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DOCTORAL THESIS

ORGANIC MATERIALS AND THEIR HUMIC SUBSTANCES AS SOURCES OF FREE AND IMMOBILIZED ENZYMES FOR SOILS AND PLANTS

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SUMMARY

RESUMEN

Durante las últimas décadas se han dedicado grandes esfuerzos a incrementar la producción de alimentos en un intento de aliviar una de las necesidades de la sociedad: aportar a la población actual y futura, la cantidad y calidad de alimentos necesaria que demanda. Esto ha supuesto una presión enorme y desproporcionada sobre los recursos naturales, especialmente el agua y el suelo. Factores negativos relacionados con las condiciones climáticas semiáridas (erosión y desertificación), son la causa de procesos de degradación del suelo. En España, el riesgo de desertificación afecta a cerca de 31,5% de la superficie total, y es particularmente grave en la cuenca del Mediterráneo (página Web del Ministerio del Medio Ambiente, Medio Rural y Marino). Está claro que los procesos de degradación de los suelos es un grave problema para su conservación y protección, ya que tal degradación impide el cumplimiento de sus funciones principales: i) que actúe como un medio de crecimiento, soporte físico, y depósito de agua y nutrientes para las plantas; ii) la regulación del flujo de agua a través del medio ambiente y iii) el impuslso de una cierta capacidad para mitigar los efectos nocivos de los contaminantes que llega al suelo por medios físicos, químicos y biológicos.

Cuando un suelo está expuesto a procesos de degradación, su estado biológico es el primero en ser afectado, y ello repercutirá sobre su capacidad productiva. La fracción biótica de la materia orgánica, formada por el conjunto de organismos vivos, juega un papel fundamental en el suelo, ya que es en última instancia, responsable del estado de dicha materia orgánica y, en términos generales, del desarrollo y funcionamiento de un ecosistema terrestre. La escasa vegetación de los suelos en zonas áridas y semiáridas como consecuencia principalmente de la baja productividad y el posterior abandono de los mismos, supone un escaso aporte de restos vegetales, lo que unido a los procesos de mineralización de la materia orgánica en este tipo de suelos, condiciona un nivel bajo de materia orgánica en los mismos. El indicado bajo nivel de materia orgánica actúa como un factor clave en la degradación de esos suelos, lo que incide en una disminución gradual de su productividad natural..

Desde el Protocolo de Kyoto de 1992, que identifica los suelos como posible sumidero de carbono, se ha avanzado mucho. En la reciente Estrategia Temática para la Protección del Suelo, que es un preámbulo para una nueva directiva europea (http://ec.europa.eu/environment/soil/pdf/ com_2006_0231), se reconoce que el suelo debe jugar un papel ecológico decisivo, tanto en un contexto agrícola como natural. Se identifica además a la pérdida de materia orgánica como una de las principales causas de la degradación del suelo. Un informe sobre la materia orgánica y la biodiversidad dentro de la Estrategia Temática Europea (Van Camp, 2004) menciona que la materia orgánica exógena, es decir, aquella materia orgánica que se adiciona a un suelo degradado con el fin de mejorar los cultivos o restaurar dicho suelo degradado para su uso posterior, constituye una valiosa fuente de materia orgánica y contribuye a la fijación de C en el suelo, colaborando por tanto a la disminución del efecto invernadero derivado de la liberación de CO_2 a la atmósfera. El

mantenimiento de niveles adecuados de materia orgánica en los suelos es lo que favorece el establecimiento de una cubierta vegetal estable y la posterior incorporación de los restos vegetales, junto con el establecimiento de una adecuada actividad microbiana; por ello, mantener o incrementar los contenidos de materia orgánica en los suelos es considerado uno de los métodos más eficaces en la lucha contra la erosión y los procesos de degradación asociados.

Una forma de mejorar por tanto, la fertilidad de los suelos degradados y en particular, de mejorar su actividad microbiana, es agregar materia orgánica exógena. Estas adiciones deben contribuir a proporcionar materia orgánica lábil en cantidad suficiente como para estimular la vida de los microorganismos que puedan existir en el suelo. En el informe elaborado sobre la materia orgánica y la biodiversidad dentro de la Estrategia Europea del Suelo se indica que la materia orgánica exógena, es decir, los materiales orgánicos añadidos al suelo (los cuales pueden proceder de residuos orgánicos de origen tan diferente como animal, vegetal o incluso urbano), para mejorar el rendimiento de los cultivos o para mejorar la calidad de los suelos degradados, constituyen hoy en día una fuente inestimable de materia orgánica del suelo. La aplicación de enmiendas orgánicas mejora el estado de los nutrientes del suelo, sirviendo como una fuente de macro y microelementos, mejorando sus propiedades físicas, mediante el aumento de la porosidad del suelo y la retención de agua, e incrementando el contenido de sustancias "tipo" húmico, conocidos como macroestructuras policondensadas carbonadas. Además, uno de los efectos beneficiosos de las mencionadas sustancias húmicas aportadas es que las enzimas que puedan sintetizarse pueden quedar protegidas en esos compuestos orgánicos durante largo tiempo, preservándolas así de la posible desnaturalización por el ataque de la proteolisis en el suelo.

Desde el punto de vista microbiológico, los microorganismos son responsables de importantes ciclos en el suelo y están involucrados en la descomposición de materia orgánica a nivel de ecosistema. Por ejemplo, ellos son responsables de la mineralización de C y su pérdida a la atmósfera en forma de CO_2, y por lo tanto de su pérdida y la dificultad de fijación en el suelo. Los microorganismos son también de interés en los procesos de humificación del carbono, teniendo así la posibilidad de construir una materia orgánica poco biodegradable y muy resistente a la mineralización. Esto es de particular interés cuando las enmiendas orgánicas (que pueden ser residuos frescos, poco estables, o composts, mucho mas estables), se añaden a los suelos degradados; la materia orgánica exógena que se adiciona estará disponible al ataque de las poblaciones microbianas existentes, tanto de aquellas autóctonas del suelo, como de las que se añaden a la enmienda. Hacemos hincapié pues en que los microorganismos juegan un papel esencial en la sostenibilidad de los ecosistemas del suelo.

Es importante destacar que el verdadero interés que tienen los microorganismos en cuanto a su relación con la calidad del suelo o los procesos de degradación y recuperación del mismo, no se refiere a su naturaleza y funciones específicas, sino más bien, a la estimación de la actividad microbiana que se produce en el medio ambiente donde actúan dichos microorganismos. Para determinar esa actividad

microbiana, algunos parámetros bioquímicos pueden constituir un excelente punto de partida. La importancia básica de la actividad enzimática en el suelo se encuentra en el hecho de que el funcionamiento del ecosistema no puede ser totalmente entendido sin la participación de procesos enzimáticos, ya que las enzimas determinar la velocidad de la mayoría de las transformaciones químicas que tienen lugar en el suelo. También proporcionan información sobre la capacidad potencial del suelo para llevar a cabo reacciones específicas, que son importantes en los ciclos de nutrientes.
La actividad de las enzimas relacionadas con el ciclo de los elementos (carbono, nitrógeno, fósforo o azufre) son de suma importancia en la calidad del suelo. Entre estas enzimas están las siguientes: fosfatasas, ureasas, proteasas y diferentes enzimas relacionadas con el ciclo del C como b-glucosidasas, celulosas y poli-fenoloxidasas. Por otra parte, indicadores del tamaño de la comunidad microbiana (carbono de la biomasa microbiana) o de su actividad (respiración basal, el ATP y la actividad deshidrogenasa) puede dar idea precisa del impacto de la adición de enmiendas orgánicas sobre la actividad microbiana de los suelos enmendados.
Como hemos dicho, las enzimas catalizan todas las reacciones bioquímicas y son una parte integral del ciclo de los nutrientes en el suelo. En general, están asociados con la proliferación de células viables, pero las enzimas pueden ser excretadas a partir de una célula viva o ser liberadas a la solución del suelo a partir de las células muertas. Una vez que las enzimas extracelulares han abandonado la protección de la célula, están expuestas a un ambiente inhóspito en el que la desnaturalización no biológica, la adsorción, la inactivación y la degradación por microorganismos proteolíticos, todo conspira para dañar a las enzimas, a menos que puedan sobrevivir debido a una nueva protección por parte de coloides minerales u orgánicos como el humus, manteniéndose así mucho mas resistentes a la proteólisis que las enzimas libres. En general, las enzimas inmovilizadas en coloides minerales u orgánicos pueden cambiar de estado, así como sus propiedades y naturaleza, (por ejemplo, pueden cambiar su cinética, su estabilidad, o la movilidad de las enzimas), ya que sufren asociaciones de tipo físico o químico junto con los mencionados coloides.
Los materiales orgánicos pueden incidir sobre la rizosfera de las plantas, no sólo por colaborar para conseguir una mayor actividad microbiana de esa zona, sino también por promover diferentes efectos apoyados en la relación directa que pueda formarse entre las sustancias húmicas y la proliferación celular de dicha rizosfera. Es bien sabido que la sustancia húmica promueve el crecimiento de las raíces como un comportamiento hormonal mediante la inducción de la ATPasa de las células de las raíces. En este sentido, las propiedades físicas y químicas de las sustancias húmicas (por ejemplo, hidrofobicidad y el tamaño de peso molecular) son el factor clave para producir efectos fisiológicos sobre las plantas.

OBJETIVO FINAL

El principal desafío de los países mediterráneos es restaurar la fertilidad y productividad de sus suelos agrícolas degradados ubicados en zonas áridas y semiáridas, mediante la adición de materia orgánica exógena (enmienda orgánica), consiguiendo así frenar los procesos de degradación y desertificación, e incentivado

la introducción de C exógeno en el suelo que será capaz de aumentar la calidad biológica, bioquímica y microbiológica del mencionado suelo. Con la materia orgánica exógena añadida al suelo, basada en muchas ocasiones en residuos orgánicos que sean de calidad, se incluyen sustratos que pueden incentivar la síntesis de algunas enzimas, y por esta razón, los ciclos de elementos como C, N, P o S, consiguiendo así aumentar la fertilidad del suelo y su productividad. El uso de residuos orgánicos (posiblemente tratados) procedentes de origen animal, vegetal o incluso urbano (lodos de depuradora) representa un "valor añadido" para los suelos donde se adicionan, tanto desde el punto de vista económico como ecológico. El objetivo de la presente Tesis Doctoral es el estudio de residuos orgánicos sin estabilizar o estabilizados mediante compostaje, y su valorización desde diferentes puntos de vista: i) como fuente de enzima total de interés en los ciclos de elementos; ii) como fuente de enzimas inmovilizadas en las sustancias húmicas, quedando por tanto dichas enzimas estabilizadas y resistentes a la desnaturalización térmica, iii) conocer el efecto de las diferentes enmiendas orgánicas estudiadas (basadas en residuos orgánicos de diferente origen: urbano, animal o vegetal), sobre la calidad bioquímica, biológica y microbiológica de los suelos degradados donde se adicionan; iv) determinar los cambios de tipo químico estructural de las sustancias húmicas (ácidos húmicos y ácidos fúlvicos) obtenidas de los suelos degradados cuando son enmendados con materiales orgánicos, v) por el efecto sobre el crecimiento de las raíces de las plantas de maíz, debido a las sustancias húmicas del suelo modificado, con esto los desechos orgánicos.

La presente Tesis Doctoral se compone de 5 experimentos correspondientes a los objetivos mencionados, que se describe sucintamente en los párrafos siguientes:

Experimento 1. Actividad enzimática contenida en materiales orgánicos, compostados y sin compostar.

La evaluación de materiales orgánicos residuales como una fuente de actividad enzimática total e inmovilizada sobre una matriz orgánica como las sustancias húmicas, es un área de investigación poco explorada, especialmente en el caso de materiales orgánicos derivados de los residuos municipales. Las enzimas catalizan las reacciones de la mayoría de los suelos esenciales para la funcionalidad del suelo. La ventaja de las enzimas inmovilizadas sobre sustancias húmicas como complejos humo-enzimático (enzimas inmovilizadas) con relación a las enzimas libre es que aquellas enzimas con interés en agricultura quedan protegidas dentro de los coloides húmicos, siendo entonces más resistentes a agentes desnaturalizantes y otras condiciones adversas del suelo. Esto explica el interés en el uso de residuos orgánicos (que se producen en una cantidad cada vez más, siendo además económicos) como fuente de enzimas inmovilizadas, que podrían concentrarse y utilizarse para mejorar la calidad de los suelos degradados o contaminados. El objetivo de este estudio fue (i) evaluar el potencial de lodos de depuradora (SS) y la fracción orgánica de los residuos sólidos urbanos (RSU), así como sus respectivos compost (CSS y CMSW) como fuente de enzimas totales, (ii) determinar la proporción de enzimas que se encuentran inmovilizados en la matriz de humus de estos materiales orgánicos, y (iii) determinar el efecto del proceso de compostaje

sobre las actividades enzimáticas totales y sobre las actividades enzimáticas inmovilizadas extraídas de los materiales orgánicos. Las actividades enzimáticas deshidrogenasas (DHA), ureasa, proteasa-BAA, fosfatasa alcalina (ALP), β-glucosidasa (GLA) y la difenol-oxidasa (OPO) se determinaron tanto en los materiales orgánicos y en sus extractos húmicos (excepto DHA). Los lodos de depuradora mostraron el mayor valor total de la ureasa, proteasa BAA, fosfatasa alcalina y actividad de DHA (12,7 μ mol NH_4^+-N g^{-1} h^{-1}, 40,6 μ mol NH_4 +-N g^{-1} h^{-1}, 75,7 μ mol PNP g^{-1} h^{-1} y 0,3 μ mol INTF g^{-1} h^{-1}, respectivamente). Para los materiales orgánicos, un porcentaje bajo de actividad enzimática ligada al humus sobre aquella total pudo ser determinada (0,6% - 22%), excepto para el caso de la actividad de DPO. El proceso de compostaje de los residuos orgánicos (proceso para estabilizar su materia orgánica) tuvo un efecto positivo sobre la ureasa, proteasa-BAA, DPO y sobre la inmovilización de la ALP en la matriz de humus, como se refleja en la mayor proporción de actividad enzimática inmovilizada sobre la actividad total, en los composts que en los respectivos materiales sin compostar (materiales frescos).

Experimento 2. Termoestabilidad de enzimas extraídas de residuos orgánicos y de sus extractos húmicos.

El objetivo de este estudio fue evaluar la estabilidad térmica (a 70 ° C de temperatura, durante 1 h) de enzimas inmovilizadas sobre extractos húmicos los cuales se obtuvieron a partir de lodos de depuradora frescos y compostados (SS y SSC), así como de residuos sólidos municipales (residuos orgánicos domésticos, RSM y MSWC). Después de un tratamiento térmico a 70 ° C, hay actividad β-glucosidasa en todos los extractos húmicos, siendo dicha actividad en las muestras de SS, SSC, MSW, y MSWC, el 35%, 68%, 17% y 12% respectivamente en comparación con muestras sin tratar térmicamente. Por el contrario, la enzima o-difenol oxidasa se vio estimulada incluso por el tratamiento térmico en las muestras de SS, pero en los extractos húmicos, esta actividad disminuyó en un 75-81%. Actividad de la ureasa en todos los extractos húmicos disminuido en un 70% o más sólo a 40 ° C, mientras que para los residuos orgánicos, esta disminución se observó después del tratamiento a 70 ° C. La actividad fosfatasa alcalina (AP) se vio afectada por el tratamiento térmico sólo en RSU y MSWC. En los extractos húmicos, la actividad AP disminuyó gradualmente hasta cero, excepto para el extracto de RSU, donde el 45% de actividad se mantuvo después del tratamiento a 70 ° C. En general, la estabilidad térmica de las enzimas en extractos húmicos fue menor que los materiales que se habían extraído.

Experimento 3. Múltiples factores de evaluación de los efectos de las enmiendas orgánicas sobre la actividad microbiana y el comportamiento de los complejos humus-enzima de un suelo semiárido.

Los objetivos de este estudio es comparar la respuesta bioquímicas de los microorganismos del suelo a diferentes enmiendas orgánicas y las dosis, así como la

dinámica de sus complejos enzima-humus frente a la actividad enzimática total. El experimento se llevó a cabo bajo condiciones de laboratorio utilizando microcosmos de suelo (500 g) enmendado con dos dosis diferentes (5 y 10 g) de diferentes materiales durante 360 días: lodos producidos en una planta de tratamiento de aguas residuales (SS), compost obtenido a partir de que dicho lodo (CSS), la fracción orgánica de residuos orgánicos domésticos (RSU), y compost del residuo orgánico anterior (CMSW). Las fracciones de carbono analizadas, tales como el carbono orgánico total (COT), carbono soluble en agua (WSC), y carbono de la biomasa microbiana (MBC), experimentaron un aumento en comparación con los resultados obtenidos en el suelo control sin enmienda; algo similar sucede con la enzima deshidrogenasa, y otras enzimas hidrolíticas (β- glucosidasa y ureasa). Las enzimas inmovilizadas sobre los extractos húmicos del suelo mostraron diferentes comportamientos en comparación con la actividad total, en función del origen de la materia orgánica que sugieren neo-formación de humus-enzima complejos.

Experimento 4. Influencia de la estabilidad y el origen de las enmiendas orgánicas sobre la humificación en suelos semiáridos.

La aplicación de enmiendas orgánicas al suelo es una estrategia ampliamente aceptada para mantener la fertilidad de dicho suelo, debido precisamente al mantenimiento y aumento de sus niveles de materia orgánica. El objetivo de este estudio fue evaluar el efecto de la aplicación al suelo de residuos orgánicos de diferentes fuentes, y con diferente grado de estabilización, sobre la humificación de la materia orgánica, medida por los cambios químicos y qumico-estructurales de sus ácidos húmicos y fúlvicos; las técnicas empleadas en este estudiado han sido la polarización cruzada de ángulo mágico con resonancia magnético nuclear de C13 (CPMAS-13C-NMR), junto con la espectroscopia de infrarrojo con transformada de Fourier (FT-IR). Se llevó a cabo un experimento de microcosmo de 360 días de duración, colocando contenderos adecuados de 500 g de un suelo semiárido enmendado con diferentes materiales: lodos procedentes de una planta de tratamiento de aguas residuales, lodos de depuradora después de ser sometidos a proceso de compostaje, la fracción orgánica de residuos orgánicos domésticos no estabilizados, y los anteriores residuos orgánicos domésticos pero sometidos a un proceso de compostaje. Se observó que la incorporación de las enmiendas orgánicas a un suelo degradado aumenta la cantidad de los ácidos húmicos en dicho suelo, independientemente de la procedencia de dichos ácidos húmicos, mientras que la cantidad de ácidos fúlvicos se mantuvo en valores muy similares a los encontrados en el suelo que actuó como control (suelo no enmendado). Los ácidos húmicos de los suelos enmendados sufrieron modificaciones con relación al suelo control, mostrando un enriquecimiento en N y en compuestos aromáticos de C, junto con un aumento en los grupos carboxílicos; todo ello supuso un aumento de las características aromáticas e hidrófobas de los suelos enmendados frente al suelo control. Después de la incubación de 360 días, algunos de los compuestos lábiles añadidos con las enmiendas orgánicas se han incorporado en los ácidos húmicos del

suelo, quedando entonces protegidos de la degradación microbiana, lo que contribuye a la acumulación de materia orgánica del suelo. La aplicación al suelo de residuos orgánicos representa una opción de gestión de residuos que resulta fundamental para zonas semiáridas y su pool de carbono.

Experimento 5. Mejora en el crecimiento de raíces provocada por ácidos húmicos procedentes de residuos orgánicos, compostados y no compostados.

