the 4th month onwards, PEC and HAL content remained more or less stable.

Absolute extracellular β-glucosidase activity significantly increased and reached peak levels in the 4th month (Fig. 3a). Although this extracellular enzyme activity later decreased in the 5th month, it remained above the level recorded at the beginning of the vermicomposting process. The maximum level recorded by extracellular β-glucosidase activity coincided with the peak in microbial activity

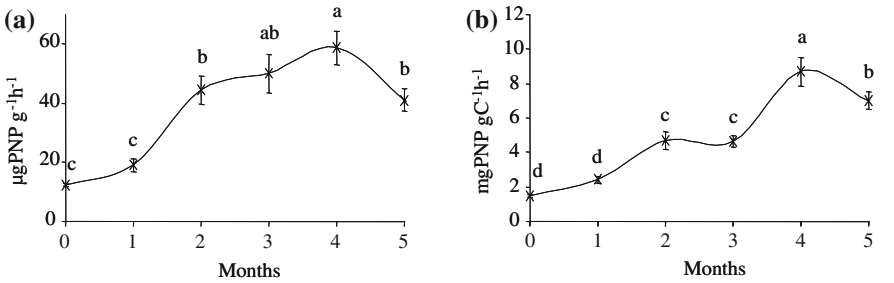


Fig. 3 Absolute (**a**) and specific (**b**) extracellular β-glucosidase activity during the vermicomposting process. Values are means of fifteen replicates. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

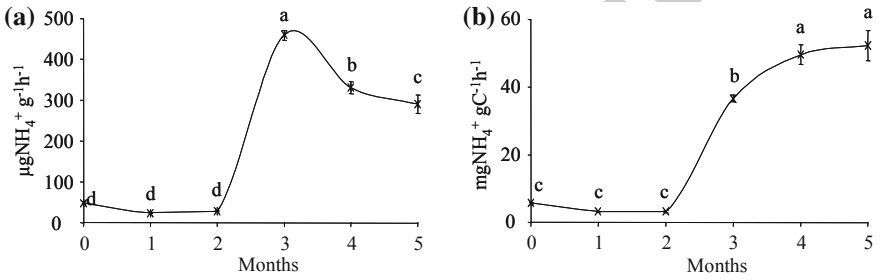


Fig. 4 Absolute (**a**) and specific (**b**) extracellular urease activity during the vermicomposting process. Values are means of fifteen replicates. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

(Fig. 1). This suggests that extracellular β-glucosidase activity greatly depends on microbes which are responsible for enzyme synthesis, whereas that the reduction in earthworm biomass, PEC, and HAL observed from the 4th month onwards did not affect this extracellular enzyme.

In contrast with absolute extracellular β-glucosidase activity, absolute extracellular urease activity (Fig. 4a) behaved differently, recording a sharper increase in the 3rd month, coinciding with growth in earthworm biomass (Fig. 1), and then fell significantly at the end of the vermicomposting process. This pattern of enzyme growth suggests that urease activity in organic extracts was strongly influenced by the earthworm population. On the other hand, despite the reduction in PEC and HAL recorded in the 5th month, the level of absolute extracellular urease activity was higher than that recorded at the beginning of the process. This suggests that a fraction of this extracellular enzyme may bind to humic matter during the vermicomposting process, thus able to remain active in the 5th month despite the high NH_4^+–N concentrations recorded in the substrate (Table 1), which inhibit extracellular enzyme activity (McCarty et al. 1992).

The absolute extracellular activity of acid phosphatase and protease showed a similar trend during the vermicomposting process (Figs. 5a and 6a). In both cases,

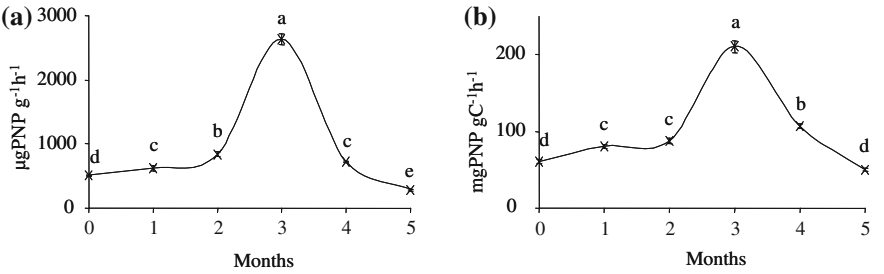


Fig. 5 Absolute (**a**) and specific (**b**) extracellular acid phosphatase activity during the vermicomposting process. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

enzyme activity increased significantly and reached a peak in the 3rd month, coinciding with maximum earthworm biomass and PEC content levels, and then decreased sharply to below the values recorded at the start of the process. As with the pattern for absolute extracellular urease activity, the highest level of acid phospatase and protease activity was recorded in the 3rd month, suggesting that the large earthworm population transformed the biological composition of the fresh organic matter through enrichment with microorganisms and extracellular enzymes. In line with these findings, Parthasarathi and Ranganathan (1999, 2000) reported that, compared to initial wastes, the casts freshly produced by earthworms have higher microbial, phosphatase and protease activity as well as larger microbial populations. The decrease in both types of absolute extracellular enzyme activities from the 3rd month onward could be explained by the reduction in earthworm biomass and the environmental constraints of adverse physico-chemical conditions (increased pH) and product inhibition (NH_4^+–N) observed in the vermireactor (Fig. 1 and Table 1).

The specific extracellular activity (activity per unit of extracted carbon) of theses enzymes showed a similar pattern to absolute extracellular activity (Figs. 3b, 5b and 6b), except in the case of specific extracellular urease activity (Fig. 4b), which unlike its absolute extracellular activity (Fig. 4a), continued to increase from the 3rd month up to the end of vermicomposting period. Benítez et al. (2000) concluded that the fact that both absolute and specific extracellular enzyme activity increase during vermicomposting gave rise to several assumptions: 1) the association of enzyme with humic substances did not affect activity enzyme sites, 2) the humic-enzyme complex is capable of resisting the microbial or earthworm attack as well as extracellular proteases and others inhibitory and degrading effects, and that 3) extracellular enzymes in vermicompost are more dependent on the type of humic compound than the quantity of C in the extract (García et al. 1993).

Slight increases in PEC and HAL was observed in mature vermicompost (MV) as compared with those recorded in fresh vermicompost (V5) (Table 2). In comparative terms, PEC and HAL content in the mature vermicompost (MV) was

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 9/11

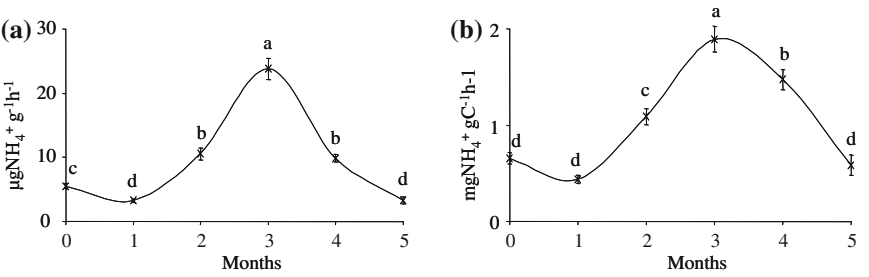


Fig. 6 Absolute (**a**) and specific (**b**) extracellular protease activity during the vermicomposting process. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

Table 2 PEC, HAL, absolute and specific extracellular hidrolases in the sheep manure (S), after the vermicomposting period (V5), and in the mature vermicompost (MV)

		S	V5	MV
PEC g kg^{-1}		2.1 ± 0.1^{c}	5.7 ± 0.3^{b}	6.3 ± 0.1^{a}
HAL g kg^{-1}		0.6 ± 1^{b}	4.3 ± 0.2^{a}	4.4 ± 0.03^{a}
β-glucosidase	Absolute (μg PNP g^{-1} h^{-1})	1.7 ± 0.2^{c}	41 ± 4^{a}	11 ± 0.4^{b}
	Specific (mg PNPg C^{-1} h^{-1})	0.8 ± 0.1^{c}	7 ± 0.5^{a}	1.8 ± 0.1^{b}
Acid phosphatase	Absolute (μg PNP g^{-1} h^{-1})	7.1 ± 1.5^{c}	287 ± 14^{a}	211 ± 4^{b}
	Specific (mg PNP gC^{-1} h^{-1})	2.9 ± 0.7^{c}	50 ± 1.3^{a}	34 ± 0.7^{b}
Protease	Absolute (μg NH_4^+ g^{-1} h^{-1})	14 ± 0.9^{a}	3.2 ± 0.5^{b}	0.7 ± 0.1^{c}
	Specific (mg NH_4^+ g C^{-1} h^{-1})	7.2 ± 0.4^{a}	0.6 ± 0.1^{b}	0.11 ± 0.01^{c}
Uréase	Absolute (μg NH_4^+ g^{-1} h^{-1})	$4.1 \pm 0.^{9}c$	291 ± 23^{a}	29 ± 1.6^{b}
	Specific (mg NH_4^+ g C^{-1} h^{-1})	2.0 ± 0.4^{c}	52 ± 4^{a}	4.6 ± 0.2^{b}

In each parameter, different letters indicate significant differences ($P < 0.05$)

3- and 7.7-fold higher, respectively, than the levels recorded in sheep manure (S). However, the absolute and specific extracellular enzyme activities were significantly lower in MV than those recorded in V5 (Table 2). It is important to note that most of the humic-enzyme complexes generated by the vermicomposting process were unable to resist denaturation, inactivation, and degradation caused by the air-drying of the vermicompost during the maturation phase (Table 1). Burns (1982) reported that the formation of humic complexes stabilizes enzymes and ensures their persistence, which would otherwise be impossible under adverse extracellular environmental conditions. On the other hand, it is well known that air-drying leads to inactivation of extracellular enzymes (Dick 1994). Since extracellular enzyme activity depends mainly on free enzymes in the vermicompost solution and a smaller fraction linked to humic substances, the drying of the vermicompost caused inactivation of free extracellular enzymes in the organic extract, while stabilized enzymes were protected against the adverse effects of low water content. Absolute and specific extracellular enzyme activities measured in air-dried mature vermicompost thus resembles the activity of enzymes closely linked to

Layout: T1 Standard SC | Book ID: 188219_1_En | Book ISBN: 978-3-642-21161-4
Chapter No.: 22 | Date: 20-5-2011 | Page: 10/11

humus colloids. We found that the levels of absolute and specific extracellular β-glucosidase, urease, and acid phosphatase activity were significantly higher in the mature vermicompost than in the initial sheep manure used (Table 2). However, extracellular protease enzyme complexes produced during the vermicomposting process were not sufficiently stabilized and were thus strongly degraded by drying during the maturation phase, as suggested by the lower extracellular enzyme activity recorded in the mature vermicompost as compared to the initial sheep manure.

4 Conclusions

Extracellular hydrolytic enzyme activities measured in the pyrophosphate extract during the continuous-supplying vermicomposting process showed a pattern that has not previously been reported. Extracellular β-glucosidase activity was related to the microbial activity, while extracellular urease, acid phosphatase, and protease activities were influenced by the earthworm biomass observed during the vermicomposting period. After the maturation period, all extracellular enzyme activity measured in the pyrophosphate extract eventually decreased due to air-drying as the free extracellular enzymes and enzymes weakly linked to humus colloids were denatured or inactivated. Therofore, the assayed continuous-supply vermicomposting system was unefficient for enhancing the formation of stabilised humus-enzymes complexes. Despite this fact, the mature vermicompost obtained showed greater extracelular enzyme activities (β-glucosidae, acid-phosphatase and urease) as compared with those recorded in the sheep manure used as an initial layer for tomato-fruit waste bioconversion.