Antecedentes y objetivos: Además de efecto general de los residuos orgánicos sobre la calidad del suelo, así como sobre el crecimiento de plantas, el efecto hormonal directo sobre el crecimiento de dichas plantas debido al uso de ácidos húmicos extraídos de materiales orgánicos es un tema de claro interés. En el presente trabajo se estudió la interacción directa entre los ácidos húmicos y el crecimiento de las raíces de plantas de maíz. Se pretende conocer si el diferente origen de los materiales orgánicos susceptibles de proporcionar ácidos húmicos, influye sobre el mencionado crecimiento de raíces. Acidos húmicos extraídos de cuatro diferentes materiales orgánicos (lodos de depuradora frescos, compost de lodos de depuradora, fracción orgánica procedente de residuos orgánicos domésticos, y compost confeccionados a partir de los residuos orgánicos domésticos), se caracterizaron químicamente mediante análisis elemental, cromatografía de par iónico (ICP), cromatografía de exclusión (HPSEC), resonancia magnético nuclear de 13C en estado sólido (13C-CPMAS-RMN), junto con la determinación del ácido indolacético. Posteriormente, diversos efectos morfológicos sobre el maíz (crecimiento de la raíz principal, crecimiento de las raíces laterales, zona de la raíz, el peso seco de las raíces y la actividad de H +-ATPasa de la membrana plasmática) fueron también analizados. Todas las muestras de ácidos húmicos supusieron una mejora en el crecimiento de las raíces de maíz mediante la actividad de la bomba de protones en las vesículas de dicho maíz; esto fue mas patente cuando los ácidos húmicos se extraían de muestras de residuos compostados (con materia orgánica mas estabilizada), los cuales contienen más grupos carboxílicos y un carácter más hidrofóbico, provocando preferencialmente efectos morfológicos y bioquímicos beneficiosos. La dinámica de formación asociaciones húmicas hidrofóbicas en la rizosfera de las plantas, puede liberar la hormona auxínica como promotora del crecimiento de dichas plantas, mejorando algunas de sus actividades bioquímicas (ATP-asa de la membrana plasmática. Por tanto, los residuos orgánicos representan una fuente de ácidos húmicos que pueden resultar útiles en el uso como promotor del crecimiento de raíces de las plantas.

Por todo lo expuesto, podemos decir que la *conclusión general* es que las enmiendas orgánicas (basadas en residuos orgánicos), pueden mejorar la calidad biológica de los suelos donde se adicionan, suponiendo una adecuada fuente de enzimas libres para el suelo. Asimismo, las enmiendas orgánicas proporcionan sustancias húmicas capaces de inmovilizar enzimas del tipo hidrolasas, colaborando por tanto en los ciclos de elementos importantes para los procesos de la vida del suelo. Con relación a la planta, las sustancias húmicas derivadas de enmiendas orgánicas también proporcionan ATPasa de la membrana plasmática, y promueven el crecimiento de las raíces.

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ABSTRACT

During recent decades, great efforts have been devoted to improving food production in an attempt to alleviate one of society's needs. This has represented an enormous and disproportional pressure on natural resources, especially water and soil. Additional factors related to semiarid climatic conditions are the cause a soil degradation processes such as desertification and erosion. In Spain, the risk of desertification affects about 31.5% of the total area, and is particularly acute in the Mediterranean basin (Web page of the Ministerio del Medio Ambiente, Medio Rural y Marino). It is clear that soil degradation processes are a grave problem for the conservation and protection of soil, since such degradation prevents soil from fulfilling its key functions: i) acting as a growth medium for plants, physical support and reservoir for water and plant nutrients; ii) regulating the flow of water through the environment and iii) providing a certain capacity to alleviate the harmful effects of contaminants reaching the soil by physical, chemical and biological means.

When soil is exposed to degradation processes, its biological state is the first to be affected and so its productive capacity diminishes. The biotic fraction of organic matter, which is made up of live organisms, plays a fundamental role in soil since it is ultimately responsible for the state of the organic matter and, in general terms, for the development and functioning of a land ecosystem. The scant vegetation of soils in arid and semi-arid zones as a result mainly of low productivity and subsequent abandonment means insufficient vegetal remains reaching them. So, their organic matter level diminishes as a result of C mineralization and the absence of OM into the system. In these soils, the low level of organic matter acts as a key factor in their degradation and it has led to a gradual decrease in the natural productivity of these semiarid soils.

Since the Kyoto Protocol of 1992, which identified soils as a possible sink of carbon, there has been much progress. The recent Thematic Strategy for Soil Protection, which is a preamble for a new European directive

(http://ec.europa.eu/environment/soil/pdf/_com_2006_0231), concedes that soil may well play a decisive ecological role, both in an agricultural and natural context. It identifies *the loss of organic matter* as one of the principal causes of soil degradation. A report on organic matter and biodiversity within the *European Thematic Strategy* (Van Camp, 2004) mentions that exogenous organic matter, that is organic materials added to a soil in order to improve harvests or restore a degraded soil for subsequent use, constitutes an invaluable source of OM and contributes to fixing C in the soil, thus partially diminishing the greenhouse effect derived from the release of CO_2 into the atmosphere. The maintenance of adequate organic matter levels in soils, which favours the establishment of a stable plant cover and the subsequent incorporation of organic compounds and the establishment of microbial activity, is considered one of the most effective methods in the fight against erosion and associated degradative processes.

One way of improving the fertility of degraded soils and particularly of improving its microbial activity, is *to add exogenous organic matter*. These amendments must contribute to provide labile organic matter in sufficient quantities to stimulate the life of the microorganisms that might exist in the soil. In the information concerning organic matter and biodiversity, elaborated upon within the soil European strategy, it is indicated that the exogenous organic matter, that is, those organic materials added to the soil to improve crop yields or to improve degraded soil quality (they can derive of different organic wastes from different origin such as urban animal or agricultural wastes), currently constitute an invaluable source of soil organic matter, apart from contributing to C fixation in the soil and, therefore, to the decrease of the greenhouse effect derived from CO_2 emissions. The maintenance of suitable levels of soil organic matter, which favour the development of a stable vegetal cover, which in turn guarantee the posterior organic matter turnover, is considered one of the most efficient methods to combat soil erosion and associated degradation processes. Application of organic materials enhances the nutrient status of soil by serving as a source of macro- and micro-elements and improves its physical properties by increasing soil porosity and water retention as a result of the presence of humic-like substances, known as poly-condensed macro-molecular structures. In addition, one of the beneficial effects of humic substance is that soil enzymes bounded to humic fractions are protected long term against denaturalization by proteolysis attack in soil.

From a microbiological point of view, microorganisms are responsible for important cycles within the soil and are involved in organic matter decomposition at the ecosystem level. For example, they are responsible for the mineralization of C and its loss to the atmosphere in the form of CO_2 and, therefore, for its loss and difficulty of fixation in the soil. Microorganisms are also of interest in the humification of C and the possibility of constructing a resistant and poorly biodegradable organic matter. This is of particular interest when organic amendments (ranging from organic wastes to composts) are added to degraded soils. This exogenous organic matter will be available to the attack of the different existing microbial populations, both the autochthonous and those added with the amendment. We emphasize that microorganisms play an essential role in the sustainability of soil ecosystems.

It is important to emphasize that the value that microorganisms hold, as regards their relation with soil quality or processes of degradation, or recovery there from, does not concern their type or specific functions, but, rather, in estimating the microbial activity occurring in the environment under study. For this, biochemical type parameters may constitute an excellent starting point. The basic importance of enzyme activity in soil lies in the fact that ecosystem functioning cannot be totally understood without the participation of enzymatic processes, since enzymes determine the rate of most chemical transformations that take place in the soil. They also provide information on the potential capacity of the soil to carry out specific reactions, which are important in nutrient cycles.

Enzyme activities related to the cycle of elements (carbon, nitrogen, phosphorus or sulphur) are of paramount importance in soil quality. Among these enzymes we propose are: phosphatases, ureases, proteases and different enzymes related to C-cycling such as glucosidases, celluloses and poly-phenoloxidases. Indicators of the size of microbial community (microbial biomass carbon) or its activity (basal respiration, ATP and dehydrogenase activity) will give an accurate idea of the impact of the addition of these wastes on the microbial activity and abundance in desertificated soils. A general assessment of the changes in functional and structural microbial community will be assayed by several techniques. Enzymes catalyze all biochemical reactions and are an integral part of nutrient cycling in the soil. In general, they are associated with viable proliferating cells, but enzymes can be excreted from a living cell or be released into soil solution from dead cells. Once

extracellular enzymes have left the shelter of the cell, they are exposed to an inhospitable environment in which non-biological denaturalization, absorption, inactivation, and degradation by proteolytic microorganisms all conspire to inflict their damage of enzymes unless they survive by new protection on the mineral and/or humic association.These enzymes bounded to mineral or organic colloids (immobilized enzymes) are more resistant to proteolysis rather than the free enzymes. Generally, those immobilized enzymes in mineral and/or organic colloid change in their status, properties and nature, (such as kinetics, stability and mobility of enzymes), since they are associated physically and chemically to other surrounding chemical compounds.

The organic materials can display a key role in rhizosphere not only through enzyme protection but also different effects such as the direct relationship between the plant cell proliferation and its humic substance. It is well-known that humic substances promote root growth as a hormonal behavior by inducing the ATPase in root cell with the proton ingredient. In this sense, the physical and chemical properties of humic substances (e.g. hydrophobicity and size of molecular size) are key factors to produce physiological effects in plants.

FINAL GOAL

The principal challenge of Mediterranean countries is to restore the fertility and productivity of degraded agricultural soils in arid and semi-arid areas by adding exogenous organic matter (organic amendment) for combating processes of degradation and desertification. This exogenous organic C will be able to increase the biological, biochemical, and microbiological quality of the soil. The exogenous organic matter added to the soil will include substrates which can incentivize the synthesis of some enzymes, which involve in the cycles of elements such as C, N, P, or S, and finally increase soil fertility and productivity as a consequence. The use of organic wastes (possibly treated) of animal, vegetal or even municipal (sewage sludge) origin in the amendments would represent an “added value” from both an economic and ecological point of view. The aim of the present PhD Thesis is the study of unstabilized and composted organic wastes from different points of view: i)

as the source of total and immobilized enzymes; ii) as humic matrix for stabilizing enzymes protecting them from thermal denaturalization ; iii) by their effect on biochemical, biological and microbiological quality of degraded soils when they are amended with organic materials (organic wastes of different origins such as urban, vegetal or animal) iv) by the changes in the chemical structures of humic substances when degraded soils are amended with organic materials; v) by the effect on plant root growth due to the humic substances of amended soils, with these organic wastes.

The present Doctoral Thesis is comprised of 5 experiments or studies corresponding to the above-mentioned objectives which are succinctly described in the next paragraphs.

Experiment 1. *Total and immobilized enzymatic activity of organic materials before and after composting.*

The evaluation of residual organic materials as a source of enzymatic activities immobilized in the humus matrix is a little explored research area, particularly in the case of organic materials derived from municipal wastes. Enzymes catalyze most soil reactions essential for soil functionality. The advantage of humus enzyme-complexes (immobilized enzymes) with regards to free enzymes is that the fanner are protection the humic colloid, being more resistant to denaturing agents and other adverse soil conditions. This explains the interest in use of organic wastes (which are produced in an increasingly amount and consequently are economical) as a source of immobilized enzymes, which could be concentrated and used to improve the quality of degraded or contaminated soils. The goal of this study was (i) to assess the potential of sewage sludge (55) and the organic fraction of municipal solid waste (MSW), as well as their respective composts (CSS and CMSW) as sources of enzymes, (ii) to determine the proportion of such enzymes which are immobilized in the humic matrix of these organic materials, and (iii) to determine the effect of the composting process on the total and the immobilized enzymatic activities of the organic materials. Dehydrogenase (OHA), urease, protease-BAA, alkaline phosphatase (ALP), □-glucosidase (GLA) and *o*-diphenol oxidase (OPO) activities were determined both in the organic materials and in their humic extracts (except DHA). Sewage sludge had

the highest total values of urease, protease-BAA, ALP and DHA activity (12.7μmol NH_4^+-N g^{-1} h^{-1}, 40.6μmol NH_4^+-N g^{-1} h^{-1}, 75.7μmol PNP g^{-1} h^{-1} and 0.3μmol INTF g^{-1} h^{-1} respectively. For both organic materials, a low percentage of total enzymatic activities were linked to humus (0.6%- 22%), except in the case of DPO activity. The SS and MSW composting process had a positive effect on the urease, protease-BAA, DPO and ALP immobilization in the humic matrix, as reflected by the higher immobilized activity/total activity ratios in the compost than in the fresh materials.

Experiment 2. *Thermostability of selected enzymes in organic wastes and in their humic extract.*

The objective of this study was to evaluate the thermostability up to 70 °C for 1 h of selected enzymes present in fresh and composted sewage sludge (SS and SSC) or municipal solid wastes (MSW and MSWC) and their humic extract. After a thermal treatment at 70 °C, no β-glucosidase activity in any humic extract was detected, whereas in SS, SSC, MSW, and MSWC, it was respectively, 35%, 68%, 17%, and 12% compared to thermally untreated samples. By contrast, o-diphenol oxidase activity was even stimulated by thermal treatment in SS samples, but in the humic extracts, this activity decreased by 75–81%. Urease activity in all humic extracts decreased by 70% or more just at 40 °C, whereas for organic wastes, this decrease was observed after treatment at 70 °C. Alkaline phosphatase (AP) activity was affected by thermal treatment only in MSW and MSWC. In humic extracts, AP activity decreased gradually to zero except for the MSW extract, where 45% activity was retained after treatment at 70 °C. In general, thermostability of enzymes in humic extracts was lower than the materials they were extracted from.

Experiment 3. *Multi-factorial assessment of the effects of organic amendments on the microbial activity and the behavior of humus-enzyme complexes in a semi-arid soil.*

The objectives of this study are to compare the biochemical responses of soil microorganisms to different organic amendments and doses and the dynamics of humic-enzyme complexes versus total enzyme activity. The experiment was carried

out under laboratory conditions using soil microcosms (500 g) amended with two different doses (5 and 10g) of different materials during 360 days: sewage sludge from a wastewater treatment plant (SS), compost from that sludge (CSS), the organic fraction of municipal solid wastes (MSW), and compost from MSW (CMSW). The different carbon fractions, such as the total organic carbon (TOC), water-soluble carbon (WSC), and microbial biomass carbon (MBC), increased compared to the control soil without amendment, as well as dehydrogenase and hydrolytic enzymes (β-glucosidase and urease) activities. The immobilized enzymes in the soil humic extracts exhibited different behaviors compared to total activity, depending on the origin of the organic material which suggest neo-formation of humic-enzyme complexes.

Experiment 4. *Influence of stability and origin of organic amendments on humification in semi-arid soils.*

The application of soil organic amendments is a widely accepted strategy to maintain soil fertility by maintaining and increasing the levels of soil organic matter. The objective of this study was to evaluate the effect of land application of organic wastes of different sources and stabilization degrees on the soil organic matter humification, measured by changes in the chemical and structural characteristics of humic and fulvic acids by cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance (CPMAS ^{13}C-NMR) and Fourier-transform infrared (FT-IR) spectroscopy. A microcosm experiment lasting 360 days was performed with 500 g of a semiarid soil amended with different materials: sewage sludge from a wastewater treatment plant, composted sewage sludge, the organic fraction of municipal solid wastes, and composted municipal solid wastes. The addition of the organic amendments increased the amount of humic acids in the soil in all cases, whereas the amount of fulvic acids remained very similar to that of the control soil. The humic acids of the amended soils were characterized by an enrichment of N and aromatic C compounds, along with carboxylic groups, which increased their aromatic and hydrophobic characteristics. After the 360-d incubation, some of the labile compounds added with the organic amendments had been incorporated into the soil humic pool and protected from degradation, contributing to the buildup of soil

organic matter. Land application of organic wastes represents a key waste management option in semiarid

Experiment 5. *Root growth promotion by humic acids from composted and non-composted urban organic wastes.*

Background and Aims: Besides general effect of organic residues on soil quality and plant crop, hormonal direct effect on plant growth by extracted humic acids of organic materials is interesting and profitable theme. In the present work, we studied on direct interaction between humic acid and root growth, depending on different origin of organic materials. All extracted humic acids of four organic materials (sewage sludge, compost sewage sludge, municipal solid waste, compost municipal solid waste) were characterized chemically by elemental analyses, ion pair chromatography (ICP), size exclusion chromatography (HPSEC), solid-state nuclear magnetic resonance (^{13}C-CPMAS-NMR) and quantification of IAA. Later, different morphological effects on maize (principal root growth, lateral root growth, root area, root mitosic site, root dry weight and H+-ATPase activity of plasma membrane) were analyzed. All humic acids samples promoted root growth and proton pump activity in maize vesicles, especially those composted samples, which contained more carboxylic groups and had a more-hydrophobic character, produced preferentially morphological and biochemical effects. The conformational dynamics of humic hydrophobic associations in the rhizosphere may release auxin-like plant growth promoters and enhance plant biochemical activities. These organic wastes represent a renewable source of humic acid for use as plant root promoter.

For all the above, we can say as the general conclusion that the organic amendments can be an adequate source of enzymes for degraded soil, improving soil biological quality; in addition, humic substance from organic materials can produce the beneficial association with enzyme activities as immobilization support as well as the plant enzyme of ATPase-plasma membrane.

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Chapter 1:

INTRODUCTION

Sustainability today

Sustainability is one of the most popular words in the 21st century. Originally, it emerged from the Brundland Report published by the UN in 1987, and the groundwork of the "Commission of Sustainable Development" was established in Rio De Janeiro at the Earth Summit in 1992. By that milestone meeting, public awareness about environmental issues had been aroused.

Since then, a number of global congresses on this theme have taken place, such as that which spawned the Kyoto protocol in 1997, awaking in citizens a consciousness of climate catastrophe, energy disputes, depletion of natural resources, global food security for a rapidly-increasing population, deforestation, and desertification. All these issues, which are integrated together in sustainability, have been discussed by different experts seeking comprehensive, sustainable systems.

The definition of sustainability in relation to soil management could be linked with soil quality. Soil quality appears to be an ideal indicator of sustainable land management (Herrik 2000). There is a parallelism between soil quality and sustainable agriculture. The Soil Science Society of America (1997) definied soil quality as the capacity of specified kind of soil to function within natural or managed ecosystem boundaries to sustain biological productivities, maintain environmental quality and promote the plant and animal health. Another organization has suggested that sustainable agriculture should involve the successful management of resources to satisfy the changing human needs while maintaining or enhancing the quality of the environment and conserving the natural resources (Technical Advisory Committee to the CGIAR, 1988).

At the latest milestone congress, in Copenhagen in December 2009, carbon trading, cap and share, and carbon emissions fees were the subjects of all-night meetings for the politicians and scientists from 196 countries gathered at this UN conference. It can be highlighted that 1020 million food-insecure people are concentrated in regions where soils are severely depleted of their soil organic carbon and nutrient reserves and are strongly degraded. More than ever, the alarm of global food security and global climate change related to an unbalanced carbon cycle has swept through all countries within the last decade.

The links between food security, climate change, and soil organic carbon

On a global level, agriculture accounts for about 14% of the total greenhouse gas emissions (GGE), including 47% of methane emissions (CH_4) and 84% of the nitrous oxides (N_2O). From 1990 to 2005, overall N_2O emissions from agriculture rose by 14%, being from soil by 21% and from manure management by 18%. In the same perido methane emission from enteric fermentation rose by 12% (Bates, 2010). On a local level, N_2O emission from livestock manure processing in Japan accounts for 18% of the total domestic N_2O emissions (Maeda et al., 2010), while 90% of the total NH_3 emission in Europe is from agriculture, which includes field-applied N fertilizers and livestock manure (Hayashi et al., 2009). Simultaneusly, in China, Brazil, and India, the middle-class population is increasing at a high rate, leading to increased consumption and production of meat: this is one of the reasons why the price of grain has risen and why, from now on, there will be an increasing need for grains for livestock and humans (Carfantan, 2009).

Yet, the twin crisis of climate change and food insecurity could be combated by restoration of the soil organic carbon (SOC) and the improvement in soil quality (Lal, 2011). By sequestrating carbon (C), agricultural soils can be an important sink for atmospheric CO_2, simultaneously increasing crop productivity. Cropland soils comprise 1.7 billion hectares globally (Angela et al., 2005) and approximately 10% of the global C pool is stored within agricultural soils (Abid and Lal, 2008). Above all, it is necessary to understand the relationship between soil and productivity.

The global food security issue that derives from the high rate of population growth in the world induces pressures on agriculture management: as a consequence, more-severe soil conditions arise due to intensive agriculture, such as loss of organic matter, depletion of nutrients, and soil erosion (Lal, 2010). Furthermore, unpredictable meteorological factors increase the uncertainty and the disturbance of agricultural management: for instance, insufficient soil moisture at planting and subsequent erratic rains in the main growing areas of Morocco and Tunisia in 2010 adversely affected wheat yields in these countries. Also, dry weather in Argentina in 2010, linked to the La Niña phenomenon, delayed field operations and early crop development (FAO, 2010). These effects result in socio-economic hazards, such as high cereal prices in global markets, and increase high-cost, external-input

agriculture due to the purchase of more irrigation water and chemical fertilizers under drought conditions in semi-arid and arid regions.

Humanity now confronts a crucial challenge: to develop methods of agriculture that sequester carbon, enhance soil fertility, preserve ecosystem services, and utilize water resources more efficiently in order to preserve the future supply, while productively employing a steadily-compounding supply of human labor. These are the prerequisites of "sustainable agriculture" (Bates, 2010).

The importance of soil as a natural resource

In the first place, it is necessary to understand deeply "what agriculture is" through a scientific approach. To deal with this, Lal (2010), a well-known soil scientist specializing in erosion and carbon sequestration, reminded us of the words of a scientist from centuries ago: "The first step in the science of agriculture is the recognition of soils and of how to distinguish that which is of good quality and that which is of inferior quality". This wisdom, from the 12th century and attributed to a soil scientist of Moorish Spain, Ibn-Al-Awam, still holds true today in regard to the essence of agricultural management, even though the systems and technology employed have developed and become more sophisticated over the intervening centuries.

Soil is composed by inorganic material (clay, silt, and sand), organic material, air, water, and macro- and microorganisms. Thus, the soil ecosystem can be defined as an interdependent life-support system.

In natural conditions, soils tend to maintain an equilibrium between their pedogenetic properties and the natural vegetation developed (Papendick and Parr, 1997). However, this equilibrium can be easily disturbed, especially by human intervention. Agricultural activity, for example, may in certain circumstances seriously damage this soil equilibrium and decrease considerably its natural quality. Particularly in the SE Spain, inappropriate agricultural practices are accompanied by the adverse environmental and climatic factors typical of a semiarid region: climate, relief, lithological substrate, and plant cover (Albaladejo et al., 2008). Both intensive agricultural practices and the agricultural use of marginal lands, which are very susceptible to environmental degradation and unsuitable for crop production, have led to the use of unsuitable land management techniques. This, in turn, has led to a loss of soil quality and fertility and the subsequent abandonment of the land. A

key factor in the degradation of these soils is the loss of natural plant cover, allowing increased water erosion and salinization processes to occur. This further aggravates the effects of the environmental conditions of this semiarid region (Garcia et al., 1995), leading to a loss of soil quality and fertility and the subsequent abandonment of the land for crop production purposes. The principles of soil conservation have been known for centuries and in many countries recognition of the dangers of soil degradation has prompted national soil conservation programs.

Soil quality

Our ability to assess the health or quality of soils and to identify the key soil properties which can serve as indicators of soil health/quality has become a major issue for food and fiber producers throughout the world. The search for indicators which can be used as quantitative tools to assess the quality/health of the soil has thus become a major challenge for both scientists and land managers. Many properties may be used to define soil quality and, once these have been quantified, the most-suitable strategies for soil management can be undertaken. Chemical and physical soil parameters such as organic matter (OM), nutrient status, run-off measurements, or aggregate stability have been used to measure soil quality (Papendick and Parr, 1997). However, these parameters change very slowly as consequence of a pressure in soil and so many years are required to measure significant changes. On the other hand, biological and biochemical properties (microbial activity) are generally reported as more difficult to measure, predict, or quantify; but this soil properties are very sensitive to any disturbance occurring in the soil ecosystem, thereby providing immediate and accurate information on changes in soil quality. They are intimately linked with the maintenance of soil structure and fertility and are potentially more sensitive to changes in the soil than indicators based on physical and chemical properties. Biological indicators therefore may provide an "early warning" of system collapse and allow us to react before irreversible damage occurs. This is because soil microbial activity has a direct influence on ecosystem stability and fertility (Smith et al., 1993; Ceccanti and Garcia, 1994). The microbial activity of soil encompasses a broad spectrum of activities and it may be determined

by both general (e.g. CO_2 evolution, heat output, and rates of nucleic acid synthesis) and specific (e.g. enzyme activities) criteria (Nannipieri, 1996).

With regard to the term "soil quality", many soil scientists describe it with different definitions (Doran and Parkin, 1994; Doran and Safley, 1997; Karlen et al., 1997; Schloter et al., 2003). Such quality definitions are contextual, subjective, value laden, outcome driven, and infinite in possibilities (Sojka and Upchurch., 1999). It is difficult to establish a standard definition, since, according to the context (ecological, urban, agricultural, or industrial), the term "soil quality" is changeable. The simplest definition for soil quality is the soil capacity to function (Karlen et al., 1997) Some authors (Papendic and Parr, 1992) defined it as "the capacity of the soil to produce healthy crop, resist erosion, and reduce the impact of environmental stresses on plants". Indeed, most definitions make reference to both agronomic (crop productivity) and environmental (sustainability) aspects. Adding to those two aspects, Doran and Parkin (1994) also highlighted the importance of the link between soil quality and plant, animal, and even human health. This expanded version of soil quality definition is: "the capacity of specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation.".

To evaluate soil quality, versatile aspects (physical, chemical, biological, and biochemical) are used to indicate the character, which influences strongly the biogeochemical processes and their transformation (conversion) in time, space, and intensity. The following parameters, representative of different properties, are key to the determination of soil quality (García et al., 2000):

- *Biological and biochemical parameters*: microbial biomass C, ATP, soil respiration, and enzymatic activities.
- *Chemical parameters*: pH, electrical conductivity (EC), total organic C, C fractions, mineral content, and total extractable NPK.
- *Physical parameters*: Soil texture, bulk density, water-holding capacity, moisture, and temperature

Since the present Ph D. Thesis is focused mainly on the relationship between the microbial activities and the humic fraction, the description of soil quality parameters in the next section highlights certain points that are linked to this theme.

Soil biological and biochemical parameters

Soil ecosystem has a great resilience capacity, which is, an important characteristic of natural ecosystems to resist changes and to return to a state of equilibrium after suffering disturbance. When a soil is in such a balanced state, it has a wide spectrum of biological activities and functions which can be described as normal. When a soil is exposed to degradative processes, its biological state is the first to be affected and so its productive capacity diminishes. The biotic fraction of OM, which is made up of microorganisms, plays a fundamental role in soil since it is ultimately responsible for the state of the OM and, in general terms, for the development and functioning of a land ecosystem. Microorganisms, therefore, influence ecosystems and soil fertility since they help set in motion biogeochemical cycles and decide their structure (Harris and Birch, 1989). They have a great influence on many OM oxidation, hydrolysis, and other transformation reactions and these in turn are reflected in the natural cycles of C, nitrogen, phosphorus and other elements, thus establishing (or not) the ideal conditions for the development of a stable plant cover. This is essential if a soil is to be of sufficient quality to maintain a suitable degree of natural fertility. However, such biological activity is influenced by many factors which, in the long run, depend on soil structural characteristics such as moisture content, temperature, and clay content.

Soil microbial activity has been used as an indicative parameter to gain insight into soil quality. It not only promotes nutrient availability to plants but is also involved in the mineralization and mobilization of pollutants. Thus, microbial activity is of vital importance in biogeochemical cycling. Basically, microbial activities are regulated by the nutritional conditions, temperature, and water availability. Other important factors affecting microbial activities are the proton concentrations and oxygen concentraion. The suite of methods used to determine soil microbial activities embraces biochemical monitoring of the metabolic processes of microbial communities.

Since hydrolytic enzymes (urease, phsophatase, and β-glucosidase) are involved in nutrient cycling, and their activity can be measured as an indication of the biochemical status of the soil. Our present knowledge of soil enzymology derives from previous studies. The first known report on enzymes in soils was presented by Woods in 1899. However, little progress ensued, due to a lack of appropriate methodologies and understanding of the nature of soil enzymes. Much information

about different enzymes and their activities in soils has been collected since 1950 and theoretical approaches and technical methods have developed; as a consequence, in the 1970s and 1980s, soil enzymatic methodology was established.

Their close relationship to soil biology, rapid response to changes in soil management, and ease of measurement are the reasons why soil enzymes have been suggested as potential indicators of soil quality (Bandick and Dick, 1999). Doran and Parkin (1990) mentioned that the soil enzymes as an index would integrate chemical, physical, and biological characteristics and could be used to monitor the effects of soil management on long-term productivity.

Since each enzymatic activity relates a specific substrate and a specific reaction (Nannipieri 1990), it is not recommendable to address the general state of nutrients in a soil through only one enzymatic assay. Thus, measurements of several enzyme activities must be measured for a more deeply knowledge of the soil status.

Chemical parameters in soil

In parallel with enzymatic assays, several C fractions are measured to determine soil quality. The labile C fraction of OM, such as water-soluble C or the so-called dissolved organic C, is considered the most biodegradable and is susceptible to mineralization (Cook and Allan, 1992 ab). The total water-soluble C (WSC) is used as an index of the mineralization of organic C and it is composed mainly of labile and easily-degradable compounds (Garcia et al., 1994). and one of these compounds are carbohydrates which represent the active soil organic component and a readily-available source of energy for microorganisms (Saviozzi et al., 1999).

The total organic C (TOC) is an important parameter with respect to obtaining the overall picture of soil microbial activity. Sparling (2001) reported that the biological and biochemical parameters are more sensitive, faster, and simpler with regard to the "diagnosis" of soil quality, so that more-useful information can be obtained than with other indicators such as TOC. However, in order to complete the knowledge about the soil biological status the correlations with TOC and other biochemical parameters, such as microbial biomass C, are reported (Bastida. et al., 2007).

Soil OM and humic substances are specifically described and discussed in more detail in another section. Other chemical parameters, such as pH, EC, macro and

micronutrients, heavy metals and organic pollutants, are also important factors for understanding the soil integrity. From the agronomic standpoint, information on soil NPK concentrations is the basis of field operations. However, these properties tend to be considered as more inherent than biological parameters.

Physical parameters of soils

Physical parameters are indispensable for understanding soil quality (Schoenholtz et al., 2000) and are vital factors for soil microorganisms. The importance of the soil physical properties depends on the research line being pursued: for instance, soil physicians or scientists using remote-sensing may consider them as more detectable and accessible with regard to understanding the exogenous influence of factors such as climatic change. From the biological and chemical points of view, the meanings are interpreted differently. More than a "consequence", they play a role in setting up some "causes" of the soil quality changes. The interaction between soil physical properties and microbial activities is very close (Masto et al., 2006), implying a great dependence of these activities on the soil physical conditions.

Soil organic matter (SOM)

The soil organic matter (SOM) plays a great role in determining soil structure and this, in turn, has an influence in process such as erosion resistance, water penetration, and root development (IPCC 2001). Also, it is the source for macronutrients, such as nitrogen, sulfur, and phosphorus, and micro-nutrients. From the agronomic point of view, enhancing the soil organic C (SOC) content pool to the optimal range improves crop and pasture yields through several processes (Lal 2010): i) increased available water capacity, ii) improved plant nutrient supplies, iii) restoration of soil structure, and iv) minimized risk of soil erosion.

The SOM constitutes the largest pool of organic C on the Earth's surface with an estimated C amount of 2100 Pg (1 Petagram (Pg)=10^{15} g), which is three times larger than the amount stored in above-ground vegetation (Jimenez et al., 2011). SOM is composed of: 10 to 20% carbohydrates, primarily of microbial origin; 20%

nitrogen-containing constituents, such as amino sugars; 10 to 20 % aliphatic fatty acids; and the rest of organic compounds being aromatic (Paul and Clark, 1989). The soil C levels are determined by the balance between OM inputs, primarily as plant residues, and root exudates, and OM losses due to mineralization, erosion, and leaching (Six et al., 2006).

Intensive agricultural practices generally produce a lost of SOM. For most soils, SOM can only be maintained at high levels by inclusion of a sod crop in the rotation, by no-tillage practices, or by frequent addition of large quantities of organic residues (e.g., animal manure).The quantity of SOM is determined mainly by the balance between primary productivity, humification and mineralization rate. In general, the presence of clay and silt increases the SOM content for a particular set of environmental interactions, and the presence of minerals such as those in certain volcanic soils also produces great retention of SOM.

In terms of SOM storage, two key factors exist: moisture and temperature.. Under moist conditions - as in tropical regions - the rate of C accumulation through photosynthesis is greater than the opposite process of decomposition: as a consequence, high C accumulation occurs in the top soil. By contrast, the environmental conditions of semiarid region have as consequences a reduced plant cover, increased mineralization rate of SOM and erosion which is translated into a low SOM accumulation.

Humic substances, an important part of soil organic matter

A fraction of SOM, which is relatively stable and recalcitrant, is called *humus*, and this fraction of SOM can persist in soils for decades, hundreds, or even thousands of years. Humic substances generally represent 1/3 to 3/4 of the total SOM and consist of three different fractions: Humic acids (HAs), fulvic acids (FAs), and humin materials, classified on the basis of their solubilities in aqueous acids and alkalis (Stevenson, 1994).

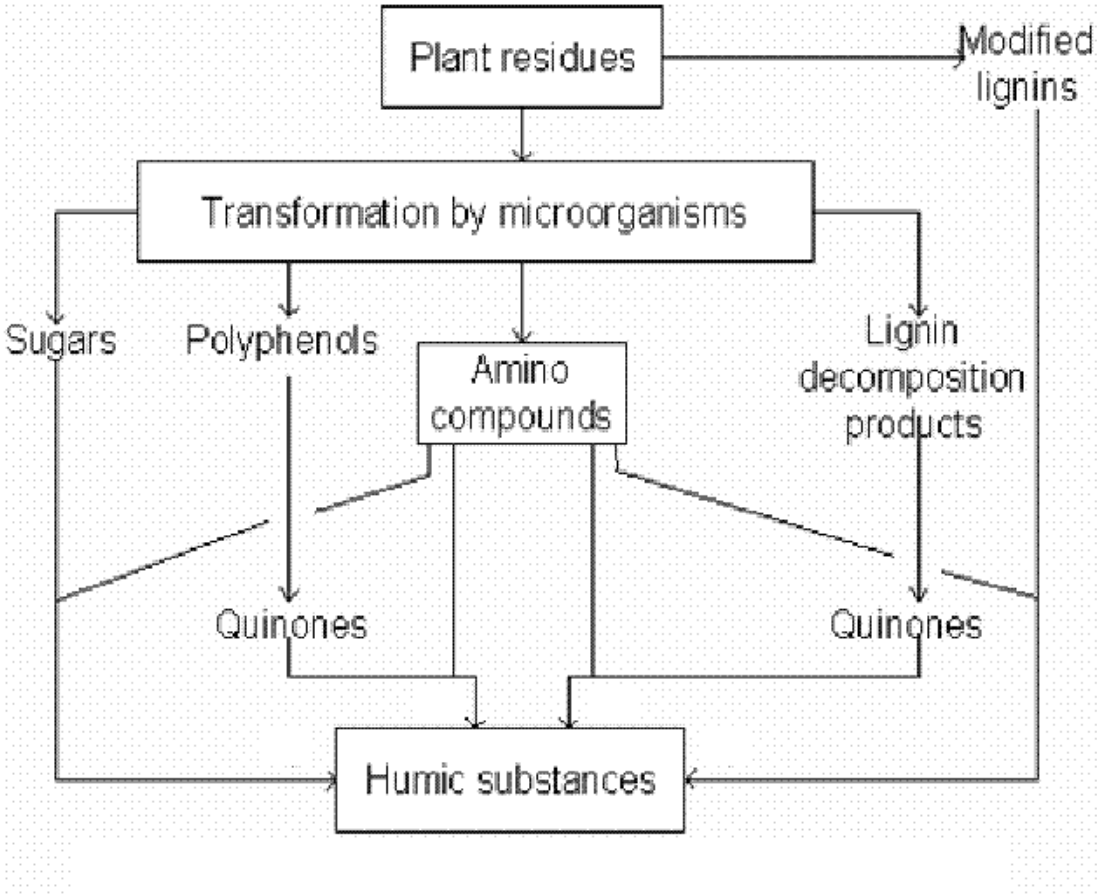
Despite major research efforts for the last 40 years and substantial progress in humus chemistry, the molecular structure of the humic substances is not completely known. Fulvic acids are the most-easily-soluble fraction and can be extracted with water under all pH conditions, while humic acids form the largest part of the exactable humic substances, which are insoluble in water at pH<2. The remainder, which is insoluble in water at any pH, is known as the humin fractions.

Several different pathways exist for the formation of humic substances during the decomposition of animal and plant debris in soil. Modification of lignin structure to produce humic substances represented the classic theory for many years, based on the fact that lignin is not fully utilized by microorganisms and the remnants become part of the soil humus. This hypothesis was strengthened by analytical data showing that humus monomers resemble the constituents of lignin. The main modification in the chemical structure of lignin involves the loss of methoxyl (OCH_3) groups along with the generation of *o*-hydroxyphenol and oxidation of aliphatic side chains to form COOH groups. However, the majority of present-day researchers favor a mechanism involving quinones: during humus synthesis, lignin still plays a significant role in producing quinones from polyphenols, derived from non-lignin C sources (e.g., cellulose) or from phenolic aldehydes and acids, released from lignin decomposition. The major difference with respect to the classic theory is that the initial material consists of low-molecular-weight organic compounds, from which large molecules are formed through condensation and polymerization. Even though considerable controversy exists around the theory of humic formation, it is accepted that extracellular enzymes plays great roles in oxidizing high-molecular-weight aromatic molecules in both pathways. One of the main beneficial effects of humic substances, in terms of sustainability, is on the longevity of C molecules. Wang and Chang (2001) studied a number of Taiwanese soils and calculated that the mean residence time of stable organic substances ranged from approximately 140 to 2200 years. The lowest average residence time (143 to 1749 years) was recorded for fulvic acids, while the mean residence times of humic acids and humins were slightly longer and similar to each other, with a range from 253 to 2200 and from 293 to 2173 years, respectively.