Acknowledgements This study supported by Junta de Andalucía (Project P05-AGR-00408). M. Fernández-Gómez thanks the Science and Innovation Ministry for his FPU doctoral grant. Finally, we would also like to thank Michael O'Shea for proofreading.

References

Benítez E, Nogales R, Elvira C, Masciandaro G, Ceccanti B (1999) Enzymes activities as indicators of the stabilization of sewage sludges composting by *Eisenia andrei*. Bioresour Technol 67:297–303

Benítez E, Nogales R, Masciandaro G, Ceccanti B (2000) Isolation by IEF of humic–urease complexes from earthworms processed sewage sludges. Biol Fertil Soils 31:489–493

Benítez E, Melgar R, Nogales R (2004) Estimating soil resilience to toxic waste by measuring enzyme activities. Soil Biol Biochem 36:1615–1621

Benítez E, Sainz H, Nogales R (2005) Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresour Technol 96:785–790

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427

Dick RP (1994) Soil Enzyme Activities as Indicators of Soil Quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining Soil Quality for a Sustainable Environment. American Soil Association-Soil Science Society of America Special Publication No. 35, Madison,WI, pp 107–124

Edwards CA (1995) Historical overview of vermicomposting. BioCycle 36:56–58

Elvira C, Sampedro L, Benítez E, Nogales R (1998) Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: a pilot scale study. Bioresour Technol 6:205–211

García C, Henández T, Costa F, Ceccanti C, Ganni A (1993) Hydrolases in the organic matter fractions of sewage sludge: changes with composting. Bioresour Technol 45:47–52

García C, Hernández T, Costa F (1997) Potencial use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plant Anal 28:123–134

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

MAPA (1986) Métodos oficiales de análisis. Plantas, productos orgánicos fertilizantes, suelos, agua, productos fitosanitarios y fertilizantes inorgánicos, vol Vol. III. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid

McCarty GW, Shogren DR, Bremner JM (1992) Regulation of urease production in soil by microbial assimilation of nitrogen. Biol Fertil Soils 12:261–264

Melgar R, Benítez E, Nogales R (2009) Bioconversion of wastes from olive oil industries using the epigeic earthworm *Eisenia Andrei*. J Environ Sci Health–Part B 44:488–495

Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Nannipieri P, Sequi P, Fusi P (1996) Humus and Enzyme Activity. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier Science, Amsterdam, pp 293–328

Nogales R, Cifuentes C, Benítez E (2005) Vermicomposting of winery wastes: A laboratory study. J Environ Sci Health–Part B 49:659–673

Nogales R, Domínguez J, Mato S (2008) Vermicompostaje. In: Moreno J, Moral R (eds) Compostaje. Editorial Mundi-Prensa, Madrid, pp 187–2008

Parthasarathi K, Ranganathan LS (1999) Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. Eur J Soil Biol 35:107–113

Parthasarathi K, Ranganathan LS (2000) Aging effect on enzyme activities in pressmud vermicasts of Lampito mauritii (Kinberg) and Eudrilus eugeniae (Kinberg). Biology and Fertility of Soils 30:347–350

Pascual JA, Moreno JL, Hernández T, García C (2002) Persistence of immobilised and total urease and phosphatase activities in a soil amended with organic wastes. Bioresour Technol 82:73–78

Romero E, Benitez E, Nogales R (2005) Suitability of wastes from olive-oil industry for initial reclamation of a Pb/Zn mine tailing. Water, Air and Soil Pollution 165:153–165

Sims JR, Haby VA (1971) Simplified colorimetric determination of soil organic matter. Soil Sci 112:137–141

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenylphosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Xu JM, Tang C, Chen ZL (2006) The role of plant residues in pH change of acid soils differing in initial pH. Soil Biol Biochem 38:709–719

Anexo

Journal of Hazardous Materials 192 (2011) 1886–1889

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Short communication

Impact of imidacloprid residues on the development of *Eisenia fetida* during vermicomposting of greenhouse plant waste

Manuel J. Fernández-Gómez*, Esperanza Romero, Rogelio Nogales

Department of Environmental Protection, Estación Experimental del Zaidín (EEZ-CSIC), Profesor Albareda 1, 18008 Granada, Spain

ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 8 June 2011
Accepted 27 June 2011
Available online 1 July 2011

Keywords:
Vermicomposting
Imidacloprid
Plant waste
Worm development
Dehydrogenase activity

ABSTRACT

Pesticide application in agriculture causes residues in post-harvest plant waste at different concentrations. Knowledge concerning how pesticide concentrations in such waste affect earthworms is essential for recycling greenhouse plant debris through vermicomposting. Here, we have evaluated the effects of imidacloprid (IMD) residues on earthworms (*Eisenia fetida*) during the vermicomposting of plant waste from greenhouse crops in Spain. Before, the effect of different IMD concentrations on earthworms was tested using cattle manure as an optimum waste for worm development. The results after using cattle manure indicate that IMD dose $\geq$5 mg kg^{-1} hinders worm growth and even causes death, whereas IMD dose $\leq$2 mg IMD kg^{-1} allows worm growth similar to control but impedes reproduction. The results from the vermicomposting of plant waste reveal that IMD inhibits adequate worm growth and increases mortality. Although 89% worms became sexually mature in substrate containing 2 mg IMD kg^{-1}, they did not produce cocoons. IMD also affected microorganisms harboured in the substrates for vermicomposting, as indicated by the reduction in their dehydrogenase activity. This enzyme activity was restored after vermicomposting. This study provides a sound basis for the vermicomposting of pesticide-contaminated plant waste.

1. Introduction

Greenhouse crop systems are expanding worldwide, occupying more than 1.5 million hectares [1], since these systems protect crops from adverse meteorological conditions, allowing the production of high-value vegetables over the entire season. However, these profitable crops cause an environmental impact on the land where the greenhouses are located due to the enormous quantities of plant wastes discarded after the harvest, around 28.5 tonnes per greenhouse hectare and year [2]. In the region of Motril (Granada, southern Spain), where this study was conducted, this type of waste consists of leaves, stems, and roots of tomato plants, which is the predominant greenhouse crop in the region [3]. Contrary to plant waste from conventional crop systems, greenhouse plant wastes are hardly recycled through traditional composting because of its high moisture, low *C*/*N*, high salinity, and heavy pesticide load. Concerning pesticides, imidacloprid can frequently be contained in greenhouse plant wastes since this neonicotinoid systemic insecticide is marketed in 120 countries for controlling various species of whitefly and aphids in over 140 different agricultural crops [4]. In Spain, imidacloprid has been widely applied since 1992, becoming one of the most widely used products in diverse Spanish greenhouse crops (tomatoes, peppers, cucumbers, zucchini, and potatoes, etc.) [5]. Since this pesticide has been reported to persist in different concentration in stems and roots of plants, depending on its application method [6], variables amount of imidacloprid are expected in greenhouse plant wastes. Thus, greenhouse plant wastes containing imidacloprid are an important hazard to the environment because this pesticide is categorized as moderately toxic and with a high potential to leach into groundwaters [7]. Hence, the recycling of the greenhouse plant wastes is necessary to avoid soil and water pollution as well as to establish sustainable agriculture.

Vermicomposting is a biotechnological process that enables the recycling of organic wastes into fertilizers through the joint action of earthworms and microorganisms. Among the epigeic earthworm species for vermicomposting, *Eisenia fetida* is the worm most commonly used in temperate climates. Although cattle manure is the optimum substrate to culture *E. fetida* [8], previous studies have demonstrated that this worm enables the vermicomposting of other organic wastes such as greenhouse debris [9,10]. Taking into account that earthworm growth and sexual development have a key role driving waste stabilization during vermicomposting [11], the evaluation of the effect of the imidacloprid on *E. fetida* development and survival is essential to provide full information on the feasibility of vermicomposting greenhouse plant waste containing this insecticide. The impact of pesticides on *E. fetida* growth

* Corresponding author. Tel.: +34 958 181600; fax: +34 958 129600.
E-mail address: manuelj.fernandez@eez.csic.es (M.J. Fernández-Gómez).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.06.077

and reproduction has been reported by Yasmin and D'Souza [12], who compiled information on how different pesticides affect to this earthworm species. Concerning imidacloprid, previous studies reported that this pesticide is harmful to *E. fetida* [13,14]. However, to date, the literature available provides information only on effects of imidaclopird on *E. fetida* worms placed in aqueous solutions, paper-filters, or soils. Therefore, it remains to be determined how this pesticide affects this worm species during vermicomposting, a biological process in which other factors influence earthworm development.

The aim of the present study was to evaluate how different concentrations of imidacloprid residues affect *E. fetida* growth and reproduction during the vermicomposting of tomato-plant waste generated from greenhouses. The effect of imidacloprid on the microbial activity in this vermicomposting substrate was also studied by analysing dehydrogenase enzyme activity. Previously, a basic experiment was conducted with worms raised on an optimum organic substrate for *E. fetida* (i.e. cattle manure), which was fortified with four increasing concentrations of imidacloprid. This first experiment was made to assess how imidacloprid affects well-nourished worms, determining what minimum concentration of imidacloprid negatively affects *E. fetida* as well as what imidacloprid concentration impedes the worm growth (or survival) on an optimal organic waste. Thus, it was decided that imidacloprid concentrations should be assayed for the vermicomposting of greenhouse tomato-plant waste, besides the effect of this sub-optimum substrate.

2. Materials and methods

Imidacloprid (IMD) (1–[(6-cloro-3 piridinyl)methyl]-N-nitro-2-imidazolidinimina) 99% purity from Ehrenstorfer (Augsburg, Germany) was used into milliQ-water solution. Non-clitellated earthworms (*E. fetida*) were selected from a culture bank at the Estación Experimental del Zaidín (Granada, Spain). Cattle manure (75% moisture, 8.5 pH, 9 dS m^{-1} electrical conductivity, 401 g kg^{-1} total organic carbon, 15 g kg^{-1} total nitrogen) constitutes by a mixture of faeces and urine without any bedding material, was collected from a farmyard of dairy cows (Granada, Spain). Plant waste (23% moisture, 7.2 pH, 10 dS m^{-1} electrical conductivity, 386 g kg^{-1} total organic *C*, 17 g kg^{-1} total *N*) was produced by tomato crops without pesticide treatment in greenhouses located in Motril (Granada, Spain). This plant waste was air-dried, chopped to particle sizes of less than 1 cm and mixed with paper-mill sludge at a 2:1 ratio (dw:dw) in order to improve the structure and moisture conditions and thereby optimise the vermicomposting process. Paper-mill sludge (64% moisture, 8.2 pH, 0.6 dS m^{-1} electrical conductivity, 175 g kg^{-1} total organic *C*, 7.3 g kg^{-1} total *N*) was collected from a wastewater treatment plant of a paper company also located in Motril.

Two vermicomposting processes were undertaken. Firstly, Petri dishes (10 cm diameter) were filled with 30 g of cattle manure containing IMD at the following concentrations: 0 (control), 1, 2, 5, and 10 mg kg^{-1}. Three Petri dishes per each IMD concentration were inoculated with four juvenile non-clitellated earthworms (*E. fetida*) weighing 250 mg each one. All Petri dishes were kept in darkness at 20 °C, maintaining their moisture by periodical watering at 80%. Earthworm growth, sexual development, and reproduction were successively monitored in each Petri dish over the time (1, 2, 3, 6, 10, 14, 21, 28, 35, 42, and 49 days). The second vermicomposting process consisted of using plastic containers (12 cm diameter × 13 cm high) which had perforated bottoms for aeration and drainage. Containers were filled with 200 g of the mixture of plant waste and paper-mill sludge contaminated with 0 (control), 2, 4, and 8 mg IMD kg^{-1} plant waste. A thin layer of cattle manure (1 cm) was placed on top of each treatment and 25 non-clitellated earthworms (total worm biomass ~5000 mg) were placed within. All containers were kept under the same conditions as described above. Earthworm development was monitored weekly for 15 weeks. Dehydrogenase enzyme activity was analysed in the substrate containing IMD before worm inoculation and also at the end of vermicomposting period, following the method described by García et al. [15].