Paul and van Veen (1978) reported that humic materials are more resistant to degradation than non-humic materials. Their C dating data indicated that the FAs

have turnover times of hundreds of years, whereas those of humins and HAs usually approach thousands of years. The long turnover times of humins and HAs can be attributed to their intimate association with the soil mineral colloids. Anderson and Paul (1984) investigated the long-term cycling of organic C in cultivated soils by radiocarbon dating. They found that the organic C of the coarse clay–fine silt was consistently the oldest fraction, concurring with the predominance of strongly-aromatic HAs in these particle-size fractions and the higher degree of resistance of humic material to acid hydrolysis.

Figure 1. Mechanisms for the formation of soil humic substances (Stevenson, 1984)



As well as C-containing molecules, nitrogen compounds can also persist greatly in soil by association with the humic fractions of SOM. Some authors (Knicker and Hactcher, 1997; Zang, 2000) suggested the long-term protection of proteins in soil might be attributable to their incorporation into hydrophobic domains of SOM. Verma (et al., 1975) measured the mineralization rate of ^{14}C-labeled protein, and found that mixtures of protein and "model" humic polymers decomposed at 37% of the rate of pure protein. A similar experiment in soil showed that mixing of labeled protein and model humic polymers reduced protein decomposition by up to 76% over 12 weeks (Martin et al. 1978). Recent NMR-based evidence for the *in vitro* covalent coupling of peptides to phenolic components of the SOM (Hu and Hatcher, 2003) suggests a way to find covalent phenolic-protein condensation products in nature.

Effect of humic substances on soil-plant system

The contribution of humic fractions to the enhancement of soil quality is manifested in many ways, such as buffering of soil pH, enhancement of cation exchange capacity, and chelation of micronutrients (Stevenson, 1982). Also, from the environmental point of view, the adsorption of pesticides and hydrocarbons, leading to soil rehabilitation after contamination, is another beneficial function of humic substances. Besides these functions in soil, there are direct effects of humic substances on plant growth: these are observed mostly as easily-measurable parameters such as leaf chlorophyll concentration, shoot and root dry weight, the number of lateral roots, and the number of flower buds (Rautham and Schnitzer, 1981; Chen, 1996). Generally, it is thought that humic substances supply the plant with its principal nutrients or act as chelating agents to transport micronutrients; however, their effects have not been explained fully because of the lack of understanding of the mechanism of plant growth promotion. The acceleration of metabolism by humic substances is a hormone-like effect. Especially, the rapid proliferation of root cells produced by humic acids has been observed by many authors (Dell'Agnola and Nardi, 1987; Piccolo et al., 1992; Mosuclo et al., 1993, 1996, 1999, and 2005; Nardi et al., 1997 and 1999; Pizzeghello et al., 2001 and 2002; Canellas et al., 2002, 2009, and 2010ab; Zandonadi et al., 2010) and the interactions of functional groups in the humic matrix with the electrochemical gradient and

ATPase of the cell membrane are thought to play a role in triggering the accelerated plant growth.

Humic substances is a term that refers to the combined humic and fulvic acid content found in naturally occurring decomposed plant and animal residues dating back thousands of years or more. They are complex organic molecules that are formed by the transformation of plant debrise. Humic substances can help improve soil structure and make important contributions to soil stability and soil fertility leading to exceptional plant growth and micronutrient uptake. There is basic agreement on the benefits of humus. In soil, humic and fulvic acid helps improve water retention, and soil structure. Humic acids readily form complexes with inorganic trace minerals in a form that can be more easily utilized by plants. When applied to clay soils, humic and fulvic acid can help break up compacted soils, allowing for enhanced water penetration and better root zone growth and development. When applied to sandy soils humic substances improve their water holding capacity thus improving root growth and enhancing the sandy soil's ability to retain and not leach out vital plant nutrients

Soil microbial activity is stimulated by humic acid by virtue of providing the indigenous microbes with a carbon source for food. As we know, soil microbes are responsible for mineralization nutrients such as nitrogen and phosphorus so that they can be available for the plant's roots. Microbes continue to transform soil organic matter and thus to increase humic substances incorporated to the matrix of soil. There is evidence of a positive effect of humic substances on the growth of various groups of microorganisms. There is also evidence that some of the humate materials contain large populations of *actinomycetes,* which are capable of degrading a wide range of substances including celluloses, humicelluloses, proteins, and lignin.

Humic acid (in general, humic substances) also plays a critical role in the ability of the plants to uptake nutrients from soil. It is especially beneficial in freeing up otherwise unavailable nutrients in the soil so that they are made available to the plant as needed. For example, humic acid can separate aluminum and phosphorus molecules, making the phosphorus available for the plant. Humic acid helps chelate mironutrients, increasing their bio-availability to plants

Humic acids are reported to increase the permeability of plant membranes, so promoting the uptake of nutrients. Humic acid can give sugars in plants. Sugars are

especially important during flowering as plants require an increased amount of carbohydrate to produce bud. Humic acids promote vigor, disease resistance and root development in plants. These increases result in faster growth. The HAs assist roots by increasing the cell permeability, increasing soil water retention, reducing water evaporation, and promoting the growth of beneficial microbial colonies in the root zone. The creation of a healthy, microbial-active root zone environment creates stronger plants that uptake nutrients better.

Environmental stresses can cause irreparable damage to plants; stunting or delaying growth, prohibiting flower production or even causing death. Free radical molecules result from stress such as high heat or temperature fluctuations, too high humidity, pesticide applications and nutrient deficiencies or toxicities. The bio-stimulant activity of HAs has a consequence the production of anti-oxidants that combat these free radicals making plants more resistant to these and other environmental stresses. Humic acid remains in the cells providing ongoing protection.

Beneficial bacteria and fungi reproduction created in the presence of humic substances biologically increase plant growth. The microbial activity produced by these bacteria and fungi are excellent root simulators. All this translates into healthier, stronger more pest resistant plants. Humic acid is an environmentally safe bio-stimulant that can be used throughout the entire growth cycle (Nardi et al., 1997; Canellas et al.2002, 2009 and 2010; Dobbss et al., 2010).

Soil degradation in semiarid environments

During recent decades, great efforts have been made to increase food production. This has led to great pressure being put on natural resources, particularly water and SOIL. An additional problem affects large areas of Mediterranean basin of Spain: soil degradation and desertification, usually as a result of aggressive human activity in combination with adverse conditions. These factors, have led to a gradual decline in the natural fertility of the soils.

In the International Desertification Convention (UNCCD, 1994), *desertification* is defined in terms of a reduction or loss of biological or economic productivity resulting from land use or from human activities and habitation patterns. Such a definition is not easy to use operationally, so desertification is usually expressed in terms of measurable physical or biological conditions or processes which can be used as surrogates for productivity loss. The convention specifically includes soil erosion by wind or water, deterioration of the physical,

chemical, and biological properties or economic value of soil, and long-term loss of natural vegetation and biodiversity. The principles of soil conservation have been known for centuries and, in many countries, recognition of the dangers of soil degradation has prompted national soil conservation programs. However, recent regional and global assessments of human-induced soil degradation processes such as: erosion, salinization, acidification, heavy metal pollution, and SOM decline indicate that the productive capacity of millions of hectares of land continues to decrease each year, thus warning us of the ecological collapse of the world's productive soils. At a local level (and, if possible, at a higher level), we need to be able to assess how land use or human activities are affecting the capacity of the land to remain productive, how such practices are reducing or improving the health of the soil, and finally how they are contributing to degradation and desertification processes.

Crop productivity is linked closely to SOM (Bauer and Black, 1994). Many agricultural soils used by the small-landholders in the tropics and sub-tropics, mostly located in developing countries, are exposed to a risk of depletion of the SOM pool (The Dang and Klinnert, 2001; Lal, 2004). To make matter worse, these soils are strongly prone to degradation by erosion, structural breakdown, reduction in microbial biodiversity, and overall deterioration in soil quality. Under these conditions, crop yields depend on the vagaries of rainfall and must cope with additional biotic (pests and pathogens) and abiotic (drought and high temperature) stresses (Lal, 2011).

According to a United Nations survey, drylands cover more than one-third of the Earth's land surface, and approximately 70% of drylands now suffer from degradation. Many factors contribute to the degradation process. To simplify them, Lal (2010) pinpointed three classes of soil degradation processes which are closely related:, 1) Soil erosion and desertification, 2) Salinization, and 3) Nutrient depletion.

In the Thematic Strategy for Protecting Soils recently published by the European Commision (http://ec.europa.eu/environment/soil/pdf/com_2006_0231), which is the precursor of a new directive (Directive COM(2006) 232), the loss of OM is identified as one of the main threats for soil degradation. The report on OM and biodiversity within this Strategy (Van camp, 2004) points out that exogenous OM, such as organic materials that are added to the soil in order to improve cultivation and soil quality or to counteract degradation for subsequent use, constitutes an inestimable source of OM for soils, contributing at the same time to fixation of C in the soil and, therefore, to diminishing the greenhouse effect resulting from the

release of CO_2 into the atmosphere. The maintenance of suitable levels of OM in the soil favors the development of a stable vegetation cover, which, in turn, ensures the future supply of organic elements to that soil, and is considered one of the most-effective methods in the fight against erosion and associated degradative processes.

The loss of OM and the degradation of soil structure are related closely with the loss of a soil´s agricultural potential and with an increasing risk of erosion. This phenomenon has been defined as a diminution or destruction of the soil´s biological potential, leading, in extreme conditions, to desertification (El Tayeb and Sukinjs, 1989).

Since in Spain and many other European countries, particularly the most southerly, the traditional sources of OM (peats and manure) are scarces, it has been proposed that the problems concerning organic wastes and soils may be lessened by considering them both together. The possible solution would involve using the OM contained in given organic wastes as organic amendments in degraded soils, thus improving their fertility and eliminating the wastes rationally through recycling.

Organic wastes

Of all the different types of waste that are generated, biological organic wastes (not plastic wastes, oils, synthetic organic, etc.), among those of municipal origin, represent one of the most significant as regards the environment since they are produced continuously and in a generalized way: therefore, they deserve particular attention. The obligation to collect and treat municipal solid wastes and sewage sludge, as laid down by several European directives, implies a substantial economic cost; therefore, it is necessary to give them a value within the framework of sustainable development. Even though the most-problematic waste is of domestic origin, its volume is similar to that of industrial origin, but while the producers of industrial wastes are responsible for treating them adequately, the responsibility in the first case is that of local authorities.

At this point, we should pause to clarify the type of organic wastes that will be considered in this PhD. Thesis and for which we shall look for different ways of adding value. Viable alternatives will be proposed only for wastes that are NOT considered “toxic or dangerous”; that is, the quantities of persistent organic such as polychloridebipheniles (PCBs), dioxins, polycyclic aromatic hydrocarbons (PAHs)

and inorganic contaminants (basically heavy metals) that they contain are low enough to not invalidate the proposals. The protection of soil and water and the prevention of risk to human health will be the underlying precepts of this study.

The study will be framed in a European context and will take into consideration legislation concerning the application of OM to soils, the emission of greenhouse effect gases, etc., which already exists in Austria, the UK, Sweden, and Holland. However, the project will also take into consideration specific problems that countries like Spain may be suffering in their extremely-degraded soils because of their scarce OM content, and the possibility of rectifying this deficit through the addition of suitable organic wastes in controlled conditions.

Enhancing Soil Organic Matter by organic wastes addition

Like crop residues, which contain substantial amounts of plant nutrients, other types of organic wastes, such as those derived from the urban and industrial sectors, also have the capacity to contribute to the improvement of soil quality. As a particular case, in our region, in the South-East of Spain, where there is a severe problem regarding degradation of agricultural soils, massive amounts of organic residues are produced in the municipal zones where the population has increased greatly in recent years. There are a number of works on the incorporation of composted organic wastes into the semi-arid soils of this region (García et al., 1992 and 1993; Pascual et al., 1997, 1998ab, and 1999; Moreno et al., 1998; Ros et al., 2003 and 2006; Tejada et al., 2006, 2007, and 2010; Bastida et al., 2006, 2007 and 2008).

Hence, the composting of organic wastes is an alternative to their disposal in landfills and is of great interest among a growing number of communities in many countries (Renkow and Rubin, 1998). Any urban community considering the establishment of recycling and composting facilities needs to weigh up several factors, such as the environmental impacts of landfills vs. composting.

Composting

Composting of agricultural residues and urban wastes such as municipal solid wastes and sewage sludge is a commonly-used practice in modern society, not only as an

alternative method of waste disposal, but also to provide organic soil amendments. Application of composted waste increases the soil nutrient status by serving as a source of macro- and micro-elements and enhances its physical properties by increasing soil porosity and water retention, due to the presence of humic-like substances produced during the composting process (Gigliotti et al 2003). In term of the greenhouse gas emission (GGE) and climate change, composting reduces the dependency on landfill, which produces 13% of the global anthropic CH_4 emissions (IPCC, 2001; Watzinger el al., 2008), while field-applied compost incorporated into a crop soil has a lower potential for NH_3 volatilization than fresh manure amendments (McGinn and Sommer, 2007; Maeda et al., 2010).

Composting is a biotechnological process by which different microbial communities initially degrade OM into simpler nutrients and then complex organic macromolecules of humic-like substances are gradually produced, forming an organic fertilizer known as compost (Hsu and Lo, 1999). The breakdown of OM during composting is a stepwise degradation of complex substances to simpler compounds under aerobic conditions and a succession of thermophilic and mesophilic steps. In this sense, oxygen, temperature, and moisture are essential to the dynamic of microbial succession during the composting process. Besides these main physic-chemical factors of composting process, the chemical structures of the original materials, such as the proportions of different elements (C:N ratio), also determine the decomposition rates during these two phases of the composting process: rapid (sugars, starches, and proteins), slow (cellulose, hemicellulose, fats, waxes, and resins), or very slow (lignin). Materials being high in cellulose and lignin take longer to decompose whereas materials being high in sugars, other carbohydrates, and lipids take less time to produce stabilized compost (Epstein, 1997).

The biodegradation process during composting can be depicted in many ways, but, basically, it consists of two major stages (Esptein, 1997): the active composting phase (also called the “thermophilic phase”) and the curing phase. During the active composting phase, easily-decomposable compounds are broken down quickly and pathogens can be eliminated by reaching temperatures of up to 60-70 ^{0}C, which favors the activation of the bacterial community in the dynamic of succession. In this

phase, a quick, short decline in pH takes place due to the formation of organic acids, while a reduction of the C/N ratio and an increase in the fulvic acids content occur.

In contrast, during the curing phase, the compounds less susceptible to degradation are broken down such as fatty acids which can be a phytotoxic hazard for plant growth, while humic acids are produced, as long-chain or ring-form polymers. The temperature, pH, and C/N ratio tend to stabilize gradually until the end, producing the optimum conditions for growth of fungi in the composting pile.

Many different organic wastes have been regarded as original raw materials for composting; however, the types of fundamental chemical structures in all these materials are largely similar and are comprised, basically, of three carbonaceous constituents: cellulose, hemicellulose, and lignin.

A number of agricultural and municipal residues contain complex C compounds derived from cell walls, such as cellulose and hemicellulose. These constituents of organic materials are decomposed in the thermophilic phase by a dynamic microbial succession of bacteria and fungi, which secrete various specific enzymes for different substrates. One of the enzymes that participates in the first phase of composting is β-glucosidase: it catalyzes the hydrolysis of cellobiose. By the degradation of carbohydrate polymers such as cellulose and hemicellulose, water-soluble C (the reaction products) accumulates; consequently, the highest β-glucosidase activity is frequently observed in the middle of the thermophilic phase (Cayuela et al., 2008)

Lignin is the third-most-abundant constituent of plant tissues. Certain plants, the woody species in particular, contribute large amounts of lignin to the material degraded through the activities of the soil microflora. Lignin degradation is a process in which three different fungal groups of decomposers, called white-rot, soft-rot, and brown-rot, are involved. White-rot organisms are the main group involved in lignin breakdown and possess the ability to completely mineralize lignin into CO_2 and H_2O. Other fungal groups (soft rot fungi) cannot completely degrade the lignine structure. Temperature has a profound influence on the rate and extent of breakdown: around 30 ^{0}C is considered optimum (Alexander, 1977). The decomposition of lignin proceeds either in the presence or in the absence of O_2, but the loss rate of lignine in both circumstances is characteristically less than that observed for cellulose, hemicelluloses, and other carbohydrates.

Decomposition of organic residues in soil

The action of microorganisms and larger fauna in the soil leads to the decomposition of all types of organic residues. By definition, decomposition is a process through which soil microorganisms transform organic materials from identifiable plant, animal, and microbial residues into CO_2, inorganic nutrients, and humus. During the decomposition process: a) Some of the C contained in the organic material is used for microbial metabolism and is released to the atmosphere as CO_2, through aerobic respiration, and methane, in anaerobic respiration; b) Some of the nutrients in the organic material are converted from organic forms into inorganic forms, through mineralization; c) The remainder of the organic material is condensed into degradation-resistant organic polymers. These resistant or recalcitrant materials adhere to soil particles and are considered to be a pool of humified SOM, or humus.

Various organic compounds enter the soil and undergo decomposition by the soil microorganisms. These organic compounds, present in root exudates and secretions, such as sugars, amino acids, and organic acids, are readily absorbed by microbial cells and they are important for microbial growth in the rhizosphere. The more-complex organic substrates in mucilages, mucigels, lysates, and cells sloughed from plant roots can also be decomposed to provide energy and nutrients for rhizosphere microbes. Microorganisms are generally short-lived and can themselves be decomposed by succeeding populations. When an organism dies, its cells lyse and gradually disintegrate; in this way the cell membranes break down, releasing carbohydrates, proteins, lipids, and other substances that can be used by living microbial cells. Bacterial cell walls are composed of peptidoglycans, which are decomposed more easily than cellulose or chitin fungal cells.

In the terrestrial ecosystems, plant debrise constitute a major input of organic substrates for the microorganisms. In native prairies, the die-back of grasses and legumes at the end of the growing season results in a layer of dead plant debris (leaves, stems, and root) at the soil surface. Considerable below-ground residues are also deposited each year, from root death. In forest ecosystems, fine roots die each year and are available for decomposition by microorganisms.

In agricultural systems, the unharvest remnants of crops are subject to decomposition. They include leaf, stem, and root tissues not removed from the field. Depending on the tillage practices in the agro-ecosystem, the residues may be decomposed rapidly, by a bacterial food chain, or slowly, by a fungal food chain. Conventional tillage involves plowing and disking the soil to depths of 10-20 cm. This type of tillage stimulates bacteria-mediated decomposition in the soil because it fragments and mixes residues more thoroughly within the soil. Tillage tends to break apart macroaggregates and disrupts macrospores, creating a soil matrix with more microaggregates, smaller pores, and water availability that favors bacterial growth. At the same time, tillage disrupts fungal hyphae and can temporarily reduce fungal activity. Reduced tillage incorporates crop residues lightly, to a depth of 5-10 cm, while no-till operations leave the crop residues on the surface, similar to the litter layer that develops in natural ecosystems. Residue incorporation in a non-till agroecosystem occurs through the action of soil fauna, such as earthworms, which can fragment litter and redistribute it in the soil profile.

The decomposition rate of these organic substrates is affected by multiple factors: 1) Climate: temperature and rainfall exert a very-important control on soil microbial activity; 2) Soil conditions: pH, soil texture, and EC; 3) Biotic factors: the size, diversity, and activity of the microbial biomass affect the decomposition rate, as do interactions between soil microorganisms and some larger biota; 4) Characteristics of the organic residues (substrates): physical size and composition; 5) Agricultural management: such as tillage and chemical products.

Soil enzymes and their ecological functions

Organic residues of animal, agricultural, or urban origin can be added to some terrestrial ecosystems. Land application of different organic wastes (sewage sludge, animal manure, agricultural wastes, composts, etc.) occurs primarily in agroecosystems. Not only does this practice permit the recycling of the nutrients contained in these wastes, but it can also increase the soil organic C concentration through two mechanisms: i) organic wastes contains about 50% organic C, which can be decomposed and humified into the soil organic C pool by microorganisms; and ii) these nutrient-rich materials promote crop productivity, which increases both the harvested biomass and the residues remaining on the soil after harvest.

The breakdown of molecular bonds in complex C compounds is achieved by microbial enzymes. Enzymes are catalytic proteins; in terms of the energy balance in a microbial cell, the cost of their synthesis should be lower than the gain obtained when energy and nutrients liberated from OM become available for microbial growth. The maximum efficiency is achieved when microbial cells absorb small organic compounds such as simple sugars, with labile and energetic C bonds, that can be transported through the extracellular membranes with minimal energy expenditure and hydrolyzed by enzymes within the cell. Such organic compounds constitute an extremely-small part of the C stored in the detritus of soils and sediments. Most of the energy reserves are in large organic polymers, which first must be attacked by extracellular enzymes excreted from the microbial cell into the soil environment.

The secretion of extracellular enzymes is a high-risk investment for microorganisms, since: i) biosynthesis of these enzymes is energetically expensive, as they are typically large and complex molecules; ii) the substrates of these enzymes are usually large organic polymers with a complex tertiary structure, which presents a challenge for their entry into the enzyme's active site. Commonly, the joint action of a diverse array of enzymes is required to depolymerize the three-dimensional organic complex, converting sections into linear chains that can then be acted upon by enzymes that break specific bonds; iii) it is possible that some proportion of the secreted enzymes will not achieve their objective (bio-cathalisis), due to physical interference by the soil matrix, insufficient enzyme or substrate concentration, sub-optimal pH, or negative chemical interactions with soil particles or other molecules in the soil solution.

Despite these barriers, it has been shown that extracellular enzymes are readily stabilized in soils, binding to clay and OM surfaces. As long as the active site is not blocked, these enzymes remain capable of acting on their organic substrates in the soil microenvironment for some time after their secretion, even longer than the lifespan of the microbe which produced them.

Immobilization and stabilization of soil enzymes.

All soil metabolic processes are driven by enzymes (Badalucco and Kuikman, 2001). The main sources of enzymes in soil are roots, animals, and microorganisms. Once enzymes are produced and excreted from microbial or root cells, they face harsh conditions; most may be decomposed rapidly by organisms, while others may be adsorbed onto soil organo-mineral colloids and possibly be protected against microbial degradation. The fraction of the soil extracellular enzyme activity which is not denaturized and/or inactivated through interactions with the soil fabric is called naturally stabilized or immobilized. The protection of these immobilized enzymes is based basically on two associations in the soil: interaction with inorganic mineral colloids, such as clay surfaces, and association with organic complexes such as humic molecules. The extracellular enzymes involved in such interactions in the soil show enhanced resistance to thermal and proteolytic degradation (Nannipieri et al., 1996).

The first evidence that soil enzymes are more stable than those added to soil was obtained in 1940 by Conrad, who concluded that OM in soils protects enzymes (urease) against microbial degradation. Now it is generally accepted that extracellular enzymes in soils are immobilized within a network of not only organic but also mineral complexes. The study of Richard Burns on the localization and behavior of soil enzymes, and their interaction with organic and inorganic compounds, published in 1978 and 1982, is a well-known work and many authors have explored and applied his theory as a basis framework until the present time. According to his established theory, enzyme locations are characterized by ten distinct categories, depending on the type of association. Simplifying them, they can be classified into five types:

1. *Enzymes associated with living cells.* There are four sub-categories, by location of enzyme function.

i) The location of enzymes could be within the cytoplasm, linked to the cell wall of a viable cell (extracellular enzymes). Many of these enzymes are associated with central aspects of metabolism, such as glycolysis and the Krebs cycle: they cannot function outside the cell since they depend on various co-factors, on being located adjacent to other enzymes, or on some physiological property of the cell.

ii) Those enzymes attached to the outer surface of the viable (living) cell yet whose active sites extend into the ambient medium. Also enzymes which are embedded in

the extracellular gums of plant roots and of microorganisms are included in this category.

iii) Those secreted by living cells during normal cell growth and division and which are found in the aqueous phase of the soil.

iiii) Enzymes, released from lysed cells, whose original functional location was on or within the cell and which may survive for a short period in the aqueous phase of the soil. Many hydrolases are included in this category.

2. *Enzymes associated with non-proliferating cells.* These enzymes exist within fungal spores, protozoan cysts, plant seeds, and bacterial endospores.

3. *Enzymes associated temporarily with soluble or insoluble enzyme-substrate complexes.*

4. *Enzymes attached to entire dead cells or cell debris.*

5. *Enzymes immobilized on mineral or humic colloids*. These enzymes have a long half-life (relative to enzymes in the soil aqueous phase). In the case of immobilization with minerals, the adsorption on the external surfaces or within the lattices of 2:1 layer silicates could take place. In the case of immobilization with humic colloids, more-possible processes are adsorption, entrapment, or co-polymerization during humic matter genesis. The assignment of enzymes to these categories is not unequivocal, since some enzymes can belong to various categories at the same time while other enzymes can change with time from one category to another.

The important role of soil enzymes in element cycles

Most organic substrates entering the soil are complex polymers and must be decomposed by extracellular enzymes before the simple monomeric compounds can be taken up and used to support the metabolic processes of soil microorganisms. Extracellular enzymes are specific in the chemical bonds that they can break. This means that an enzyme can only bind to certain sites on the organic substrate before beginning the hydrolysis process. If an organic substrate becomes saturated with enzymes at all available adsorption sites, then additional extracellular enzymes produced by the microbial cell will diffuse further away. As proteins, extracellular

enzymes tend to bind to mineral surfaces and organo-mineral complexes if they do not encounter and bind to an appropriate substrate. Sometimes, a bound enzyme becomes inactivated because the three-dimensional protein structure cannot fold or bend in the required manner. In other cases, enzymes bound to soil surfaces retain their catalytic ability long after the organism that produced them has died. Soil OM is considered to be biochemically stabilized when there is insufficient enzyme activity to completely degrade the fresh material to CO_2. If no organisms in a soil system are capable of producing the cellulase enzymes required for cellulose depolymerization, for instance, then any cellulose present in the soil would be considered to be biochemically stabilized.

Oxidoreductases and hydrolases have been the most studied enzyme activities of soil because of their role in the oxidation and release of inorganic nutrients from organic matter (Dick and Tabatabai, 1993). Dehydrogenases are cofactor-requering enzymes, and they function only intracellularly (Nannipieri et al., 1990). Their activity represent the energy transfer through the respiratory chain (Nannipieri, 1994). For this reason, dehydrogenase activity in soil provides an index of overall microbial activity. In soils of the Mediterranean areas, dehydrogenase activity is a marker of microbial activity (García et al., 1994). Some authors have criticised this approach (Nannipieri et al., 1990, Beyer et al., 1992) because this enzyme activity measurement is affected by numerous factors such as soil type, pH, artifacts, etc (Von Mersi and Schiner, 1991; Camiña et al, 1998).

The study of different hydrolase enzyme activities is important since they indicate the potential of a soil to carry out specific biochemical reactions, and are important in maintaining soil fertility (Tabatabai, 1971; Skujins, 1976; Burns, 1982). Urease is a microbial enzyme, which hydrolyses the C-N peptidic bonds of linear amides of urea type nitrogenated substrates, producing CO_2 and NH_3 (Tabatabai, 1982). Urease acts in the hydrolysis of organic to inorganic nitrogen using urea-type substrates,The protease which hydrolyses Nα-benzoyl-L-argininamide (BAA) acts, as does urease, in the hydrolysis of proteins to ammonium using simple peptidic substrates The mineralization of organic phosphorous that is to mention that it is the transition into inorganic phosphorous which is assimilable by plants is catalysed by extracellular enzymes (phosphatases) from bacteria, fungi, protozoa or root exudates (Nannipieri et al., 1990). β-glucosidase catalyzes the hydrolysis of β-glucosides in the soil or in decomposing plant residues (Hayano and Tubaki, 1985), such hydrolysis being of

fundamental importance for microorganisms to obtain energy from the soil (Eivazi and Zakaria, 1993).

Enzymes-humus complex

Soil is an inhospitable environment for extracellular enzymes in that non-biological denaturalization, inactivation, and degradation by proteolytic microorganisms all conspire to take their toll of enzymes once they have left the protection of the cell (Burns, 1978). However, those which retain their high stability and resistance against thermal and proteolytic degradation are usually associated with clay or humus particles in the soil.

There is general agreement that more than 90% of the total nitrogen in the soil is present in the organic fraction (Stevenson, 1994). However, only 5% of this organic nitrogen is mineralized annually, implying clear stability against microbial decomposition. The nitrogen in the organic fraction has great association with humic substances, which act as a storehouse and supplier of nitrogen for plant roots and microorganisms. A relatively-large amount of aminic nitrogen present in the soil is incorporated in humified matter, up to 50% of which is thought to be present as peptides and proteins (Varanini and Pinton, 2001).

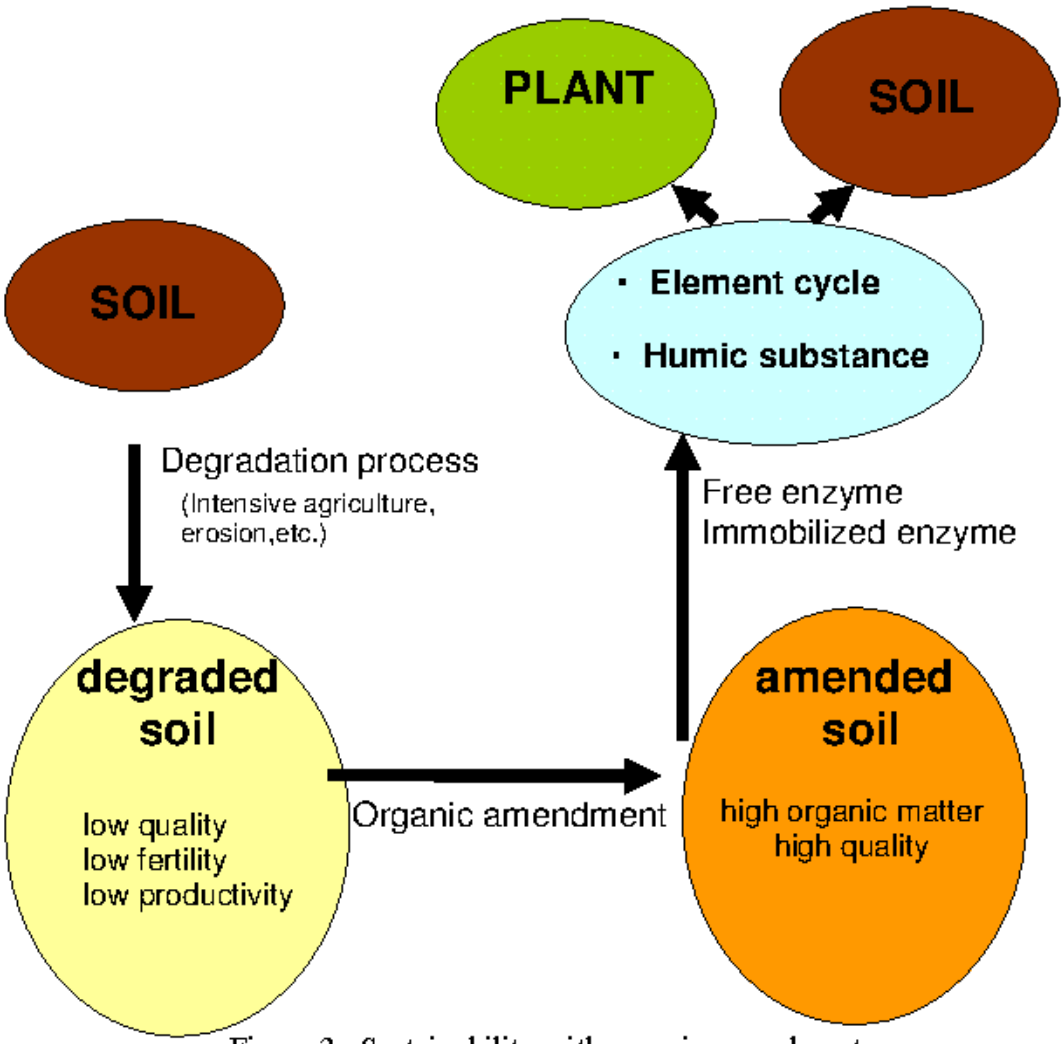


Figure 2. Sustainability with organic amendments

Humic acid and fulvic acid contain a number of functional groups that possibly interact with enzyme proteins in certain bonds that protect against denaturation. Several chemico-physical mechanisms of the humic-enzyme association are observed, including physical entrapment, hydrogen bonding, ionic bonding, and formation of an electron donor-acceptor complex (Boyd and Mortland, 1982; Gosewinkel and Broadbent, 1986; Ruggeiro and Radogna, 1988; Garzillo et al., 1996).

One of the main research priorities for pioneers of the soil enzymology field was the extraction of enzyme-humic complexes from soil and their kinetic characterization. This research was pursued mostly from the 1970s until the end of the 1980s, along with studies of enzyme immobilization by clay colloids. Immobilized enzymes

usually have a long-term and operational stability, being very stable toward physical, chemical, and biological denaturating agents. Furthermore, they may be recovered at the end of the metabolic process in the soil (Gianfreda and Rao, 2004). Extraction with the humic complex has been reported for the following enzymes: urease (Burns et al., 1972; Petit et al., 1976), diphenoloxidase (Mayudan and Sarkar, 1974a and b), peroxidase (Bartha and Bordeleau, 1969; Bollag et al., 1987), catalase (Pérez-Mateos et al., 1988), lacase (Leonowicz and Bollag, 1987), protease (Ladd, 1972; Mayudan et al., 1975, and hydrolases such as phosphatase, arylesterase, and β-glucosidase (Sarkar et al., 1980; Cacco and Maggioni, 1976). In subsequent research, emphasis was placed on the artificial synthesis and/or purification of the soil humic-enzyme complex. The synthesis of the humic-enzyme complex was carried out in the presence of divalent cations such as Ca^{2+}, and different types of enzymes have been subjected to this approach, principally for studying the interaction between the enzyme and humic substances (Ladd and Butler, 1975; Maignan, 1982; Sarkar, 1986; Serban and Nissenbaum, 1986; Ruggiero and Radogna, 1988). At the end of the 1980s, after the theory of the humic-enzyme complex had been verified and its importance for soil sustainability underlined, the methods of purification and extraction of the complex as well as the identification of chemical compounds in the matrix were developed through the great efforts of the scientists, using new equipment such as IEF and pyrolysis-gas-chromatography (Ceccanti et al., 1986 and 1989; Bonmati et al., 1998; García et al., 1992 and 1995).

While many varieties of kinetic experiments were performed, a new horizon in soil remediation involving enzyme immobilization is being developed. The oxidoreductases, one of the main extracellular-enzyme groups, -participate in the oxidation of toxic aromatic compounds by transforming them into less-toxic substances in the soil. For example, partial oxidation of recalcitrant pollutants such as polycyclic aromatic hydrocarbons (PAHs) by extracellular oxidative enzymes gives rise to products of increased polarity and water solubility and thus with a higher biodegradability (Meuleberg et al., 1997). A number of articles on this subject have been published by Bollag since the 1970s and by Gianfreda since the 1990s, respectively, and their work has advanced our understanding of the mechanism and processes of soil bioremediation in relation to extracellular enzymes. Some of their work is focused on the enzymes immobilized with clay and humic colloids in the

long-term (Gianfreda and Bollag, 1994, Gianfreda et al., 1993; Naidja et al., 1997; Dec et al., 2000; Ahn et al., 2002; Dúran et al., 2002; Rosas et al., 2008).

Lately, advanced technology in the molecular biology field has contributed to work on the mechanisms of the interactions between proteins, the chemical structures of organo-mineral colloids, and microorganisms in soils. Since the end of the 1990s, there has been growing recognition among soil scientists that it is necessary to go beyond DNA and protein analysis for an improved understanding of soil functionality. Different protocols involving the simultaneous extraction of RNA, DNA, and protein from soil have been released (Duarte et al., 1998; Griffiths et al., 2000; Hurt et al., 2001; Weinbauer et al., 2002). A shift in research themes has occurred, to a new wave of soil proteomics and DNA studies, which provide more-detailed information for the understanding of the basic components (DNA, RNA, and proteins) of enzymes and microorganisms. For the foreseeable future, these will remain the preferred molecular biology tools, together with the usual biochemical and chemical techniques.

The resistance against denaturation of immobilized enzymes inhumic matrix

The stability against enzyme denaturation by exogenous factors (e.g. temperature and attack by other enzymes) is a topic which shed lights on soil sustainability. There is general accordance that enzyme complexation with organic colloids in soil increases their persistence against thermal denaturation (Rowell et al., 1973; Ladd and Butler, 1975; Sarkar and Burns, 1984) as well as against proteolytic attack by enzymes such as pronase or trypsin (Petit et al., 1976; Nannipieri et al., 1982 and 1986; Sarkar and Burns, 1984). On the other hand, some scientists reported a loss of enzymatic activity due to complexation between enzymes and organic colloids (Vuroinen et al., 1996; Kandeler, 1990). These reductions were attributed to possible inhibition by the following factors:

1) The presence of monomer aromatic compounds or quinones, which may remain in the aqueous phase - like the rest of the residual compounds - during the process of polymerization (Rao et al., 2010).

2) The location of adsorption, where the protein part of the enzyme is exposed, such as on the colloid surface (Boyd and Mortland, 1985).

3) The shield formed by the solid support around the enzymes in the complex. This protection prevents the preolytic attack of proteases, which hydrolyzes the enzymes, and, at the same time, reduces the mobility of these enzymes.

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Chapter 2:

OBJECTIVES

Premise

In natural conditions, soil tends to be in equilibrium state after the long process of the soil formation, denominated as "*edaphogenesis*", which is taken place over

millenniums. After attained to the maximum evolution of this process, the soil can be found, to some extent, with the plant covers which continuously input certain quantity of organic materials (plant debris) and nutrients. At the same time, this plant cover has a great contribution to hold same or even better quality of soil structure and to serve for the protection against deteriorative process of soil erosion. In this sense, it can be deuced that the soil in equilibrium sustains successful microbial activity and perform its functions correctly under a dynamic ecosystem. However, that equilibrium of soil, attained through long run process, can be subjected to some disturbances. The anthropogenic activity, considered as one of those disturbances, influences the sustainable equilibrium status of soil. The effect could be positive or negative depending on climatic conditions, biodiversity, organic matter content, type of vegetation, soil characteristics, intensity and timing of anthropologic activity.

One important characteristic of the soils of the Mediterranean region is that they are indeed submitted to erosion and desertification processes and they have low organic matter content. Intensive cultivation, continual ploughing, inappropriate management of soil and forest fires, during centuries, have had an important effect on humification processes and on the soil organic matter (SOM) content. One way of improving the fertility of degraded soils and particularly of improving its microbial activity, is to add exogenous organic matter. By this term we mean that the amendment must contribute to provide labile organic matter in sufficient quantities to stimulate the life of the microorganisms that might exist in the soil. For this purpose, organic fraction of municipal solid wastes and sewage sludge may be appropriated source of organic matter. *The characteristics of this sub-product or organic wastes have beneficial effects in soil. Thus, from a physical point of view, these soil organic amendments increase soil sponginess, contribute to improve the nutritional quality of the soil and, most importantly, and its labile organic fraction which is beneficial in order to improve the microbial activity, increasing, in this way the soil potential fertility and biogeochemical cycles of the most important elements.*

There are uncountable kinds of organic materials that are applied to the degraded soil as carbon and nutrient resources. And, many of them are derived from the wastes and residues produced in the agriculture, industrie, and municipalities. After being undergone the amendments of those reused organic materials, soil statues to afresh step forward to the equilibrium state. In this condition, the improvement of soil

quality is renewably taken place in physical, chemical, biological and biochemical aspects. One of those is a reinforcement of material inputs, which enhances the association between immobilized enzyme and humic substances in soil and this effect can increase the resistance against enzyme denaturalization for a long term. Not only new input of humic substance in rhizosphere contributes the soil enzymes that play great role on the nutrient cycles, but also contributes the plant growth directly by inducing the root cell proliferation.