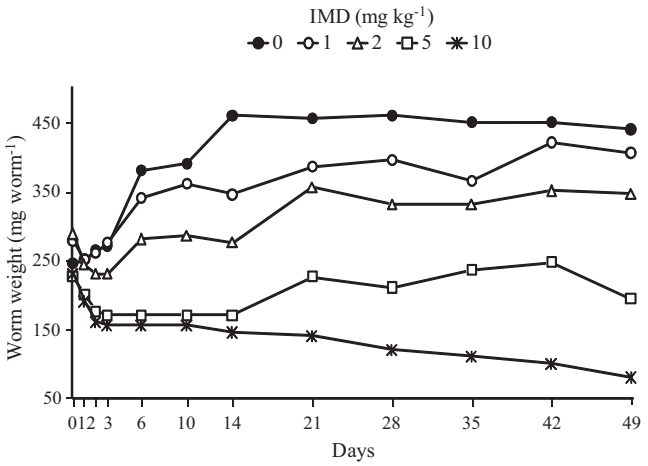


Fig. 1. Changes in the weight of *Eisenia fetida* individuals over time in cattle manure containing imidacloprid (IMD) at 0, 1, 2, 5 and 10 mg kg^{-1}.

Repeated-measures ANOVA analyses were performed to evaluate the effect of time on the worm development during the vermicomposting processes, determining the interactive effect of the time with the assayed IMD concentrations. The Kruskall–Wallis and Mann–Whitney tests were applied to assess the differences in dehydrogenase activity among treatments due to the non-normality of these data. Statistical analyses were conducted at >95% confidence level ($P < 0.05$) using SPSS® Windows (Chicago, Illinois, USA).

3. Results

The weight of the worms fed on cattle manure significantly varied over time ($F = 6.99$, $P < 0.01$), depending on the IMD concentration contained in this substrate ($F = 2.96$, $P < 0.01$) (Fig. 1). Thus, *E. fetida* worms raised on cattle manure containing concentrations of IMD ≥2 mg kg^{-1} were unable to significantly increase their weight over time, while a significant increase in the individual worm biomass was recorded in cattle manure containing 1 mg IMD kg^{-1} ($F = 2.35$, $P = 0.03$), which was similar to that in the control without IMD. By contrast, a significant reduction in the individual worm biomass was observed over time in the manure containing 10 mg IMD kg^{-1} ($F = 5.50$, $P < 0.01$). Fig. 2 offers an overall view of the effect of increasing concentrations of IMD in worm weight, revealing that the logarithmic worm-weight gain (with respect of initial worm weight), at 14, 35, and 49 days, significantly fit a negative linear regression in relation to IMD concentrations at ($P < 0.01$). In this figure the regression slope decreases almost two-fold from 14 to 49 days. This indicates that high doses of IMD strongly hindered worm growth as opposed to low doses, which allowed worms to increase their initial weight over time. With regard to sexual development, in contrast to control, none of the worms growing in manure with IMD developed a clitellum, thus remaining sexually immature. Only the higher IMD concentrations, 5 and 10 mg kg^{-1}, caused worm mortality of 25% and 80%, respectively.

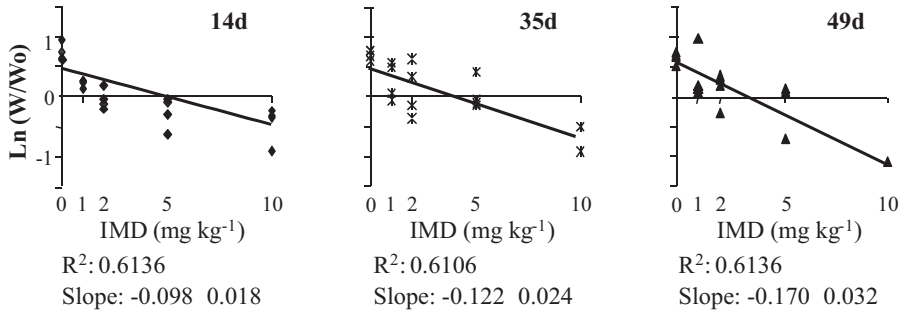


Fig. 2. Logarithmic worm-weight gain at 14, 35 and 49 days (worm weigh [W]/initial worm weight [W0]) in cattle manure containing imidacloprid (IMD) at 0, 1, 2, 5 and 10 mg kg^{-1} manure.

When *E. fetida* was used to vermicompost the plant waste mixed with paper-mill sludge, the mixture without IMD did not provide good nourishment, resulting in a sub-optimal worm development as compared with cattle manure. Indeed, a strong reduction of 31% in the number of worms ($F = 18.89$, $P = 0.05$) was recorded after one week in the control without IMD (Fig. 3a). Plant wastes containing IMD made this decline significantly stronger in the initial worm population, for reductions of 89% at 2 mg IMD ($F = 179.56$, $P < 0.01$), 81% at 4 mg IMD ($F = 75.94$, $P = 0.01$, and 95% at 8 mg IMD kg^{-1} plant waste ($F = 720.14$, $P < 0.01$) over a week. To offset the substrate influence on worm development and to continue assessing the effect of IMD, we restocked the dead worms in the 2nd week, reaching the same worm number (25) and biomass (~5 g) as at the beginning of the experiment. Then, during the next 10 weeks, an overall significant decline in the worm population was registered in all the vermicomposting processes ($F = 74.83$, $P < 0.01$), in which decreasing tends were similar to each other (Fig. 3a). This reduction in worm number coincided with overall increases in the weights of surviving worms from the 2nd until the 10th week ($F = 32.12$, $P < 0.01$; Fig. 3b). The average weight gain recorded over time was significantly different among the substrates with different IMD concentrations ($F = 5.10$, $P = 0.03$). Thus, individual worm biomass rose by 245% in control after seven weeks, while gains of 120, 116, and 32% were found in substrates containing 2, 4, 8 mg IMD kg^{-1} plant waste, respectively. With respect to the sexual development of *E. fetida*, worms became mature in the substrate without IMD by the 3rd week, while this occurred at the 6th week in the case of substrates containing any IMD concentration. At the 7th week, 43, 47, and 13% of the worms displayed a clitellum in the vermicomposting substrates containing 2, 4, or 8 mg IMD kg^{-1} plant waste, respectively, while 83% of control worms reached maturity. At the 10th week, the percentage of mature worms found in the vermicomposting substrate containing 2 mg IMD kg^{-1} plant waste was similar to that in the control without IMD (89%), but only 58 and 39% maturity was found in the case of the substrates containing 4 and 8 mg IMD kg^{-1} plant waste, respectively. Despite that some worms reached sexual maturity in the substrates containing IMD, they did not produce cocoons. By contrast, great numbers of cocoons were detected in the substrate control, with newly hatched earthworms appearing after the 11th week. Consequently, the worm population recorded in the vermicomposting substrate without IMD at the end of the vermicomposting was significantly higher period compared with that in the substrates with IMD ($F = 7.59$, $P = 0.01$) (Fig. 3b).

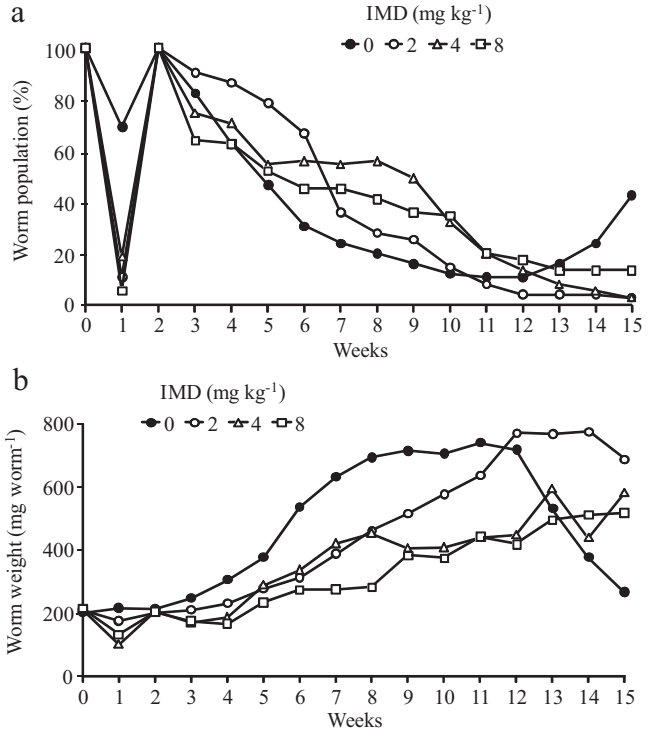


Fig. 3. Percentage of worm number in relation to initial worm number (a), and weight of surviving-individuals of *Eisenia fetida* (b) during the vermicomposting of greenhouse tomato-plant waste containing imidacloprid (IMD) at 0, 2, 4, 8, and 10 mg kg^{-1}.

Before worm inoculation, the dehydrogenase activity in the vermicomposting substrates containing the assayed IMD concentrations was significantly lower than control without IMD ($\chi^2 = 8.13$, $P = 0.04$) (Fig. 4). The IMD concentration of 8 mg kg^{-1} plant waste caused the greatest reduction in this activity, which diminished 60% as compared to that of the control. After vermicomposting, dehydrogenase activity values in the vermicomposts resulting from plant wastes containing IMD were similar to those of the vermicompost from the control ($\chi^2 = 4.44$, $P = 0.22$).

4. Discussion

The results for worms optimally fed on cattle manure suggests that IMD concentrations ≥2 mg kg^{-1} depresses *E. fetida* growth, whereas at concentrations ≥10 mg IMD kg^{-1} the high mortality caused impedes vermicomposting. The 25% mortality noted after 7 weeks in manure containing 5 mg IMD kg^{-1} differs from the results of studies using artificial soil contaminated with 3.5 mg IMD kg^{-1}, in which 50% of mortality in *E. fetida* population was reported after a week [13]. This suggests that the toxicity of IMD for *E. fetida* could

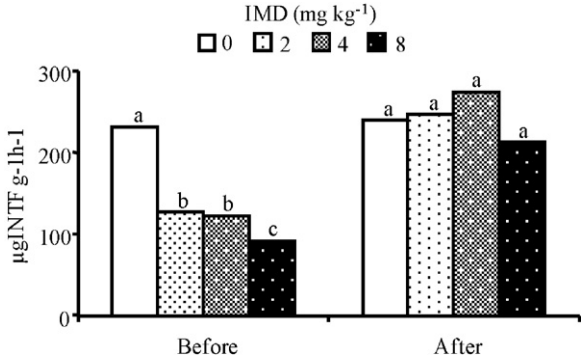


Fig. 4. Dehydrogenase activities in greenhouse tomato-plant waste containing imidacloprid (IMD) at 0, 2, 4, 8, and 10 mg kg^{-1}, before and after their vermicomposting. Different letters indicate significant differences ($P < 0.05$) among the dehydrogenase activities of the initial substrates or among the dehydrogenase activities of the end vermicomposts.

be tempered by vermicomposting process if optimum substrates are used.