General Goal

The general goal of this PhD. Thesis is to study the effects on the fertility and productivity of degraded agricultural soils in arid and semi-arid areas by the adding exogenous organic matter (organic amendment). This is successful strategy for combating processes of degradation and desertification, and introducing exogenous C in the soil which will be able to increase the biological, biochemical and microbiological soil quality. The exogenous organic matter added to the soil will include substrates which can incentive the synthesis of some enzymes by microbial cells, These in turn, catalyze the cycles of elements as C, N, P or S, thus increasing soil fertility and the use of organic wastes (possibly treated) of animal, vegetal or even municipal (organic fraction of domiciliary wastes and sewage sludge) origin in the amendments would represent an "added value" from both an economic and ecological point of view.

Specific objectives:

On biological and biochemical characteristics of organic wastes:

- To study the total and immobilized enzyme activities in different organic materials. The goal of this study was (i) to assess the potential of sewage sludge and the organic fraction of municipal solid waste, as well as their respective composts as sources of enzymes, (ii) to determine the proportion of such enzymes which are immobilized in the humic matrix of these organic materials, and (iii) to determine the effect of the composting process on the total and the immobilized enzymatic activities of the organic materials. Dehydrogenase, urease, protease-BAA, alkaline

phosphatase, β-glucosidase and *o*-diphenol oxidase activities were determined both in the organic materials and in their humic extracts (except dehydrogenase activity).

- To evaluate the thermostability up to 70 °C for 1 h of selected enzymes present in fresh and composted sewage sludge or municipal solid wastes, and their humic extract..

On soil biochemical quality and cycle of elements:

- After applying the organic amendments into the semi-arid soil in laboratory condition and to monitor their influence on biochemical and chemical soil property. The objectives of this study are to compare the biochemical responses of soil microorganisms to different organic amendments and doses and the dynamics of humic-enzyme complexes versus total enzyme activity

- To prove increase of the immobilized enzymes in humic substance after one year amendment.

On humic and fulvic acids in amended soil

- The objective of this study was to evaluate the effect of land application of organic wastes of different sources and stabilization degrees on the soil organic matter humification, measured by changes in the chemical and structural characteristics of humic and fulvic acids by cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance (CPMAS ^{13}C-NMR) and Fourier-transform infrared (FT-IR) spectroscopy. To study the chemical composition of soil humic substances after organic amendments and compare with non-amended soil.

On plants:

- To study the effect of organic residues on soil quality and plant crop, hormonal direct effect on plant growth by extracted humic acids of organic materials is interesting and profitable theme. In the present work, we studied on direct interaction between humic acid and root growth, depending on different origin of organic materials. All extracted humic acids of four organic materials (sewage

sludge, compost sewage sludge, municipal solid waste, compost municipal solid waste) were characterized chemically by elemental analyses, ion pair chromatography (ICP), size exclusion chromatography (HPSEC), solid-state nuclear magnetic resonance (^{13}C-CPMAS-NMR) and quantification of IAATo observe the influence of different structures of humic substances from humic substances on plant root growth.

- To study the mechanism of the relationship between the humic substance and the root cell proliferation.

Chapter 3:

Total and immobilized enzymatic activity of organic materials before and after composting

Total and immobilized enzymatic activity of organic materials before and after composting

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ABSTRACT

The evaluation of residual organic materials as a source of enzymatic activities immobilized in the humus matrix is a little explored research area, particularly in the case of organic materials derived from municipal wastes. Enzymes catalyze most soil reactions being essential for soil functionality. The advantage of humus enzyme-complexes (immobilized enzymes) with regards free enzymes is that the former are protected in the humic colloid, being more resistant to denaturing agents and other adverse soil conditions. This explains the interest of use organic wastes (which are produced in an increasingly amount and consequently are economical) as a source of immobilized enzymes, which could be concentrated and used to improve the quality of degraded or contaminated soils. The goal of this study was (i) to assess the potential of sewage sludge (SS) and the organic fraction of municipal solid waste (MSW), as well as their respective composts (CSS and CMSW) as sources of enzymes, (ii) to determine the proportion of such enzymes which are immobilized in the humic matrix of these organic materials, and (iii) to determine the effect of the composting process on the total and the immobilized enzymatic activities of the organic materials. Dehydrogenase (DHA), urease, protease-BAA, alkaline phosphatase (ALP), β-glucosidase (GLA) and *o*-diphenol oxidase (DPO) activities were determined both in the organic materials and in their humic extracts (except DHA). Sewage sludge had the highest total values of urease, protease-BAA, ALP and DHA activity (12.7 μmol NH4+-N g-1 h-1, 40.6 μmol NH4+-N g-1 h-1, 75.7μmol PNP g-1 h-1 and 0.3μmol INTF g-1 h-1, respectively), which decreased with composting, but MSW had the highest total value of DPO activity. For both organic materials, a low percentage of total enzymatic activities were linked to humus (0.6-22 %), except in the case of DPO activity. The SS and MSW composting process had a positive effect on the urease, protease-BAA, DPO and ALP immobilization in the humic matrix, as reflected by the higher immobilised activity/total activity ratios in the compost than in the fresh materials.

Keywords: compost, enzymatic activity, immobilized enzymes, sewage sludge, urban wastes.

1. Introduction

There is growing interest in the recycling of human-generated organic residues, in order to minimize environmental problems resulting from their accumulation in landfills. Large quantities of organic wastes (17x106 Mkg/year) are an issue in Spain (Orozco-Barrenetxea et al., 2004). In addition, many agricultural soils have degraded quality. Addition of these organic amendments to the soil could eliminate wastes as well as improve soil quality and ultimately plant production (Costa et al., 1991). A number of soil chemical and microbiological properties

of these type of amendment have been assessed to identify substances that might prohibit plant growth and/or characteristics that might enhance plant growth through improvements to soil quality (García et al., 1992a; Pascual et al., 1997). From this point of view, some characteristics of organic materials which limit their agricultural use are: the content of heavy metals, organic C fractions, plant nutrients, phytotoxic compounds such as polyphenols and salts. Enzymatic activity has also been assessed in organic amendments but primarily in relation to the impact these amendments have on nutrient cycling once in the soil (García et al., 1993; Ayuso et al., 1996)

Soil microbial populations and their enzymatic activities play an important role in soil fertility and quality because most organic matter and nutrient transformations are mediated by such activities (Nannipieri et al., 1990). The hydrolytic activity of enzymes such as urease, protease, phosphatase and □-glucosisdase are essential in the N, P, and C cycles, respectively, where they catalyse transformations of the organic forms of the aforementioned elements to forms more readily available to microorganisms and plants. Other oxidoreductase enzymatic activities have an important role in the oxidation of soil organic matter (dehydrogenase activity) or in the formation of humic substance precursors (o-diphenol oxidase). Unlike the rest of the aforementioned enzymes dehydrogenase is exclusively intracellular and its activity depends on various enzymes. Dehydrogenase has a fundamental role in the cell metabolism of all microorganisms and therefore serves as an indicator of the overall microbial activity (Nannipieri et al., 1990).

The use of organic materials as a source of immobilized enzymes, to improve soil quality, is a relatively undeveloped research line. There are numerous studies on the characterization of the organic materials derived from municipal solid waste or wastewater depuration process, by the enzyme activities they contain, and their importance in nutrient cycling (García et al. 1995, Ceccanti and García, 1994, Nogales et al. 1982). However, little is known concerning which fraction of this enzymatic activity is complexed with the humus of these organic materials. The link of enzymes to humus matrix affects their activity towards their substrates in a positive or negative way, their specificity of action, their stability toward physical factors such as pH and temperature and their resistance to proteolysis (Nannipieri et al., 1974; Ladd and Butler, 1975). Thus an increase of soil temperature or proteases content will have a lower effect on the tertiary structure of these immobilized enzymes in the humic matrix and in this form the activity and specificity of the enzyme will not be perturbed (Nannipieri et al., 1996). On the other side, immobilized enzymes in humic molecules are long-lived and do not depend of microbial population variations or their synthesis by living cells (Burns, 1982).

The use of immobilized enzymes, once extracted and purify, can be more suitable for degrading organic pollutant in soil than microbiological methods because the former can often be used

under extreme conditions that would be detrimental to active microbial cells (Nannipieri and Bollag, 1991). However, a key factor is the extraction and purification of these humus-enzymes complexes without affecting its activity. The aim of this study was to: 1) assess the potential of different organic materials as an enzymatic activity source, 2) determine the proportion of such enzymes which are immobilized in the humic matrix of these organic materials, and 3) determine the effect of the composting process on the total and the immobilized enzymatic activities of the organic materials.

2. Materials and Methods

Organic materials

Four different organic residues were used in this study: a sewage sludge (SS) collected from a municipal wastewater treatment plant in El Raal-Murcia (SE Spain); the compost (CSS) produced from the above mentioned sewage sludge; the organic fraction of a municipal solid waste (MSW) collected from the treatment plant of Mula (15 km from Murcia city), which receives all the household wastes produced in the metropolitan area of Murcia (300,000 inhabitants); and the compost produced from this organic material (CMSW). MSW was obtained after manual and mechanical separation of most part of the metallic, plastic and paper materials from the wastes. The composting (industrial scale) of SS and MSW was carried out in horizontal reactors, in which the material remained static but with mechanical ventilation provided. The maximum temperature reached (65° C) was maintained for a minimum of 48 hours (to guarantee disinfection of the material), after which the temperature was maintained in the range 53-60° C during most of the process. The moisture level of the material was the optimum (60%) for increasing microbial activity. To improve oxygenation inside of the sewage sludge pile during the composting process, a bulking agent (wood shavings) was added on a volumetric basis in the proportion of 1:2 (material:bulking agent). The composting process lasted 75 days for both SS and MSW. Three samples (each composed of 8-10 subsamples) of each organic material was collected and air dried ,and then each sample was milled in order to homogenize the material. The three replicates of each material were analysed and the mean value was calculated for each parameter.

Chemical analysis

The total organic C (TOC) content of air-dried samples was determined using an automatic analyzer (Thermo Finnigan Flash EA 1112).

Humic substances were extracted with a 0.1M, pH 7.1 sodium pyrophosphate solution (w/v ratio=1:10) by mechanical shaking for 24 hours. The neutral pH of the extractant was selected because the enzymatic activities detected at this pH were higher than those at alkaline pH (non published data). The centrifuged and filtered (0.2 μm Millipore membrane) extracts were dialyzed against distilled water with a membrane of 12000-14000 molecular weigh cut off and 25 Å pore diameter (Visking® dialysis tube, Serva GMBH, Heidelberg, Germany) to obtain a purified humic extract. All the C

fractions determined in the aqueous extract were also analysed (using the above mentioned methods) in the sodium pyrophosphate extracts of the materials. All analyses were carried out in triplicate.

Enzymatic activity assays.

Urease, protease-BAA, ALP, GLA and DPA activity were determined in both the original organic materials (total enzymatic activity) and the pyrophosphate extracts (enzymatic activity immobilized in the humic substances) obtained from these materials. To determine the enzyme fraction immobilized in the humic colloids, all the above enzymatic activities were measured using 1 ml of the pyrophosphate extracts. Dehydrogenase activity was only determined in the original materials, because this enzyme is intracellular and therefore not immobilised in the humic matrix (Rossel et al., 1997). All enzymatic activity determinations were carried out in triplicate.

Urease activity was determined by the buffered method of Kandeler and Gerber (1988): 0.5 ml of a solution of urea (0.48%) and 4 ml of borate buffer (pH 10) were added to 1 g of organic material in hermetically sealed flasks, and then incubated for 2 hours at 37 °C. The ammonium content of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate addition prior to incubation to determine the native ammonium content of the organic materials.

Protease-BAA was measured using the method of Ladd and Butler (1972) modified by Bonmati et al. (1998): To 0.5 g of organic material, 4 ml of 0.1 M pH 7.1 phosphate buffer and 1 ml of 0.03M N-β-benzoyl-L-argininamide (BAA) solution prepared in the afore mentioned buffer were added, incubating at 40°C for 1 hour. The ammonium content of the centrifuged extracts was determined as above by a modified indophenol-blue reaction. Controls were prepared without substrate addition prior to incubation to determine the native ammonium content of the organic materials.

Alkaline phosphatase and BLA activities were determined following the methods reported by Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1988), respectively. For both enzymatic activities, 2 ml of modified universal buffer (MUB) pH 11 and 0.5 ml of p-nitrophenyl phosphate 0.025 M (for phosphatase activity assay) or 2 ml of MUB pH 6 and 0.5 ml of p-nitrophenyl β-D-glucopiranoside 0.025M (for β-glucosidase activity assay) were added to 0.2 g of organic material. Then the mixtures were incubated at 37 °C for 1 hour. After incubation the enzymatic reactions were stopped by cooling on ice for 15 min. Then, 0.5 ml of 0.5 M CaCl2 and 2 ml of 0.5 M NaOH (for phosphatase) or 2 ml of 0.1 M Tris(hydroxymethyl) aminomethane-sodium hydroxide (THAM-NaOH) pH 12 (for β-glucosidase) were added. In the control, the respective substrates were added before the addition of CaCl2 and NaOH.

o-Diphenol oxidase activity was determined as reported by Perucci et al. (2000). To 0.5 g of organic material, 1.5 ml of catechol solution 0.2 M

(substrate), 1.5 of proline solution 0.2 M (reagent), and 2 ml of phosphate buffer (0.1M, pH 6.5) were added. The mixture was incubated at 30 °C for 10 min and the enzymatic reaction was stopped by cooling in an ice-bath and adding 5 ml of ethanol. After centrifugation, the absorbance of the supernatant was measured at 525 nm. In the control, the substrate (catechol) was added to the samples after incubation.
Dehydrogenase activity was determined as reported by Von Mersi and Schinner (1991) using p-iodonitrotetrazolium chloride as substrate and measuring the absorbance of the iodonitrotetrazolium formazan (INTF) produced in the enzymatic reaction.

Statistical analysis
Data were submitted to one way ANOVA in order to determine significant differences between the means of the organic materials. Then, a multiple range test at the 95 % confidence level was performed using Tuckey's method.

3. Results
Characterization of the materials
Chemical and physicochemical characteristics of the organic amendments are reported in Table 1. Total organic carbon percentage in the different organic materials range from 18 to 20% and therefore these materials can be considered as organic amendments which can improve organic matter content of degraded soils such as those of semiarid regions. The increase in TOC observed for SS after composting was due to the incorporation of wood shaving as a bulking agent. In general the pH, EC, and heavy metal content of the materials would not restrict the plant growth if they were added to soil. All amendments have the potential to supply essential plant macronutrients.
Enzymatic activities
The level of DHA was 3 times higher in SS than in MSW indicating a higher activity of the microbial biomass (Fig. 1). This is most likely due to sewage sludge being produced in a wastewater treatment plant, where the most important step of the effluent treatment is a biological process. The lower level of microbial metabolic activity in MSW with respect to SS may

be due to the presence of some toxic compounds which inhibited microbial activity. It is likely that these toxic products were degraded during the composting, this explains the higher DHA values of CMSW with respect to MSW. Another hypothesis is that there was a higher adsorption of INTF (product of the reaction catalysed by dehydrogenase, which was used for its determination) on the humic colloids of MSW, thus leading to underestimation of DHA (Camiña et al. 1998). Dehydrogenase is exclusively an intracellular enzyme and so its enzymatic activity in the humic extracts was not determined

Dehydrogenase activity decreased during the SS composting process, likely due to the fact that a fraction of microbial biomass was destroyed by the high temperatures reached in this stabilisation process (Fig. 1). It is also possible that the dilution effect produced by the addition of the bulking agent, together with the degradation of the more labile substrates with composting, caused the decrease in DHA observed in CSS. The DHA of MSW increased with composting, and the cause is related to the presence of inhibitors of dehydrogenase activity (inorganic N and Fe3+) in MSW, which decreased during the composting process. The inhibitory effect of high concentrations of ammonium, nitrites and Fe3+ on soil dehydrogenase activity has been reported by Trevors (1984).

Table 1. Chemical and physico-chemical characteristics of the different organic materials (values on dry weight basis)

	TOC[†] (%)	EC[¶] (mS/cm)	pH	TON[$] (%)	P_{tot} (% P_2O_5)	K_{tot} %	Cu (mg/kg)	Zn (mg/kg)	Cr (mg/kg)	Pb (mg/kg)	Ni (mg/kg)
SS	17.9	6.82	6.82	4.56	12.4	0.35	177	581	8.40	28.1	16.5
SSC	20.6	3.00	7.05	3.39	10.9	0.38	218	523	15	55.5	21.6
MSW	17.0	6.79	6.73	1.63	2.04	0.71	283	1855	107	125	120
CMSW	16.6	3.93	7.90	2.56	2.46	0.51	336	628	47.9	233	77.2
[ψ]EU limits							1000-1750	2500-4000	1000-1500	750-1200	300-400

[†] TOC; Total organic carbon.[¶]EC; Electrical conductivity.TON; Total organic nitrogen.[ψ] Heavy metal concentration limits in sewage sludge when this amendment is used in agriculture (C.E.C., 1986)

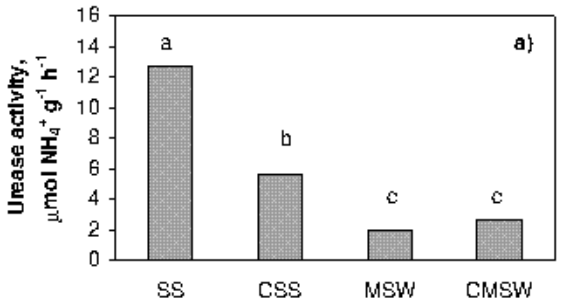

Figure 1. Dehydrogenase activity: SS, sewage sludge; CSS sewage sludge compost; MSW, organic fraction of municipal waste; and CMSW, compost obtained from the organic fraction of municipal waste. Bars followed by the same letter are not significantly different ($p<0.05$) according to the Tuckey test.

The DPO activity was significantly higher in MSW (1.3 μmol catechol ox. 10 min-1 g-1) than in SS (0.4 μmol catechol ox. 10 min-1 g-1), which indicated that there was a lower amount of the specific substrates (o-diphenolic compounds) of this enzyme in SS than in MSW (Fig. 2a). The SS had already undergone a first digestion in the wastewater treatment plant and substrates that could activate the synthesis of DPO may have been drastically reduced during this process. The DPO activity decreased significantly with the composting in the original materials (Fig. 2b). This could be explained by the fact that the dialysis of the pyrophosphate extracts eliminated a great amount of salts and other compounds which could inhibit DPO activity. Among the latter

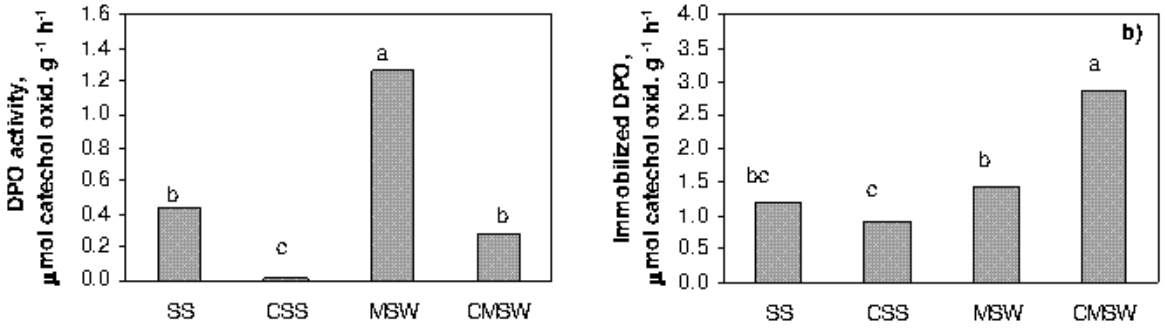

Figure 2. O-diphenol oxidase activity in the organic materials and the pyrophosphate extract. Nomenclature of samples as in Figure 1.

microorganisms were decomposed. The DPO enzymes are directly involved in humus formation, and therefore the higher the levels of this enzymatic activity the greater the intensity of humification process (Stevenson, 1982).

The DPO activity in the humic extract was higher than that determined higher immobilized activity/total activity ratios (88.6 and 10.3 for CSS and CFMW, respectively) than the materials before composting (2.8 and 1.1, for SS and FMW respectively), which indicates that a more stable organic matter was formed during composting. Thus, the humic extracts of

composts which have a higher level of immobilized DPO activity could be used as useful amendments to favour the humification process in soil.

As shown in Fig. 3a, urease activity was higher (12.7 μmol NH4+-N g-1 h-1) in SS that in MSW (2 μmol NH4+-N g-1 h-1). According to García et al. (1992b) sewage sludge has a high load of urea-like compounds that act as urease substrates, inducing urease synthesis by microorganisms. The composting process reduced the urease activity only in the case of SS, the loss of urease activity being explained by both the depletion of substrate with organic matter stabilization, and the dilution effect of adding the bulking agent. However, the composting process did not diminish the MSW urease activity, suggesting that CMSW contains nitrogen compounds which function as specific substrates of this enzyme. This trend could be explained by both the elimination of inhibitors and the production of new substrates of the enzyme caused by the depolymerization of more complex organic compounds during the composting process. This fact demonstrated the different behaviour of the organic matter of these materials during the composting process.

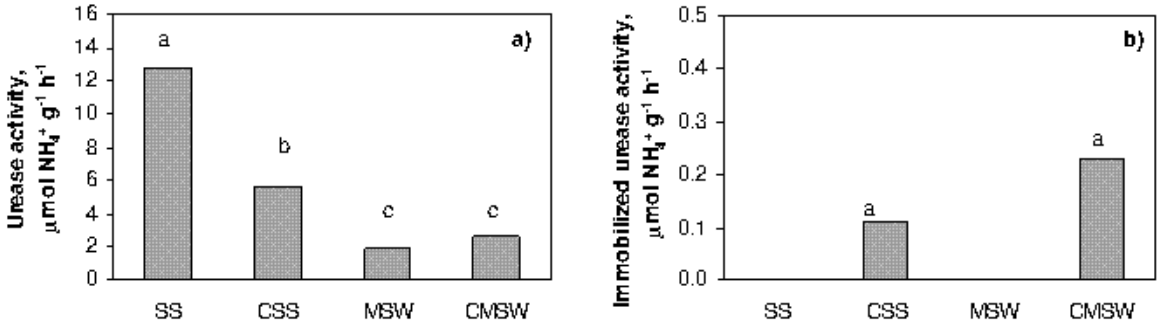

Figure 3. Urease activity in the organic materials and the pyrophosphate extract. Nomenclature of samples as in Figure 1.

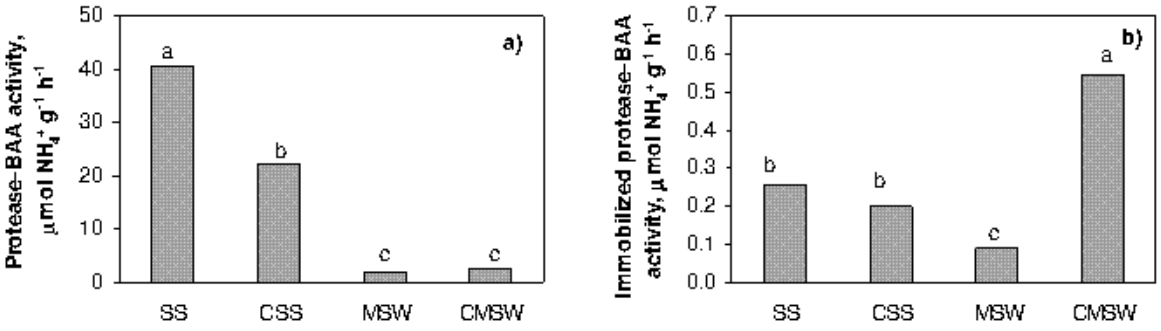

Figure 4. Protease activity in the organic materials and the pyrophosphate extract. Nomenclature of samples as in Figure 1.

No detectable urease activity was observed in the pyrophosphate extract of the non-composted materials (Fig. 3b). The activity detected in the pyrophosphate extract of the composted materials was very low in comparison with the total urease activity (2 and 8.6% respectively for CSS and CMSW). Other authors have reported low levels of urease activity in sodium pyrophosphate extracts and postulated the possible inactivation of ureases by phenolic compounds (Nannipieri et al. 1974).

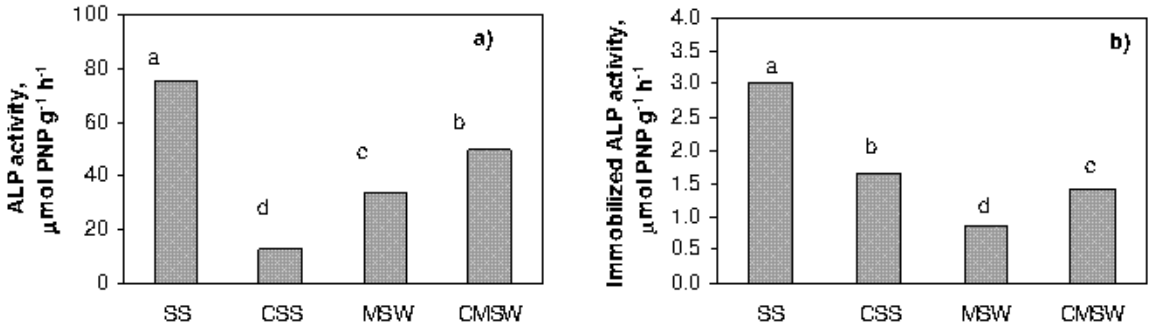

Figure 5. Alkaline phosphatase activity in the organic materials and the pyrophosphate extract. Nomenclature of samples as in Figure 1.

Protease-BAA, an enzyme which hydrolyzes N-α-benzoyl-L-argininamide to produce ammonium, was significantly greater in SS than MSW (Fig. 4a). It is necessary to bare in mind that SS has a high content of cellular proteins and polypeptides (Cuesta, 1996; García, 1990), which are capable of activating the synthesis of the enzyme. Similarly to urease activity, the protease-BAA activity of SS decreased during the composting process as a result of the loss of substrates that implies the mineralization of the organic matter during the composting process and because of the dilution effect produced by the addition of bulking agent. This trend was not observed in the case of MSW since there was already a low level of protease-BAA activity detected before the composting process. Several reasons for this can be put forward: i) there was no dilution effect of this enzymatic activity because no bulking agent was added in this case; ii) the amount of specific substrates of this enzyme did not decrease with the organic matter mineralization; iii) substrate production compensated its degradation during the composting process.

After pyrophosphate extraction of the uncomposted organic materials and the subsequent dialysis of the extracts, low levels of proteases-BAA activity were detected compared with the content of the original materials (0.6 and 0.9 %, respectively for SS and MSW) (Fig. 4b). Bonmatí et al. (1998) reported, that most of protease-BAA activity is associated with humine and this organic matter fraction is not extracted with sodium pyrophosphate (Stevenson, 1982). The low absolute values of protease-BAA bound to humus in SS did not significantly change with the composting process,

which can be explained by the dilution effect of the bulking agent, which masks the increase of protease-BAA activity that would normally be expected during the composting process. This trend was observed after the composting of MSW: organic matter stabilization involved a higher level of linkage between the enzyme and the humus. However, higher values of immobilized activity/total activity ratios of composted materials (0.009 and 0.2, respectively for CSS and CMSW) in comparison with fresh materials (0.006 and 0.04 respectively for SS and MSW) were observed.

The ALP activity was significantly higher in SS than in MSW (Fig. 5a), probably due to the higher organic phosphorous (P) content of the former derived from polyphosphates of detergents (Garcia et al., 1992b). These P compounds act as substrates of phosphatases, which catalyse their hydrolysis to inorganic P (PO_4^{3-}). After composting SS, ALP activity decreased due to a decrease in organic P and an increase in inorganic P, which is an inhibitor of this enzyme and to the dilution effect of the bulking agent (García et al., 1995). Phosphatases are adaptive enzymes synthesized by microorganisms in response to their need for inorganic P (Skujins, 1976). After composting MSW, ALP increased due to the depletion of inorganic phosphorous which would induce the excretion of phosphatase by microorganisms. Moreover, in the composting process, new humic compounds were synthesised, which suggests that this enzyme could bind to humus compounds by ionic, hydrogen or covalent binding; therefore, this enzyme is more protected from the action of denaturalising agents than in the fresh organic material (Dick and Tabatabai, 1984).

Humus-associated ALP was higher in SS than in the rest of organic amendments, suggesting that the addition of humic extracts of SS to soil may help the hydrolysis of organic P, allowing a reduction in the use of chemical fertilizers containing inorganic P (Fig. 5b). Both composts showed higher immobilization indices (immobilized activity/total activity ratio) for ALP (0.13 and 0.03, respectively for CSS and CMSW) than the respective fresh organic materials (0.04 and 0.02, respectively for SS and MSW), confirming that the organic matter stabilization that occurs with

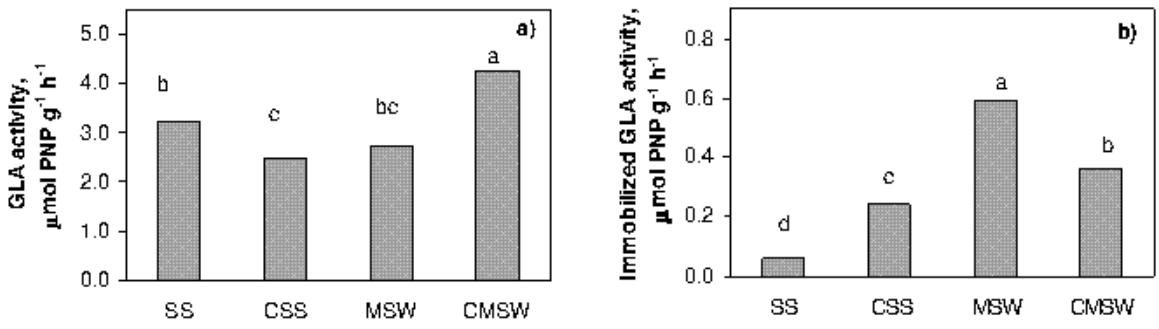

Figure 6. B-glucosidase activity in the organic materials and the pyrophosphate extract. Nomenclature of samples as in Figure 1.

composting produces humic fraction that is more able to bind the enzyme, thus preventing its denaturalisation.

No significant difference was observed between the GLA values of SS and MSW (Fig 6a). This may be due to the similar content of specific substrates of this enzyme in both organic materials. □-Glucosidase catalyses the hydrolysis of cellobiose, and the reaction products (glucose) are important as energy resources for microorganisms (Tabatabai, 1994). Cellobiose is, in turn, the product of the enzymatic hydrolysis of cellulose catalysed by other enzymes. After the composting process, the GLA of the two materials showed a similar trend to the rest of the hydrolase activities studied. Thus, a decrease of GLA was observe after SS composting as a result of the decrease in microbial activity (dehydrogenase activity diminution) and, therefore, in the need for energetic molecules. On the other hand, the GLA increased after MSW composting due to the increase in microbial activity, as measured by DHA, probably as a result to the degradation of inhibitory substances with composting. This correlation between GLA and DHA has been reported by Eivazi and Zakaria (1993).

A comparison of the values of GLA in the original materials with those in the humic extracts revealed low immobilized GLA levels in humus (1.5-22%) compared with total activity (Fig. 6b). Other authors obtained higher yields (35%) for GLA immobilized in soil humus colloids with regard to the total soil activity (Busto and Perez-Mateos, 2000). The humic substances extracted from the organic materials differ from those extracted from soil because the latter have been formed after a lengthy period of humification. Thus, it is possible that the humic substances extracted from the organic materials do not have the same capacity of β-glucosidase immobilization as those extracted from soil. An increase (from 1.5 to 9.6%) in the percentage of GLA extracted with pyrophosphate compared with the total activity of GLA was observed after SS composting, which indicates that SS stabilization improved immobilization of enzyme on humus. In the case of MSW composting, no such increase in GLA immobilization was observed, suggesting that the humic substances formed were different and did not improve the specific immobilization of β-glucosidase. (Table 3).

Conclusions

The results of this study show that the fresh and composted organic materials studied display hydrolase activity and part of this activity is associated with the humus and therefore protected. For this fact, the addition of this organic amendments to soil may improve the cycling of N, C and P. Sewage sludge with regard to MSW had a higher level of urease, protease-BAA and phosphatase total activity. Composting the organic materials had a general positive influence on the immobilization of enzymes in humus matrix. These humus-enzymes complexes would be more resistant to proteases or temperature increase being, therefore, more durable in soil to develop to their catalytic function. Consequently composts seem to be more suitable than

fresh materials to be used in soil remediation and as a source of immobilized enzymes. It can also be concluded that DPO is an enzyme very prone to linkages with organic colloids, the pyrophosphate extracts showing higher DPO activity than the organic materials. In contrast, hydrolase activities were present in low levels in pyrophosphate extracts with regard to total enzymatic activity. Additional research is necessary to determine whether the humus-enzyme complexes, extracted by the procedures described, can be applied to degraded soils in order to increase their enzyme activity, as well as to establish whether these humus enzyme complexes are stable against denaturalising agents, such as temperature and proteases.

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Chapter 4:

Thermostability of selected enzymes in organic wastes and in their humic extract

Thermostability of selected enzymes in organic wastes and in their humic extract

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ABSTRACT

The objective of this study was to evaluate the thermo stability up to 70 °C for one hour of selected enzymes present in fresh and composted sewage sludge (SS and SSC) or municipal solid wastes (MSW and MSWC) and their humic extract. After a thermal treatment at 70°C, no β-glucosidase (β-GL) activity in any humic extract was detected, whereas in SS, SSC, MSW, and MSWC it was respectively 35, 68, 17, and 12% compared to thermally untreated samples . By contrast *o*-diphenol oxidase (*o*-DPO) activity was even stimulated by thermal treatment in SS samples, but in the humic extracts this activity decreased by 75-81%. Urease (UR) activity in all humic extracts decreased by 70% or more just at 40°C whereas for organic wastes this decrease was observed after treatment at 70°C. Alkaline phosphatase (AP) activity was affected by thermal treatment only in MSW and MSWC. In humic extracts AP activity decreased gradually to zero except for MSW extract, where 45% activity was retained after treatment at 70°C. In general thermo stability of enzymes in humic extracts was lower than materials they were extracted from.

Keywords: enzyme stabilization, compost, municipal solid, sewage sludge, thermo stability, humus-enzyme complexes

1. Introduction

Many enzymes synthesized by microorganisms are leaked from living cells or released from lysed cells to their environment, but their presence in soil is ephemeral unless they are absorbed by clay colloids, or linked to humic molecules (1). Extracellular enzymes which are immobilized in soil organic or inorganic colloids are thought to play a vital role in microbial ecology. They function as stabilised catalysts for the degradation of high molecular weight substrates producing smaller molecules which can be assimilated by microorganisms (2).

Nowadays there is an increasingly interest on the possibility of increasing soil immobilized enzyme activity to transform some organic

pollutants into less toxic substances, or to release nutrients into soil solution (3). Some investigations have been conducted to immobilize enzymes on clay colloids (4, 5, 6) or on synthetic humic-like molecules (7), for understanding interactions of enzymes with humic substances (8), and to determine the stability of these enzymatic complexes, and evaluate their potential use in rehabilitation of degraded soils.

However, the stability of humus-enzyme complexes derived from organic wastes such as domestic wastes or sewage sludge has not been studied. Such organic materials are generated in large amounts by human activities, and they are, therefore, economical materials having stabilised humus-enzymes complexes for extracts with enzymatic activity. The evaluation of the stability of these complexes is necessary to determine their suitability for use in soil remediation. Enzymes are very sensitive to temperature and it has been observed that the immobilization of enzymes in humic colloids protect them from denaturation by changes in surrounding temperature (1).

The purpose of this work was: i) to determine the thermal stability of selected hydrolases (urease, alkaline phosphatase, and β-glucosidase) and an oxidoreductase (o-diphenol oxidase) in fresh and composted organic materials (a sewage sludge and a municipal solid waste); and ii) to determine the thermal stability of the same enzymes in the pyrophosphate extract obtained from the above mentioned organic materials. In this way we will be able to evaluate: i) which organic material is a better source of thermostable enzymes; and ii) whether total and extractable enzymatic activities have the same thermo stability.

2. Materials and Methods

Organic materials

Four different organic residues were used in this study: a sewage sludge (SS) collected from a municipal wastewater treatment plant in El Raal-Murcia (SE Spain); the compost (CSS) produced from the above mentioned sewage sludge; the organic fraction of a municipal solid waste (MSW) collected from the treatment plant of Mula (15 km from Murcia city), which receives all the household wastes produced in the metropolitan area of Murcia (300,000 inhabitants); and the compost produced from this organic material (CMSW). MSW was obtained after manual and mechanical separation of most part of the metallic, plastic and paper materials from the wastes. The composting (industrial scale) of SS and MSW was carried out in horizontal reactors, in which the material remained static but with mechanical ventilation provided. The maximum temperature reached (65° C) was maintained for a minimum of 48 hours (to guarantee disinfection of the material), after which the temperature was maintained in the range 53-60° C during most of the process. The moisture level of the material was the optimum (60%) for increasing microbial activity. To improve oxygenation inside of the sewage sludge pile during the composting process, a bulking agent (wood shavings) was added on a volumetric basis in the proportion of 1:2 (material:bulking agent). The composting process lasted 75 days for both SS and MSW. Three samples

(each composed of 8-10 subsamples) of each organic material was collected and air dried ,and then each sample was milled in order to homogenize the material. The three replicates of each material were analysed and the mean value was calculated for each parameter.

Extraction of humus-enzyme complexes

Humus-enzyme complexes were extracted with a 0.1M, pH 7.1 sodium pyrophosphate solution (w/v ratio=1:10) by mechanical shaking for 24 hours (9). The extracts were centrifuged and filtrated (through 0.2 μm Millipore membrane, type DVPP), then they were dialyzed against distilled water with membranes having 12000-14000 Da molecular weigh cut off and 25 Å pore diameter (Visking® dialysis tube, Serva GMBH, Heidelberg, Germany) in order to remove inorganic salts which can cause artefacts in the enzymatic activity assays.

Thermal treatments

10 g of organic wastes or 10 ml of the pyrophosphate extract were transferred to glass flasks, and placed

Table 1. Characteristics of the selected organic wastes.