In the case the vermicomposting of tomato-plant waste from greenhouse, its mixture with paper-mill sludge was initially inadequate for worm development, as indicated the worm mortality in the substrate without IMD. This may be because this mixture was not sufficiently nutritious to support the initial population added. Thus, after a reduction in the worm population, the surviving worms could properly feed on this substrate, gaining weight and they became sexually mature, giving rise to new worm offspring in the control without IMD. The presence of IMD in the plant waste intensified the reduction in worm population, and also hindered the weight gain of surviving worms, as occurred in the control without IMD. Among the different concentrations of IMD tested, only the lowest (2 mg IMD kg^{-1} plant waste) enabled worms to grow acceptably and become sexually mature. However, the presence of IMD impeded cocoon production by the sexually mature worms. This suggests that IMD prevent the *E. fetida* reproduction. This could be explained by the finding of Luo et al. [13], who reported that 0.5 mg IMD kg^{-1} in artificial soil caused significant sperm deformity in *E. fetida*.

Dehydrogenases are intracellular enzymes involved in oxidative phosphorylation, the analysis of which provides a measure of the overall activity of microorganisms [15]. Furthermore, this activity has previously been used as a reliable biomarker for analysing the negative impact of pesticides on the resident microbiota in vermicomposting [16]. Hence, the reduction in dehydrogenase activity initially caused by the presence of IMD in vermicomposting substrate indicates that this pesticide depleted its inhabiting microorganisms. This negative effect may be partially responsible for the lower worm fattening observed in the substrates containing IMD, since it is well known that microorganisms constitute a major part of the diets of earthworms [17]. The restoration of dehydrogenase activity after vermicomposting suggests that this process was able to recover the formerly depleted microbial population.

In conclusion, in practical terms, the vermicomposting of greenhouse plant-waste containing IMD concentrations ≤ 2 mg kg^{-1} is feasible. However, the presence of this insecticide in the vermicomposting substrate impedes the *E. fetida* reproduction. Further research is needed to ascertain whether the vermicomposting of organic wastes contaminated at low concentrations of this pesticide would enable the mineralization of this pesticide.

Acknowledgements

This study was financed by "Junta de Andalucía" project P05-AGR-00408. M.J. Fernández-Gómez thanks the Science and Innovation Ministry for their FPU doctoral grant (AP2006-03452). The authors thank D. Nesbitt for assisting in the translation of the manuscript into English.

References

[1] E. Espi, A. Salmeron, A. Fontecha, Y. García, A.I. Real, Plastic films for agricultural applications, J. Plastic Film Sheet. 22 (2006) 85–102.
[2] F. Manzano-Agugliaro, Gasificación de residuos de invernadero para la obtención de energía eléctrica en el sur de España: ubicación mediante SIG, INCI 32 (2007) 131–136.
[3] Ministerio de medio ambiente y medio rural y marino, Encuesta sobre Superficies y Rendimientos de Cultivos, Gobierno de España, Madrid, 2010.
[4] D. Drobne, M. Blazic, C.A.M. Van Gestel, V. Leser, P. Zidar, A. Jemec, P. Trebse, Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea), Chemosphere 71 (2008) 1326–1334.
[5] D. Hernández, V. Mansenét, J.M. Puiggrós Jové, Use of Confidor 200 SL in vegetable cultivation in Spain, Pflanzenschutz-Nachrichten Bayer 52 (1999) 364–375.
[6] R. Juraske, F. Castells, A. Vijay, P. Muñoz, A. Antón, Uptake and persistence of pesticides in plants: measurements and model estimates for imidacloprid after foliar and soil application, J. Hazard. Mater. 165 (2009) 683–689.
[7] World Health Organization, Recommended classification of pesticides by hazard and guidelines to the classification, World Health Organization, Geneva, 2006.
[8] A.J. Reinecke, S.A. Viljoen, The influence of feeding patterns on growth and reproduction of the vermicomposting earthworm *Eisenia fetida* (Oligochaeta), Biol. Fertil. Soils 10 (1990) 184–187.
[9] M.J. Fernández-Gómez, R. Nogales, H. Insam, E. Romero, M. Goberna, Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops, Waste Manage. 30 (2010) 2461–2468.
[10] M.J. Fernández-Gómez, E. Romero, R. Nogales, Feasibility of vermicomposting for vegetable greenhouse waste recycling, Bioresour. Technol. 101 (2010) 9654–9660.
[11] P.M. Ndegwa, S.A. Thompson, K.C. Das, Effects of stocking density and feeding rate on vermicomposting of biosolids, Bioresour. Technol. 71 (2000) 5–12.
[12] S. Yasmin, D. D'Souza, Effects of pesticides on the growth and reproduction of earthworm: a review, Appl. Environ. Soil Sci. 2010 (2010) 1–9.
[13] Y. Luo, Y. Zang, Y. Zhong, Z. Kong, Toxicological study of two novel pesticides on earthworm *Eisenia foetida*, Chemosphere 39 (1999) 2347–2356.
[14] Y. Zang, Y. Zhong, Y. Luo, Z.M. Kong, Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*, Environ. Pollut. 108 (2000) 271–278.
[15] C. García, T. Hernández, F. Costa, Potential use of dehydrogenase activity as an index of microbial activity in degraded soils, Commun. Soil Sci. Plan. 28 (1997) 123–134.
[16] E. Benítez, R. Nogales, C. Elvira, G. Masciandaro, B. Ceccanti, Enzyme and earthworm activities during vermicomposting of carbaryl-treated sewage sludge, J. Environ. Qual. 28 (1999) 1099–1104.
[17] C.A. Edwards, K.E. Fletcher, Interactions between earthworms and microorganisms in organic-matter breakdown, Agric. Ecosyst. Environ. 24 (1988) 235–247.

Bioresource Technology 102 (2011) 9638–9645

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Role of vermicompost chemical composition, microbial functional diversity, and fungal community structure in their microbial respiratory response to three pesticides

Manuel J. Fernández-Gómez [a,*], Rogelio Nogales [a], Heribert Insam [b], Esperanza Romero [a], Marta Goberna [b]

[a] Department of Environmental Protection, Estación Experimental del Zaidín (EEZ-CSIC), Profesor Albareda 1, 18008 Granada, Spain
[b] Institute of Microbiology, University of Innsbruck, Technikerstraβe 25, A-6020 Innsbruck, Austria

ARTICLE INFO

Article history:
Received 20 May 2011
Received in revised form 25 July 2011
Accepted 29 July 2011
Available online 5 August 2011

Keywords:
Microbial functional diversity
Pesticides
Fungal community structure
Enzyme activity
Community-level physiological profile

ABSTRACT

The relationships between vermicompost chemical features, enzyme activities, community-level physiological profiles (CLPPs), fungal community structures, and its microbial respiratory response to pesticides were investigated. Fungal community structure of vermicomposts produced from damaged tomato fruits (DT), winery wastes (WW), olive-mill waste and biosolids (OB), and cattle manure (CM) were determined by denaturing gradient gel electrophoresis of 18S rDNA. MicroResp™ was used for assessing vermicompost CLPPs and testing the microbial response to metalaxyl, imidacloprid, and diuron. Vermicompost enzyme activities and CLPPs indicated that WW, OB, and DT had higher microbial functional diversity than CM. The microbiota of the former tolerated all three pesticides whereas microbial respiration in CM was negatively affected by metalaxyl and imidacloprid. The response of vermicompost microbiota to the fungicide metalaxyl was correlated to its fungal community structure. The results suggest that vermicomposts with higher microbial functional diversity can be useful for the management of pesticide pollution in agriculture.

1. Introduction

Vermicomposting is a low-cost biotechnology which enables the recycling of a variety of wastes from different nature through the combined action of earthworms and microorganisms. Vermicomposts were early reported as bioactive amendments housing microbial communities relevant to improve soil fertility (Kale et al., 1992). Apart from being excellent organic products for agriculture, vermicomposts can be considered useful materials for restoring pesticide contaminated soils as they enhance the adsorption of pesticides reducing the environmental risk of pesticide leaching towards groundwaters (Romero et al., 2006). Fernández-Bayo et al. (2009) reported that amending soils with vermicomposts fosters dissipation of pesticides, such as diuron in agricultural soils. On the other hand, vermicomposts have also been reported as suitable materials for developing bioremediation tools such as biobarriers (Moreno et al., 2009) or biocovers (Moon et al., 2010). In this sense, vermicomposts could be promising organic materials to constitute biomix layers in biobed systems, like thermophilic-composts, which have already been reported to be effective for adsorbing and degrading pesticides in biobeds (Vischetti et al., 2008). Recently, Blaszak et al. (2011) have isolated microorganisms capable of biodegrading the pesticide simazine from a vermicompost produced from manure, suggesting that vermicomposts are a source of pesticide-biodegrading microorganisms. Hence, further knowledge on the resident microbial community in a vermicompost could help to predict its potential utility for pesticide bioremediation.

It is expected that vermicomposts produced from different parental wastes have different chemical compositions and dissimilar autochthonous microbial communities. However, to date, the interrelationships between the chemical features of different vermicomposts and the functional diversity of their resident microbiota are still unclear. Moreover, there are no studies which provide information on the connection between the functional diversity of vermicompost microbiota and its responses to pesticides. Thus, it remains to be elucidated to what extent microbial functional diversity of a vermicompost could be related with its potential utility for pesticide bioremediation. Relevant information on this topic could be inferred by investigating the impact of pesticides on the microbiota of different vermicompost types along with their chemical features and microbial functional diversity. The microbial functional diversity of different vermicomposts can be assessed by

Abbreviations: CLPP, community-level physiological profile; DGGE, denaturing gradient gel electrophoresis; PCA, principal component analysis; RI, respiration index; TOC, total organic carbon; DT, vermicompost from damaged tomato fruits; WW, vermicompost from winery wastes; OB, vermicompost from olive-mill waste mixed with biosolids; CM, vermicompost from cattle manure; WSC, water soluble carbon.

* Corresponding author. Tel.: +34 958 181600x255; fax: +34 958 129600.
E-mail address: manuelj.fernandez@eez.csic.es (M.J. Fernández-Gómez).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.07.113

determining their community-level physiological profile (CLPP), which captures the ability of the vermicompost microbiota to metabolize single carbon substrates (Campbell et al., 2003; Mondini and Insam, 2005). Hill et al. (2000) reported that if CLPPs from two samples are clearly separated, then the functional diversity of their resident microbial communities can be considered different. MicroResp™ is a micro-respiration system designed for determining the CLPPs of microbial communities housed in whole-substrates, avoiding the disadvantages of other CLPP approaches based on culturing of microorganisms on plates such as the Biolog system (Campbell et al., 2003). In addition, the great versatility of this system makes it useful for assessing the effect polluting substances, such as pesticides, on the respiratory activity of microbiota inhabiting organic substrates (Campbell et al., 2003). On the other hand, the assessment of enzyme activities, such as oxidoreductases and hydrolases, in vermicomposts has also been reported useful for studying the biochemical functional diversity of vermicompost microbiota (Benítez et al., 1999; Vivas et al., 2009). Recently, Sen and Chandra (2009) reported that the changes in enzyme activities and CLPP occurred during the vermicomposting of sugarcane waste allows assessing the functional diversity of the microbiota involved in vermicomposting. Hence, the joint analysis of enzyme activities and CLPPs seems to be a suitable approach to investigate the functional diversity of microbial communities housed in diverse vermicomposts. On the other hand, the high activity of some hydrolytic enzymes in a vermicompost could be related to the potential ability of its resident microbiota to degrade certain organic substrates and xenobiotics. For instance, Fernández-Bayo et al. (2009) suggested that the high urease activity found in diuron contaminated soils, which had been amended with a vermicompost produced from spent grape marc, could be related to the hydrolysis of this nitrogen-containing herbicide as ureases catalyses the cleavage of N–C bonds in ureic compounds.

Fungal communities play a very important role as an active component of the vermicompost microbiota (Aira et al., 2006). Previous studies reported that vermicomposts house a high diversity of fungi able to degrade a variety of compounds (Anastasi et al., 2005). In addition, most fungi tolerate high concentrations of polluting chemicals, since they have a complex enzymatic machinery able to degrade complex polymers and xenobiotics such as pesticides (Bending et al., 2002). Thereby, the fungal community structure in vermicompost might be related to the response of its microbiota to pesticides. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 18S rRNA gene fragments has been reported as a useful fingerprinting technique for determining the structure of fungal communities inhabiting complex environmental samples (Vainio and Hantula, 2000). Previous studies have highlighted that DGGE is an easy, fast and reproducible technique to compare the fungal community development during the different phases of vermicomposting (Fernández-Gómez et al., 2010).

In view of the above, this study investigated the chemical features, enzyme activities (dehydrogenase, β-glucosidase, acid phosphatase, and urease), community-level physiological profile, and the fungal community structure of four different vermicomposts, exploring the relationships between these parameters and the respiratory response of the vermicomposts' microbiota to the fungicide metalaxyl, the insecticide imidacloprid, and the herbicide diuron.

2. Methods

2.1. Vermicompost collection

The vermicomposts analysed in this study were the following: a vermicompost from damaged tomato fruits (DT), one from winery wastes (WW), one from olive-mill waste mixed with biosolids (OB), and one from cattle manure (CM). All these vermicomposts were produced by using the earthworm species *Eisenia fetida*. DT was produced by vermicomposting damaged tomato fruits through an indoor continuous-flow reactor as described by Fernández-Gómez et al. (2010). Briefly, 500 g of earthworms were inoculated in a layer of sheep manure (15 kg) placed in the bottom of the reactor, and 10 kg of damaged tomatoes were applied on that layer every week for five months. Afterwards, the earthworms were removed by hand and the organic substrate was left maturing in the reactor for two months without further waste addition. WW was produced by vermicomposting of spent grape marc mixed with lees cake at a ratio 1:1 (dw:dw) by adding an earthworm biomass equivalent to 10% of the waste mass (dw) contained in the bed. Waste moisture was kept at 80–85% by periodical watering during the vermicomposting process. After six months, the earthworms were removed by hand and the resulting organic substrate was finally matured for two months without further water addition. OB was obtained from wet olive cake mixed with municipal biosolids at a ratio 8:1 (dw:dw) after six months of a vermicomposting process which was similar to that of WW. CM was commercially produced by Lumbricor S.L. (Córdoba, Spain) from cattle manure, which was vermicomposted on a large-scale windrow system for four months plus one month of maturation. All these vermicomposts were homogenised and three samples of 250 g were separately taken and ground (<2 mm).

2.2. Chemical analyses

Vermicompost pH and electrical conductivity (EC) were measured with a glass electrode using a 1:10 sample:water (dw:v) ratio. Total organic carbon (TOC) and total nitrogen (N) were determined with a LECO TruSpec CN analyzer (LECO Corporation, St. Joseph, USA). Water soluble carbon (WSC) was extracted by mechanical shaking at 60 °C for 1 h with distilled water (1:10 sample:water; dw:v). Humic acid like (HAL) and fulvic acid like (FAL) compounds were extracted from 2 g of sample by mechanical shaking at 37 °C for 2 h with 40 ml of a solution consisting of 0.1 M $Na_2P_4O_7$ and 0.1 M NaOH. This extract was subsequently acidified to pH ≈ 1 with H_2SO_4 and centrifuged at 3500 rpm to separate the HAL fraction that precipitated from the FAL fraction, which remained in solution. The HAL solution was then obtained by dissolving the precipitate in 10 ml of 0.5 M NaOH. The C content in the WSC, HAL, and FAL solutions was determined by dichromate oxidation followed by titration with ferrous ammonium sulphate.

2.3. Enzyme activity analyses

Dehydrogenase activity was determined using iodonitrotetrazolium formazan (INTF) as substrate, as described by García et al. (1997). β-glucosidase and acid phosphatase activities were analysed by determining the amount of p-nitrophenol (PNP) produced from 4-nitrophenyl-β-D glucanopyranoside (PNG) and 4-nitrophenyl phosphate (PNPP) as described by Tabatabai (1982), and Tabatabai and Bremner (1969), respectively. Urease activity was determined using urea as a substrate as described by Kandeler and Gerber (1988). Each enzyme activity was determined per triplicate using 0.2 g of vermicompost sample.

2.4. Microresp™ analysis

The community level physiological profiles (CLPPs) were determined by using the micro-respiration system Microresp™ with eleven carbon sources: five carbohydrates (L-arabinose, D-xylose, N-acetyl-D-glucosamine, D-trehalose, D-raffinose), four amino-acids (L-arginine, L-cysteine, D-lysine, glycine), and two organic acids (DL-malic acid and D-galacturonic acid), which are ecologically relevant

in soils since they are plant root exudates. Different quantities of each C substrate were dissolved in milliQ-water so that a C concentration equivalent to 30 mg glucose g^{-1} vermicompost water (11 mg C g^{-1} vermicompost water) was set in each deep-well of the Microresp™ plate after the addition of 0.025 ml of substrate solution (Campbell et al., 2003). In addition to these C substrates, the respiratory response to three pesticides was assayed in the same Microresp™ plate: metalaxyl (acylamino-acid fungicide), imidacloprid (neonicotinoid insecticide), and diuron (phenylurea herbicide). Analytical standard of metalaxyl or imidacloprid were dissolved into milliQ-water and 0.025 ml of each solution was added to vermicompost samples placed in each deep-well (0.20 g) to obtain the follow pesticide concentrations: 1, 2, 4 and 8 μg pesticide g^{-1} vermicompost, which are concentrations usually detected in soils as a consequence of pesticide application in conventional agriculture. Assayed concentrations of diuron were: 0.5, 1, 2 and 4 μg pesticide g^{-1} vermicompost. The low solubility of this pesticide (35 mg l^{-1}) prevents to test diuron concentrations over 4 μg pesticide g^{-1} vermicompost since Microresp™ is a miniature applies a small volume of solutions to analyse samples. A volume over 0.025 ml is unadvised because its can cause anaerobic conditions in the tested material (Campbell et al., 2003). Together with the solutions containing the C-substrate and the pesticides one control with deionized water was also included in the Microresp™ deep-well plate. Thus, samples from all four vermicomposts (each being incubated with eleven substrates, three pesticides with four concentrations each, plus a control) were placed together into the same Microresp™ 96 deep-well plate. The assay was reproduced in triplicate.

The patterns of C substrate oxidation in each vermicompost type (CLPPs) were determined by measuring the absorbance (590 nm) in each well of the detection plates at time zero and after 6 h of incubation with a Zenyth 3100 multimode detector (Anthos, Eugendorf, Austria) spectrophotometer, following the recommendations as described by Campbell et al. (2003). A respiration index (RI) was defined as the difference in absorbance between zero hours and 6 h of incubation in each well containing each C substrate divided by the difference in absorbance between zero hours and 6 h recorded in wells containing deionized water. RI values obtained from C substrates and pesticides were expressed per g vermicompost placed into each deep well. The RI values of each C substrate reflect the ability of the vermicompost microbiota to oxidize the substrate. In the case of pesticides, RI values >1 indicate a stimulation of vermicompost microbial respiration as compared with its basal respiration determined by addition of deionized water. Conversely, RI values <1 indicate an inhibition of the basal respiration of the vermicompost microbiota.

The Shannon's diversity index (*H*') (Shannon and Weaver, 1963) was used as an index of potential metabolic diversity since this was calculated based on the RI values of single C substrates. This index was measured for each vermicompost as follows: $H' = -\Sigma\, pi \log pi$, where *pi* is the ratio of the respiration index for each single C substrate to the sum of the respiration indices for all carbon substrates. The catabolic versatility index (*CV*) (Sharma et al., 1998) was also calculated using the RI values of single C substrates registered in each vermicomposts as follows: $CV = M/\mathrm{SD}$, where *M* is the average RI value from all RI values of every single C substrates measured in each vermicompost, and SD is the standard deviation of M.

2.5. DNA extraction and PCR-DGGE analysis

Total DNA was separately extracted from 250 mg of each one of the three samples taken from the four vermicompost types by means of the PowerSoil™ DNA Isolation kit (MO BIO Laboratories, Solana Beach, USA). DNA solutions were checked for quality by electrophoresis in 1% agarose gels stained with ethidium bromide.

A DNA extract from each sample of the four vermicomposts was subjected to PCR amplification using the primer set FR1 (5′-AICCA TTCAATCGGTAIT-3′, I = inosin) and FF390 (5′-CGATAACGAACGAGA CCT-3′) in order to amplify fragments of 390 bp of the fungal 18S rRNA gene (Vainio and Hantula, 2000). PCR reactions were performed using 2 μl of DNA solution in a total volume of 25 μl, containing 0.2 μM each primer, 1× reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20], 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin, and 0.025 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany). PCR reactions without a DNA template were included in parallel as control. Thermal cycling was performed as described by Fernández-Gómez et al. (2010), using a PCR Express cycler (ThermoHybaid, Ulm, Germany). Proper sizes of amplification products were verified by electrophoresis on 1.5% agarose gels stained with ethidium bromide and inspected under a UV-transilluminator. PCR product concentrations were determined with the PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, USA). Fluorescence was measured with an Anthos Zenyth 3100 multimode detector (Anthos, Eugendorf, Austria).

The denaturing gradient gel electrophoresis (DGGE) was conducted in an INGENYphorU System (Ingeny International, Goes, The Netherlands), after loading 100 ng of PCR products into a 8% (w/v) polyacrylamide gel in 1× TAE (20 mM Tris–Cl, 10 mM acetate, 0.5 mM Na_2EDTA). The gel contained a denaturing gradient of 30% to 60% (100% denaturants consisting of 40% (v/v) formamide and 7 M urea) as described by Fernández-Gómez et al. (2010). A 100 bp DNA ladder (Genecraft®, Germany) was used as marker. Gels were stained with silver nitrate using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Freiburg, Germany) and photographed for subsequent image analysis as described below.

2.6. Data analysis

Significant differences among the vermicomposts concerning chemical parameters, enzyme activities, and the indices *H*' and *CV*, were determined by one-way analysis of variance (ANOVA) with mean separation based on the *post hoc* Duncan's multiple-ranged test conducted at a confidence level >95% ($p < 0.05$). The correlations between these parameters were examined using Pearson correlation coefficients, and significant results are given at a high confidence level ($p < 0.01$). A two-way ANOVA was performed for assessing the effect of the pesticides on the RI values registered in the vermicomposts. When significant differences were observed for either the type of vermicomposts or the assayed concentrations of each pesticide, the *post hoc* Duncan's multiple-ranged test was conducted at a confidence level >95% ($p < 0.05$). These statistical analyses were carried out using the software SPSS® Windows Version 13.0 (IBM, Chicago, USA).

The RI values registered for every single C substrate in each vermicompost, which as a whole determine the vermicompost CLPP, were subjected to principal component analysis (PCA) using the PC-ORD program Version 5.0 (MjM Software Design, Gleneden Beach, USA). Multivariate ANOVA of PCA-axes values was applied using SPSS® Windows Version 13.0 for the statistical testing of the separation of vermicomposts along each PC. When a significant F-statistic was noted, the *post hoc* Duncan's multiple-ranged test was conducted at a confidence level >95% ($p < 0.05$) to compare samples' scores for the PCA-axis.

Comparison of DGGE patterns was carried out with the GelCompar II software (Applied Maths, Kortrijk, Belgium). After the conversion of the scanned gel, DGGE band patterns were normalized using reference positions defined by the molecular ladder to align

the bands for proper comparison. Similarity values among the band patterns recorded in the vermicompost samples were calculated by comparing their densitometric curves using the Pearson correlation coefficient. The dendrogram was calculated with the unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm setting a position tolerance of 1% for band matching. The binary matrix representing the occurrence of DGGE bands (band presence/absence) in the vermicompost samples was exported. Statistical correlations between the matrix of occurrence of DGGE bands and the matrices corresponding to the chemical parameters, the enzyme activities, the RI of the C substrates, and the RI of the pesticides measured in the vermicomposts were evaluated by applying a Mantel tests with 999 iterations. Mantel test was performed using Euclidean distances for calculating the distance matrices of the chemical parameters, the enzyme activities, the RI of the C substrates, and the RI of the pesticides; and binary distance for the matrix of occurrence of DGGE bands was used. Significant matrix correlations are given at a high confidence level ($p < 0.01$). This analysis was performed using the Vegan package for R 2.12.1 (R Development Core Team, 2007).

3. Results and discussion

3.1. Chemical features of the vermicomposts

Significant differences were observed for the chemical features examined in the vermicomposts (Table 1). The TOC and N contents in the vermicomposts were in the order of WW > OB > DT > CM. Vermicomposts with a higher organic carbon content are more adequate for pesticide bioremediation as they are capable of adsorbing more pesticides as well as providing greater amounts of decomposable carbon compounds that ca facilitate the microbial pesticide degradation by co-metabolic processes (Tsui and Roy, 2007). On the other hand, Castillo et al. (2008) reported that high levels of N contents in organic materials can promote pesticide degradation since the greater availability of this nutrient may stimulate the growth and/or activity of autochthonous microorganisms capable of degrading pesticides. These authors also reported that low levels of N in organic materials can activate the fungal lignin-degrading enzymatic system, thus promoting pesticide biodegradation by fungi. The pH value of the vermicomposts is another parameter which could have relevant influence on processes of pesticide biodegradation. The neutral pH of OB and CM can favour the activity of bacteria involved in pesticide biodegradation (Castillo et al., 2008).

The greatest water soluble C (WSC) content was recorder in DT and the lowest in CM (Table 1). Given that this fraction of organic-matter comprises the C compounds which are easily available for microorganisms (Benítez et al., 1999), then a high WSC content in vermicompost could allow the microbial pesticide degradation by co-metabolic processes. Other fractions of organic matter in vermicompost such as humic acid-like (HAL) and fulvic acid-like (FAL) compounds have been reported to be capable of adsorbing pesticides from an aqueous solution (Romero et al., 2006). In this sense, the higher HAL and FAL contents in WW and DT suggest that these vermicomposts would be the most effective in adsorbing pesticides from soil solution (Table 1). Previous studies reported that improving the adsorption of pesticides in agricultural soils through addition of vermicomposts with a high ability to adsorb pollutants is a successful strategy for decontamination purposes (Fernández-Bayo et al., 2009). Despite the binding of pesticides to vermicomposts is able to decrease their availability for microorganisms, this does not necessarily constitute a barrier against their mineralization since part of a pesticide's sorbed fraction could be still degraded through extracellular enzymes and other microbial mechanisms (Aislabie and Lloyd-Jones, 1995). On the other hand, previous studies have also reported that there is an inversely proportional dependence between the adsorption of a given substance and its solubility in water, so that pesticides having low solubility are more likely adsorbed on organic materials (Ignatowicz, 2011).

Table 1
Chemical features of vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM).

Properties[1]	DT	WW	OB	CM
TOC (g kg^{-1})	156 (2.6) c	422 (1.5) a	342 (3.1) b	98 (1.7) d
WSC (g kg^{-1})	19.1 (0.4) a	7.2 (0.6) c	10.4 (0.2) b	3.4 (0.9) d
HAL (g kg^{-1})	30.6 (0.7) b	53.9 (9.0) a	11.2 (0.4) c	15.3 (0.2) c
FAL (g kg^{-1})	17.0 (0.9) b	44.5 (2.1) a	9.2 (0.4) c	8.7 (0.5) c
N (g kg^{-1})	15.8 (0.4) c	22.5 (0.2) a	17.8 (0.5) b	10.1 (0.1) d
pH	10.4 (0.2) a	8.3 (0.2) b	7.4 (0.3) c	7.5 (0.1) c

[1] TOC: total organic carbon; WSC: water soluble carbon; HAL: humic acid like compounds; FAL: fulvic acid like compounds; N: total nitrogen. For each property (standard deviations are given in brackets for $n = 3$), different letters indicate significant differences among vermicomposts ($p < 0.05$).

3.2. Enzyme activities of the vermicomposts

A visual comparison among the enzyme activities measured in the vermicomposts is shown in the Fig. 1. Therein, the values of the four enzyme activities define a Sun-ray plot. The Sun-ray plots has been proposed as a visual fingerprint of biochemical activity in organic materials, considering the area and shape of this kind of plot reflect the potential functional diversity of the inhabiting microorganisms (Vivas et al., 2009). The Sun-ray plot displayed by DT was characterized by a high value of dehydrogenase activity, which was significantly higher than those of other vermicomposts. This suggests that DT contains the most active microbiota since dehydrogenase activity is considered as a measure of the overall microbial activity (García et al., 1997). This enzyme activity was positively correlated with the content in WSC of the vermicomposts ($r = 0.83$, $p < 0.01$), suggesting that the high microbial activity of DT was supported by its great pool of easily available carbon compounds for the resident microbiota. This is in line with the results reported by Benítez et al. (1999), who also found a significant positive correlation between dehydrogenase activity and WSC during vermicomposting. With regard to WW, this was characterized by a higher level of β-glucosidase activity as compared with its other enzyme activities. However, among all vermicompost, the greatest value of β-glucosidase activity was observed in OB. β-glucosidases are enzymes involved in the mineralization of organic materials as they catalyse the hydrolysis of terminations of the β-D-glucose chain releasing β-glucose that becomes available for microorganisms. This enzyme activity was positively correlated to the content in TOC of the vermicompost ($r = 0.73$, $p < 0.01$). Therefore, the greater β-glucosidase activity in WW and OB may be a consequence of the higher TOC content in these vermicomposts as compared with that in DT and CM. The β-glucosidase activity was also positively correlated with the acid phosphatase ($r = 0.93$, $p < 0.01$) and urease activities ($r = 0.91$, $p < 0.01$) recorded in the vermicomposts. The latter activities were also positively correlated to each other ($r = 0.99$, $p < 0.01$). Acid phosphatase and urease activities are involved in the mineralization of organic phosphomonoester and ureic compounds, respectively. Like β-glucosidases, acid phosphatases and ureases are inducible enzymes involved in the breaking down of the organic matter during vermicomposting (Benítez et al., 1999). Taking into account that a low microbial activity was found in OB, as its dehydrogenase activity value indicated, the high activity of these hydrolytic enzyme evidenced in OB might be due to the existence of a pool of extracellular enzymes. This is in agreement with Benítez et al. (2005), who

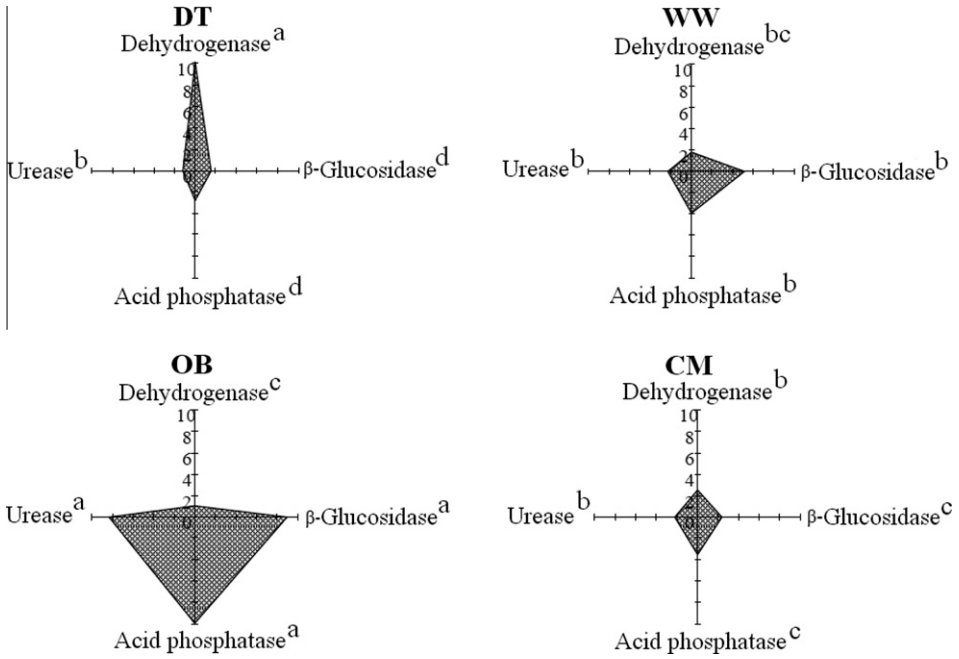


Fig. 1. Enzyme activities in the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). Dehydrogenase activity is expressed as $7\times$ (μg INTF g^{-1} h^{-1}), β-glucosidase activity as $75^{-1}\times$ (μg PNP g^{-1} h^{-1}), acid phosphatase activity as $250^{-1}\times$ (μg PNP g^{-1} h^{-1}), and urease activity as $20^{-1}\times$ (μg NH_4^+ g^{-1} h^{-1}). For each enzyme activity, different letters indicate significant differences among the vermicomposts ($p < 0.05$).

reported a high concentration of active ureases immobilized in a vermicompost from olive-cake wastes.

Given that the potential activity of hydrolytic enzymes measured in organic amendments is indicative of the biochemical functional diversity of its resident microbiota (Vivas et al., 2009), then the highest β-glucosidase, acid phosphatase and urease activities recorded in OB suggest that this vermicompost might have the largest microbial functional diversity. Conversely, the poor activity of these hydrolases recorded in DT and CM suggest a low biochemical functional diversity of their resident microbiota. In addition, the smallest Sun-ray plot area recorded in CM indicates that its microbiota was scarcely active, indicating an even lower biochemical functionality than that of DT.

3.3. Community-level physiological profiles of the vermicomposts

Principal component analysis (PCA) of the CLPPs of the vermicomposts is shown in the Fig. 2. The first and second principal components (PC 1 and PC 2) explained 80.9% and 13.6% of the variance, respectively (Fig. 2). PC 1 was inversely correlated to L-cysteine ($r = -0.99$) and DL-malic acid ($r = -0.97$) whereas PC 2 was directly correlated to the carbohydrates D-trehalose ($r = 0.93$) and D-raffinose ($r = 0.92$). Statistical analysis of the samples' scores for each PCA-axis demonstrated a significant separation of the vermicomposts in two groups along PC 1 ($F = 37.7$, $p < 0.05$): a group comprising the CLPPs of WW, OB, and DT, and another constituted of the CLPPs of CM. This indicated that the microbial functional diversity of CM is significantly different from that of the other vermicomposts. Mondini and Insam (2005) reported that PCA on CLPPs of samples from different phases of composting was a sensitive method for distinguishing among the microbial communities with different functional abilities involved in each composting phase.

Concerning the functional diversity indices determined in the vermicomposts, the value of Shannon's index (*H'*) was the lowest in CM (Table 2). This indicates that the resident microbiota in CM had comparatively the poorest ability to oxidize the diverse C substrates tested. Campbell et al. (2003) reported that a low ability of a microbial community to oxidize diverse C substrates is

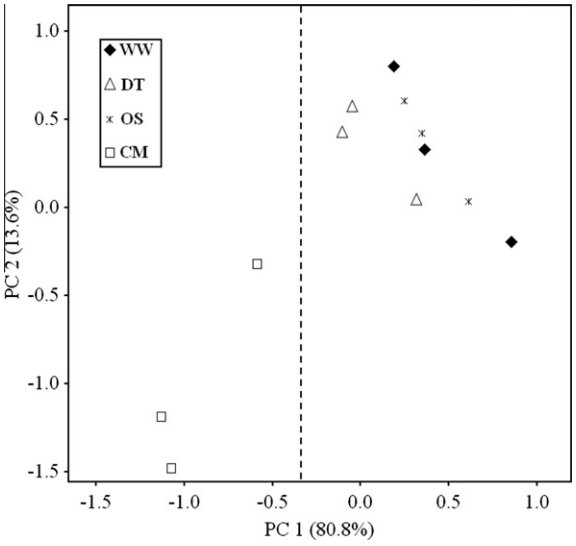


Fig. 2. Principal component analysis of the community-level physiological profile of the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). The dotted line indicates a significant separation of the vermicomposts along PC 1 ($p < 0.05$).

indicative of its limited functional diversity. Hence, according to *H'*, CM had less microbial functional diversity as compared to that of other vermicomposts. With regard to the catabolic versatility (*CV*) index, the vermicomposts were ranked as follows: WW and OB > DT > CM (Table 2). This indicates that the microbiota in WW and OB was able to oxidise every C substrate with a similar strength (Sharma et al., 1998). Conversely, the microbiota housed in DT and CM preferentially oxidised certain C substrates. Both indices were positively correlated with the vermicompost TOC (*H'*: $r = 0.75$, $p < 0.01$; *CV*: $r = 0.94$, $p < 0.01$) and N content (*H'*: $r = 0.85$, $p < 0.01$; *CV*: $r = 0.94$, $p < 0.01$), suggesting that the functional and catabolic diversity of the resident microbiota in the vermicomposts could be

Table 2
Shannon's (*H'*) and catabolic versatility (*CV*) indices calculated from the respiration of single C substrates in vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM).

Vermicompost[1]	*H'*	*CV*
WW	1.035 (0.001) a	11.178 (0.877) a
DT	1.024 (0.005) a	6.985 (0.940) b
OB	1.034 (0.000) a	10.298 (0.248) a
CM	0.958 (0.019) b	3.135 (0.523) c

[1] Different letters for each index (standard deviations are given in brackets; $n = 3$) indicate significant differences among vermicomposts ($p < 0.05$).

influenced by these chemical parameters. Thus, the low contents of TOC and N in CM might be partly responsible for the little capability of its resident microbiota to oxidize different C substrates. These results are in accordance with the observations on enzyme activities that pointed out the lowest biochemical functionality of the resident microbiota in CM (see Section 3.2).

3.4. Fungal community structure of the vermicomposts

The DGGE corresponding to the resident fungal community in each vermicompost is shown in Fig. 3a. From this picture, discrete bands were associated to dominant rRNA sequences of fungal taxa whereas the background contains subdominant sequences from rare fungal taxa, that were generally omitted from the analysis of DGGE profile (Loisel et al., 2006). Earlier studies have demonstrated that a single band may contain rRNA sequences from more than one species, besides several bands can be generated from a single species (Sekiguchi et al., 2001). In addition, Loisel et al. (2006) reported that the number of bands is usually stabilized rapidly around 35 despite an increase in the number of sequences belonging to different microorganisms, thus revealing that the analysis of the DGGE bands is inadequate for estimating the true diversity of microbial communities. Therefore, in the present study, DGGE band patterns of the vermicomposts were mainly considered as genetic fingerprints of their fungal community structure.

The cluster analysis of the DGGE profiles reported similarities (Pearson correlation coefficient) >95% among the three samples collected from each vermicompost (Fig. 3b). This suggests that the vermicomposts were homogeneous materials with a well defined fungal community. Furthermore, this confirms the reproducibility of this analysis. The comparison among the DGGE bands patterns also revealed that the fungal communities in the different vermicomposts had analogous structures (similarity $\geqslant$75%) but with relevant dissimilarities, which were responsible for the formation of two vermicompost clusters. A group was constituted of WW and OB, which clustered together with 88.3% similarity, whereas DT and CM constituted another group with 87.6% similarity. This grouping pattern could be influenced by the chemical features of the vermicomposts since a significant correlation was detected between the occurrence of DGGE bands in the vermicomposts and their chemical features, according to the Mantel test ($r = 0.88$, $p < 0.01$). Among the chemical features examined, the strongest predictor of the DGGE band patterns of the vermicomposts was their TOC content ($r = 0.72$, $p < 0.01$), greater in WW and OB than that in DT and CM (Table 1). The occurrence of DGGE bands in the vermicomposts was also correlated with their enzyme activities ($r = 0.60$, $p < 0.01$), recording the β-glucosidase activity alone the highest correlation value ($r = 0.60$, $p < 0.01$). This indicates that the chemical characteristics of each vermicompost conditioned its fungal community structure, which may be responsible for part of the hydrolase activities in the vermicomposts, mainly for the β-glucosidase activity. In agreement with this, Lazcano et al. (2008) reported a significant positive correlation between the β-glucosidase activity in a vermicompost from cattle manure and its content in ergosterol, a molecule used as a biomarker for measuring fungal biomass. By contrast, a comparatively lower correlation coefficient, but significant at lower confidence level, was found between the occurrence of DGGE bands in the vermicomposts and their CLPPs ($r = 0.20$, $p = 0.038$). This suggests that other resident microorganisms in the vermicomposts such as bacteria, protozoa, or non-dominant fungi may be considerably involved in the profile of the consumption obtained from the C substrates.

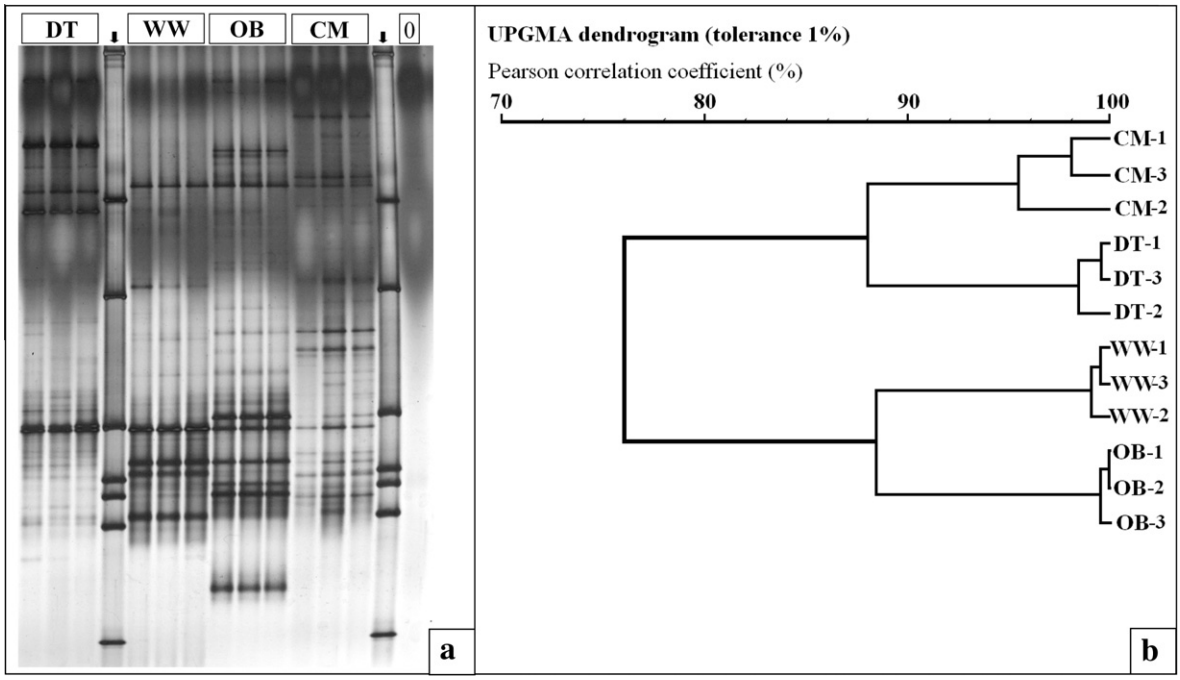


Fig. 3. Picture of the DGGE of fungal 18S rRNA gene fragments (a) and dendrogram depicting the relatedness among the band patterns (b) in the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). Black arrows indicate lanes with a molecular ladder. Lane defined as 0 includes the PCR product from control reaction without DNA template.

3.5. Response of the vermicomposts' microbiota to pesticides

All the assayed concentrations of metalaxyl caused RI values >1 in WW, DT, and OB, whereas CM registered RI values <1, which were significantly different from those in the former vermicomposts ($F = 18.5$, $p < 0.05$) (Fig. 4a). The differences observed among the RI value of the vermicomposts treated with the different concentrations of metalaxyl were not statistically significant for any vermicompost type ($F = 1.6$, $p = 0.21$). In addition, the interaction between the vermicompost type and the metalaxyl concentration was not significant ($F = 0.2$, $p = 0.99$). The RI values of the vermicomposts were affected by the assayed concentrations of imidacloprid in a similar way similar to that observed in the case of the metalaxyl (Fig. 4b). Thus, the RI values registered in CM were significantly lower than those in the other vermicomposts ($F = 5.8$, $p < 0.05$), whereas the RI values of the vermicomposts were not significantly different among the assayed concentrations of imidacloprid ($F = 0.9$, $p = 0.43$). Likewise, the interaction between both factors was not significant ($F = 0.1$, $p = 0.99$). Concerning diuron, all types of vermicomposts recorded RI values $\geqslant 1$ for all the assayed concentrations of diuron, except in the case of CM treated with 4 μg diuron g^{-1} vermicompost (Fig. 4c). No significant differences were found either among the vermicompost types ($F = 1.5$, $p = 0.23$) or among the assayed concentrations of diuron ($F = 0.9$, $p < 0.45$). No significant interaction was recorded between both factors ($F = 0.33$, $p = 0.96$).

A whole interpretation of these results suggests that the resident microbiota in WW, DT, and OB was able to maintain or even increase its basal respiration despite the presence of these pesticides at all the concentrations assayed. The increment in the microbial basal respiration of these vermicomposts could be due to various events: (a) the pesticides were directly used as a carbon resource by the vermicompost microbiota; (b) the pesticides stimulated, probably by microbial co-metabolic processes, the consumption of other carbon substrates contained in the vermicompost; and/or (c) pesticide toxicity stressed the vermicompost microbiota. However, the particular response of the autochthonous microbiota in each vermicompost and the presumable pesticide degradation cannot be assessed in details because the small quantities analysed by using MicroResp™ hinders to study other parameters. Hence, further studies in depth should be conducted to confirm whether the microbiota inhabiting WW, DT, or OB is able to use these pesticides as carbon source and even degrade them to CO_2. In contrast to WW, DT, and OB, the resident microbiota in CM showed a different response to the pesticides metalaxyl and imidacloprid, decreasing its basal respiration for all the assayed concentrations. Nevertheless, the microbial basal respiration of CM did not decrease by the assayed concentration of diuron. This may be due to the lowest solubility of this pesticide as compared with that of metalaxyl and imidacloprid. Aislabie and Lloyd-Jones (1995) reported that the solubility of a pesticide can conditioned its toxicity for microorganisms inhabiting soils.

The negative effect of metalaxyl and imidacloprid on the basal microbial respiration of CM could be related to the lowest microbial functional diversity of this vermicompost as compared with that of WW, DT, and OB (see discussion above). Thus, the resident microbiota in WW, DT, and OB could endure the pesticide presence better than that in CM probably due to their greater functional diversity. This assumption is consistent with the idea proposed by Degens et al. (2001), who found that soil microbial communities with a reduced functional diversity are less resistant to stress or disturbance by abiotic factors such as pH, heavy metals, or wet-dry or freeze–thaw cycles.

In the case of the fungicide metalaxyl, the RI values were significantly correlated with the vermicompost fungal community structure ($r = 0.25$ $p = 0.02$). Even a more robust was the correlation

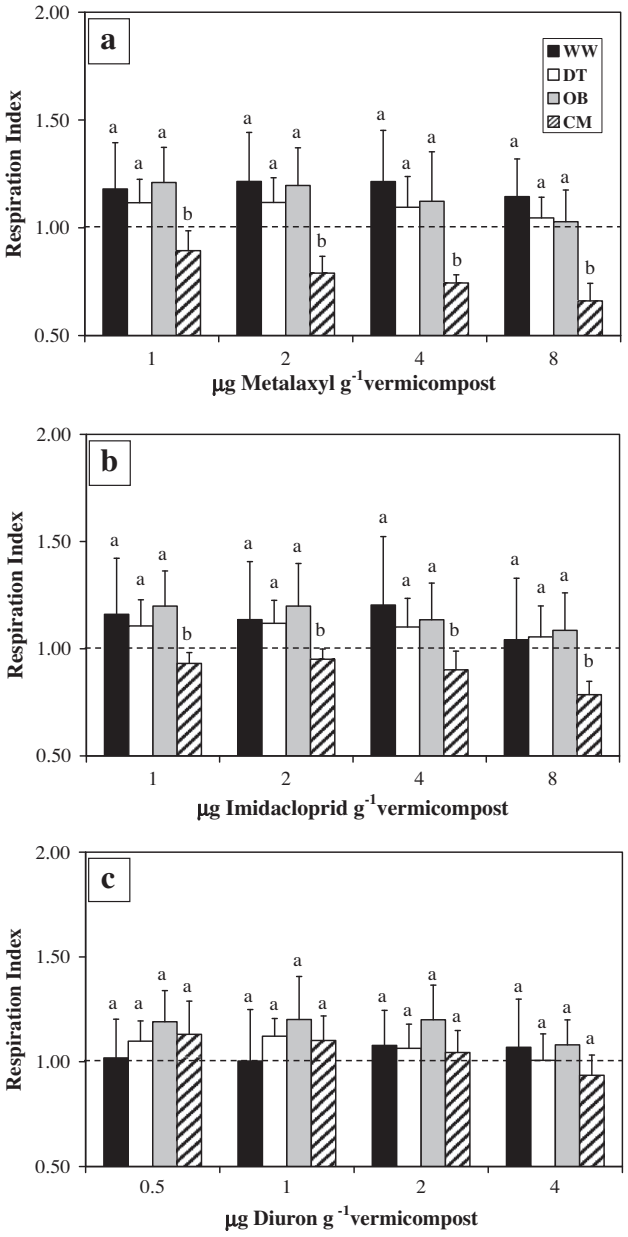


Fig. 4. Effect of pesticides on the respiration index (RI) of the vermicomposts produced from winery wastes (WW), damaged tomato fruits (DT), olive-mill waste and biosolids (OB), and cattle manure (CM). Different letters indicate significant differences among the RI values ($p < 0.05$).

found for the highest concentration of metalaxyl alone ($r = 0.32$, $p < 0.01$). This indicates that the impact of this fungicide on the basal respiration of the vermicompost microbiota was partly related to its particular fungal community. Thus, the reduction in the RI observed in CM after metalaxyl might be due to the lesser tolerance of the fungal community inhabiting this vermicompost as compared to the fungal communities of the other vermicomposts. On the other hand, earlier studies reported that as bacteria as fungi are capable of degrading metalaxyl in soil (Bailey and Coffey, 1986). Indeed, recent studies have also reported that some fungi (i.e. *Coriolus versicolor*, *Hypholoma fasciculare* and *Stereum hirsutum*) inhabiting the organic matrix of a biobed can effectively degrade metalaxyl and other pesticides (Bending et al., 2002). Thus, vermicomposts with an increased activity after metalaxyl addition, such as WW, DT, and OB, may be adequate materials to construct the organic matrix of biobeds. Furthermore, the presence in these

vermicomposts of fungal strains capable of degrading metalaxyl should be investigated by further studies. Hence, the results indicate that MicroResp™ can be used as a rapid, simple, and inexpensive technique to asses the respiratory response of vermicompost microbiota to pesticides, allowing the fast screening and selection of those vermicomposts whose microbial communities deserve in-depth analyses.

4. Conclusions

This study provides novel information about the relationships between chemical composition, microbial metabolic diversity, and fungal community structure of different vermicomposts with respect to the respiratory response of their resident microbiota to pesticides. The results suggest that vermicomposts with high functional diversity could be used as organic soil conditioner for diminishing pesticide pollution in agriculture. Moreover, WW, DT, and OB are recommended for conducting further in-depth studies in order to understand the mechanisms of tolerance and presumable degradation of these pesticides by their autochthonous microorganisms. These vermicomposts are also useful for seeking microorganisms capable of degrading the assayed pesticides.

Acknowledgements

This study was founded by the Junta de Andalucía (P05-AGR-00408) and the Marie Curie Actions (MEIF-CT-2006-041034). Manuel J. Fernández Gómez thanks the Science and Innovation Ministry for their FPU doctoral Grant and his temporary stay at the University of Innsbruck (AP2006-03452). Marta Goberna and Heribert Insam thank support by the Marie Curie Actions (MEIF-CT-2006-041034).

References

Aira, M., Monroy, F., Domínguez, J., 2006. *Eisenia fetida* (Oligochaeta, Lumbricidae) activates fungal growth, triggering cellulose decomposition during vermicomposting. Microb. Ecol. 52, 738–747.

Aislabie, J., Lloyd-Jones, G., 1995. A review of bacterial-degradation of pesticides. J. Soil Res. 33, 925–942.

Anastasi, A., Varese, G.C., Filipello Marchisio, V., 2005. Isolation and identification of fungal communities in compost and vermicompost. Mycologia 97, 33–44.

Bailey, A.M., Coffey, M.D., 1986. Characterization of microorganisms involved in accelerated biodegradation of metalaxyl and metolachlor in soils. Canadian J. Microbiol. 32, 562–569.

Bending, G.D., Friloux, M., Walker, A., 2002. Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS Microbiol. Lett. 212, 59–63.

Nogales, R., Elvira, C., Masciandaro, G., Ceccanti, B., 1999. Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour. Technol. 67, 297–303.

Benítez, E., Sainz, H., Nogales, R., 2005. Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresour. Technol. 96, 785–790.

Blaszak, M., Pelech, R., Graczyk, P., 2011. Screening of microorganisms for biodegradation of simazine pollution (obsolete pesticide Azotop 50 WP). Water Air Soil Pollut., 1–13.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. Appl. Environ. Microbiol. 69, 3593–3599.

Castillo, M.d.P., Torstensson, L., Stenström, J., 2008. Biobeds for environmental protection from pesticide use – a review. J. Agric. Food Chem. 56, 6206–6219.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biol. Biochem. 33, 1143–1153.

Fernández-Bayo, J.D., Nogales, R., Romero, E., 2009. Effect of vermicomposts from wastes of the wine and alcohol industries in the persistence and distribution of lmidacloprid and diuron on agricultural soils. J. Agric. Food Chem. 57, 5435–5442.

Fernández-Gómez, M.J., Nogales, R., Insam, H., Romero, E., Goberna, M., 2010. Continuous-feeding vermicomposting as a recycling management method to revalue tomato-fruit wastes from greenhouse crops. Waste Manage. 30, 2461–2468.

García, C., Hernández, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plan. 28, 123–134.

Hill, G.T., Mitkowski, N.A., drich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramírez, S., Lynch, S.T., Nelson, E.B., 2000. Methods for assessing the composition and diversity of soil microbial communities. Appl. Soil Ecol. 15, 25–36.

Ignatowicz, K., 2011. Selection of sorbent for removing pesticides during water treatment. J. Hazard. Mater. 169, 953–957.

Kale, R.D., Mallesh, B.C., Kubra, B., Bagyaraj, D.J., 1992. Influence of vermicompost application on the available macronutrients and selected microbial populations in a paddy field. Soil Biol. Biochem. 24, 1317–1320.

Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils 6, 68–72.

Lazcano, C., Gómez-Brandón, M., Domínguez, J., 2008. Comparison of the effectiveness of composting and vermicomposting for the biological stabilization of cattle manure. Chemosphere 72, 1013–1019.

Loisel, P., Harmand, J., Zemb, O., Latrille, E., Lobry, C., Delgenes, J.P., Godon, J.J., 2006. Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ. Microbiol. 8, 720–731.

Mondini, C., Insam, H., 2005. Effect of inoculum standardization on community level physiological profiles of compost samples. Compost Sci. Util. 13, 27–33.

Moon, K.E., Lee, S.Y., Lee, S.H., Ryu, H.W., Cho, K.S., 2010. Earthworm cast as a promising filter bed material and its methanotrophic contribution to methane removal. J. Hazard. Mater. 176, 131–138.

Moreno, B., Vivas, A., Nogales, R., Macci, C., Masciandaro, G., Benítez, E., 2009. Restoring biochemical activity and bacterial diversity in a trichloroethylene-contaminated soil: the reclamation effect of vermicomposted olive wastes. Environ. Sci. Pollut. Res. 16, 253–264.

R Development Core Team, 2007. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Viena. Available from: http://www.R-project.org

Romero, E., Salido, A., Cifuentes, C., Fernández-Bayo, J.D., Nogales, R., 2006. Effect of vermicomposting process on pesticide sorption capability using agro-industrial wastes. Int. J. Environ. Anal. Chem. 86, 289–297.

Sekiguchi, H., Tomioka, N., Nakahara, T., Uchiyama, H., 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnol. Lett. 23, 1205–1208.

Sen, B., Chandra, T.S., 2009. Do earthworms affect dynamics of functional response and genetic structure of microbial community in a lab-scale composting system? Bioresour. Technol. 100, 804–811.

Shannon, C.E., Weaver, W., 1963. The Mathematical Theory of Communication. University of Illinois Press, Urbana.

Sharma, S., Rangger, A., von Lützow, M., Insam, H., 1998. Functional diversity of soil bacterial communities increases after maize litter amendment. Eur. J. Soil Biol. 34, 53–60.

Tabatabai, M.A., 1982. Soil enzymes. In: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. ASA and SSSA, Madison, pp. 907–943.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301–307.

Tsui, L., Roy, W.R., 2007. Effect of compost age and composition on the atrazine removal from solution. J. Hazard. Mater. 139, 79–85.

Vainio, E., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104, 927–936.

Vischetti, C., Monaci, E., Cardinali, A., Casucci, C., Perucci, P., 2008. The effect of initial concentration, co-application and repeated applications on pesticide degradation in a biobed mixture. Chemosphere 72, 1739–1743.

Vivas, A., Moreno, B., García-Rodríguez, S., Benítez, E., 2009. Assessing the impact of composting and vermicomposting on bacterial community size and structure, and microbial functional diversity of an olive-mill waste. Bioresour. Technol. 100, 1319–1326.