	SS*	SSC	MSW	MSWC
Total organic C, (%)	17.9	20.6	17	16.6
	(1.5)	(0.6)	(1.2)	(1.0)
Electrical conductivity (mS cm^{-1})	6.82	3	6.79	3.93
	(0.06)	(0.03)	(0.02)	(0.02)
pH (1:5, om:water)	6.82	7.05	6.73	7.9
	(0.09)	(0.04)	(0.05)	(0.08)
Kjeldahl N (%)	4.56	3.39	1.63	2.56
	(0.2)	(0.3)	(0.2)	(0.3)
Humic substance C (%)	0.61	1.38	0.34	0.61
	(0.2)	(0.3)	(0.2)	(0.1)
Water soluble carbohydrates (mg C kg^{-1})	695	1524	141	271
	(0.14)	(0.07)	(0.06)	(0.03)
P (% P_2O_5)	12.4	10.9	2.04	2.46
	(0.2)	(0.3)	(0.4)	(0.5)
K (% K_2O)	0.35	0.38	0.71	0.51
	(0.1)	(0.1)	(0.4)	(0.09)
Cu (mg kg^{-1})	177	2.18	283	336
	(0.4)	(0.09)	(0.1)	(1.2)
Zn (mg kg^{-1})	581	523	628	1855
	(0.05)	(0.04)	(0.1)	(1.2)
Cr (mg kg^{-1})	8.4	15	47.9	107
	(0.03)	(0.02)	(0.13)	(0.14)
Pb (mg kg^{-1})	28.1	55.5	125	233
	(0.03)	(0.4)	(0.2)	(0.4)
Ni (mg kg^{-1})	16.5	21.6	77.2	120
	(0.6)	(0.3)	(0.8)	(0.2)

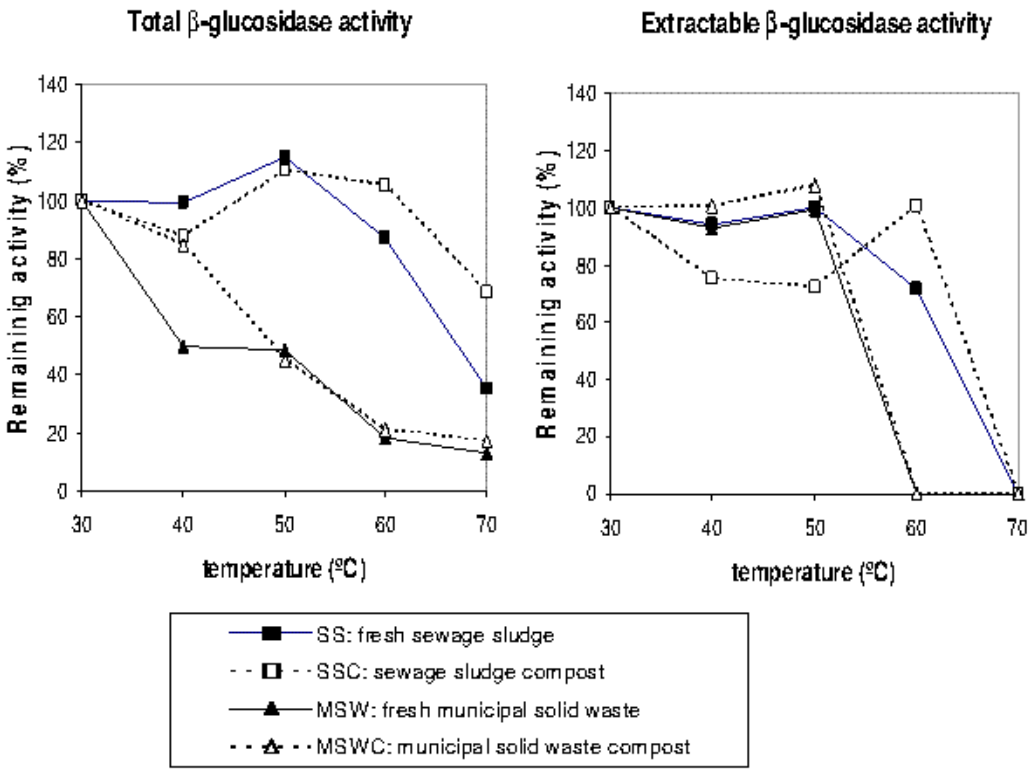
*SS (sewage sludge), SSC (sewage sludge compost), MSW (municipal solid waste), MSWC (municipal solid waste compost) Numbers in parenthesis indicate standard deviation.

inside a thermostatic bath at 30, 40, 50, 60 and 70 °C for one hour (10). All thermal treatments were carried out in triplicate.

Enzymatic activities

Urease (UR) activity was determined by the buffered method of Kandeler and Gerber (11): 0.5 ml of a solution of urea (0.48%) and 4 ml of borate buffer (pH 10) were added to 1 g of organic material in hermetically sealed flasks, and then incubated for 1.5 hours at 37° C. The ammonium content of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate addition prior to incubation to determine the native ammonium content of the organic materials. Alkaline phosphatase (AP) and β-glucosidase (β-GL) activities were determined following the methods reported by (12) and (13) respectively, using 0.2 g of organic material, and 2 ml of MUB (modified universal buffer) and incubating samples at 37° C for 1 hour. AP activity assay was performed at pH 11 using p-nitrophenyl phosphatase as substrate; β-GL activity was assayed at pH 6 using p-nitrophenyl β-D-glucopiranoside as substrate. Enzymatic reactions were stopped by cooling in ice for 15 min. Then, 0.5 ml of CaCl2 0.5 M and 2 ml of NaOH 0.5 M (for AP) or 2 ml of Tris

Figure 1. Effect of temperature increase on the total and extractable β-glucosidase activity of the different organic wastes. Bars denoted the standard mean deviation (n=3)



(hydroxymethyl) aminomethane-sodium hydroxide (THAM-NaOH) 0.1 M pH 12 (for β-GL) were added. Controls were performed as samples but adding substrate immediately before the addition of CaCl2 and NaOH. The p-nitrophenol (p-NP) formed was determined at 398 nm.

o-Diphenol oxidase (o-DPO) activity was determined as reported by (14). To 0.5 g of organic material, 1.5 ml of catechol solution 0.2 M (substrate), 1.5 of proline solution 0.2 M (reagent), and 2 ml of phosphate buffer (0.1M, pH 6.5) were added. The mixture was incubated at 30°C for 10 min and the enzymatic reaction was stopped by cooling in ice-bath and adding 5 ml of ethanol. After centrifugation, the absorbance of the supernatant was measured at 525 nm. In the control, the substrate was added after incubation and before ethanol addition.

To evaluate the activity of the extractable enzymes, all the above enzymatic activities were measured as described above but using 1 ml of the pyrophosphate extracts.

Statistical treatmentss

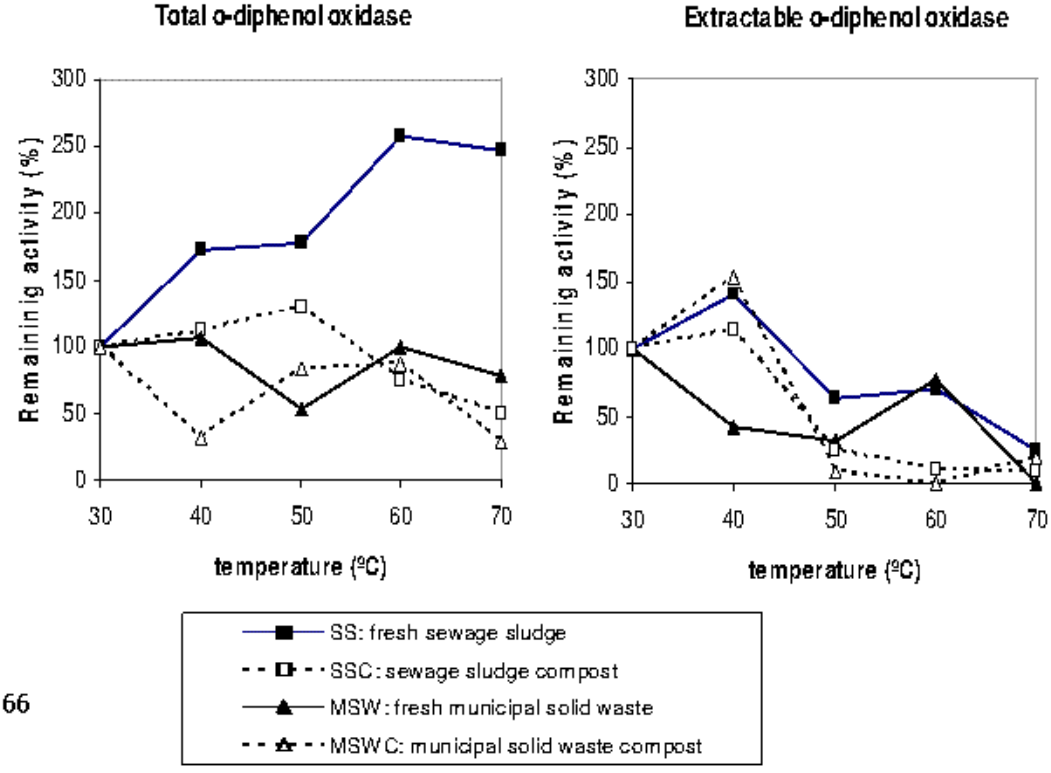
For each enzymatic activity data were submitted to a two way ANOVA using the Statgraphics v. 5.0 software package (Statistical Graphics Corporation, 1991). A Tukey multiple range test ($p<0.05$) was performed to establish the honestly significant differences (HSD) between means.

3. Results

β-glucosidase activity

Total and extractable β-GL decreased with increasing treatment temperature (Figure 1). Total β-GL in SS (both fresh and composted) showed higher stability than fresh or composted

Figure 2. Effect of temperature increase on the total and extractable *o*-diphenoloxidase activity of the different organic wastes. Bars denoted the standard mean deviation (n=3)



MSW (Figure 1). For instance, at 70oC the activity detected in SSC, SS, MSWC and MSW was a 68%, 35%, 17% and 12% respectively, compared to original activity. It should be noted that in SS and SSC β-GL activity showed only slight variations in the 30-60°C temperature range, decreasing sharply at 70°C, whereas in MSW and MSWC β-GL activity decreased gradually with temperature increase.

In all pyrophosphate extracts β-GL was thermo stable up to 50°C, but it fell to zero at 60°C for both MSW and MSWC and at 70°C for SS and SSC.

o-Diphenol oxidase activity

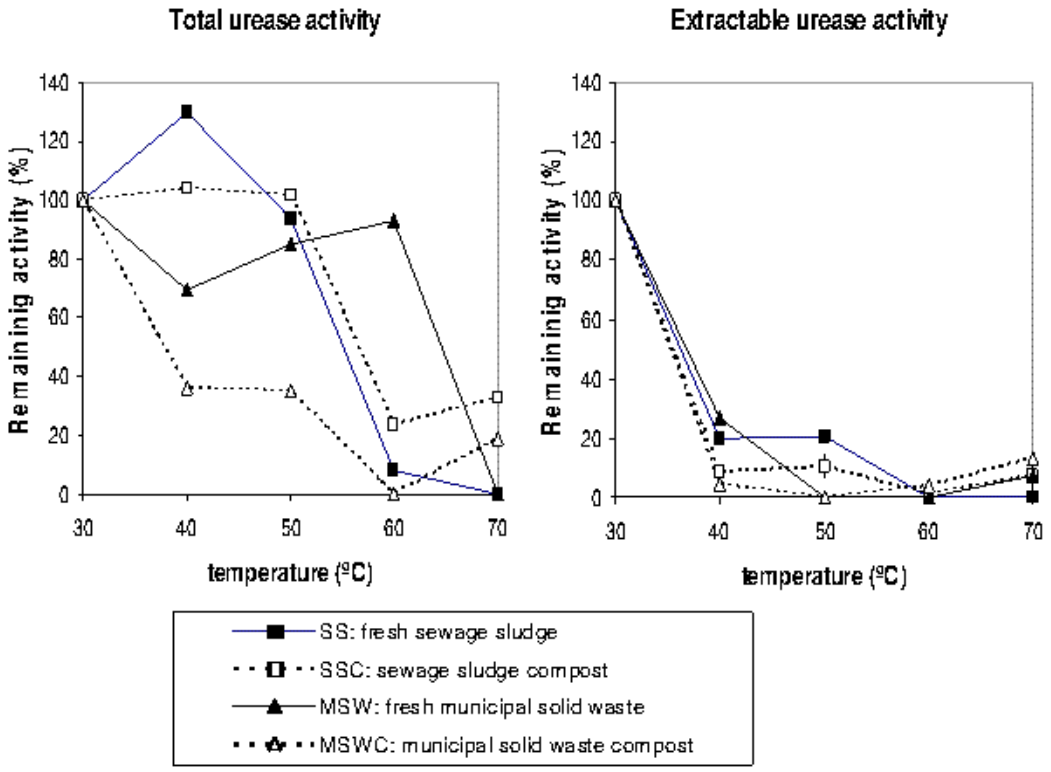
Total *o*-DPO activity decreased with increasing temperature in all the organic materials except SS, where an increase of about 2.5 times was detected both at 60 and 70°C (Figure 2).

In the pyrophosphate extracts of SS, SSC and MSWC *o* -DPO activity decreased considerably at 50 °C, being only 19% (MSWC) or 25% (SSC and SS) compared to 30 °C (Figure 2). At 70 °C *o* -DPO activity was close to detection limit. In the case of MSW, *o* -DPO activity decreased by 60% from 30 to 40 °C and disappeared at 70° C.

Urease activity

In MSWC (Figure3) UR activity decreased gradually with increasing temperature, whereas in MSW, urease activity was almost stable up to 60 °C but at 70 °C no activity was detected.

Figure 3. Effect of temperature increase on the total and extractable urease activity of the different organic wastes. Bars denoted the standard mean deviation (n=3)



By contrast UR activity in SS and SSC was stable up to 50 °C and then decreased sharply

Concerning thermo stability of the extractable UR activity, a strong diminution of this enzymatic activity was observed at 40°C in all cases. Especially, this decrease has been manifested in the case of MSWC remaining at 40 °C only 8.6 % of the activity existing at 30°C.

Alkaline phosphatase activity

Total AP activity was slightly affected by temperature in SS and SSC samples (Figure 4). In SS a decrease of 24 % of the initial activity was observed at 70 °C whereas in SSC activity values decreased slightly from 30 to 50°C, increasing afterwards to reach at 70°C values close to those observed at 30°C.

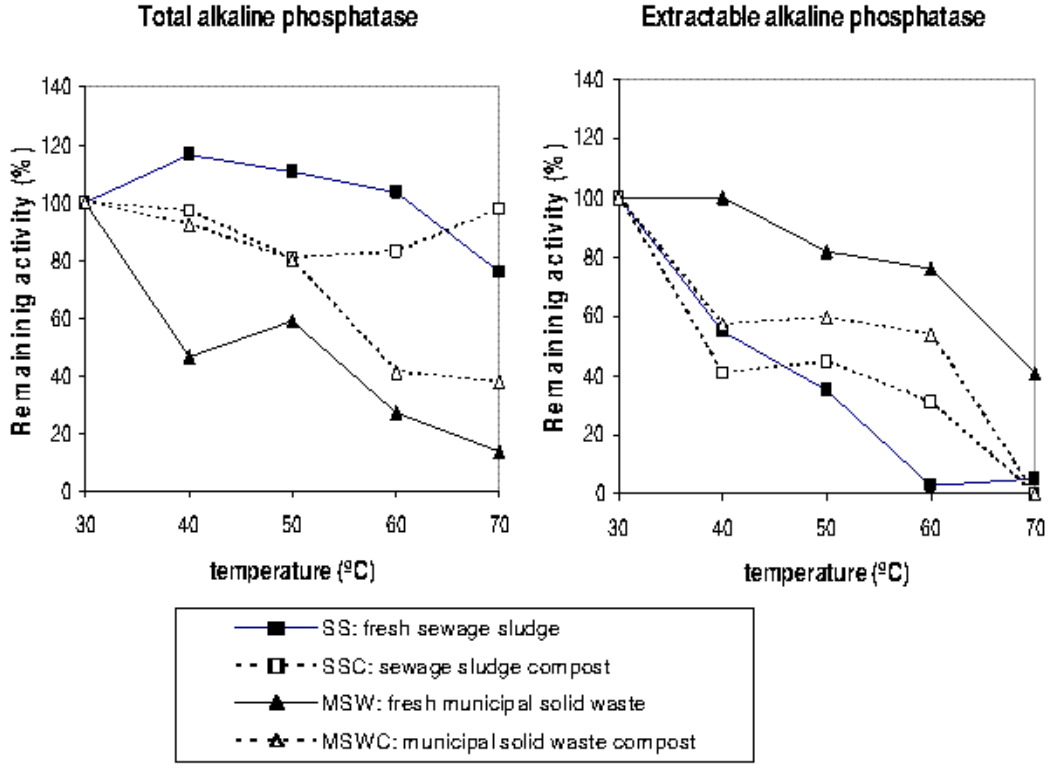
By contrast, in fresh and composted MSW, AP activity was highly affected by temperature, remaining 13.5% and 38% respectively at 70 °C of the activity exhibited at 30°C.

Extractable AP activity from both SSC and MSWC decreased from 30 to 40 °C; then remained constant up to 60 °C and at 70 °C it fell to zero (Figure 4). In MSW extract, AP activity showed no significant differences up to 60 °C, then decreasing to about 40% of the activity at 30 °C. In the case of SS activity decreased linearly to zero from 30 to 60 °C.

4. Discussion

It must be emphasized that whereas total β-GL activity was measurable at 70 °C in all studied organic wastes, in the humic extract, it

Figure 4. Effect of temperature increase on the total and extractable alkaline phosphatase activity of the different organic wastes. Bars denoted the standard mean deviation



was not detected. In fact, the latter was on average only 10% of total β-GL activity (Table 2). Other authors have reported low levels of UR activity in sodium pyrophosphate extracts and postulated the possible inactivation of ureases by phenolic compounds [13]. On the other hand, the changes in the enzyme activity with temperature can be attributable to changes produced in the tertiary structure of the protein [14].

o-DPO catalyzes the oxidation of o-diphenols to quinones, and it is thought to play a major role in soil humification. Synthesis of *o* -DPO by microorganisms is induced by different substrates, including anilines, aromatic compounds, or lignin preparations [15], and investigations have focused on the role of this enzyme in lignin degradation during composting process [16, 17]. These compounds, in a second step, are polymerized and integrated in the humus molecule [12]. The heating of SS produced an increase in *o*-DPO activity, whereas in MSW, it was slightly affected by temperature increase. In the case of the composts, a decrease in this enzymatic activity with increasing temperatures was observed. This fact highlights the influence of the organic matter nature on enzyme thermostability and may be explained by: (1) the separation of inhibitors from the enzyme with the increase of temperature and (2) the unmasking of some active sites produced when the protein conformation change with the temperature increase [1]; (3) similarly to β-GL, this can be attributable to changes in tertiary structure of the enzyme too. Other authors [17] reported activity losses of about 60% after 15 min of preincubation at 70 °C using

Table 2. Total and extractable enzymatic activities in the studied organic wastes, and percentage of extractable with regards to total activity.

		SS*	SSC	MSW	MSWC
Alkaline phophatase activity			□mol p-NP g^{-1} h^{-1}		
	Total	75.7	12.7	33.8	50.1
		(2.8)	(1.5)	(1.5)	(1.0)
	Extractable	3	1.65	0.98	1.43
		(1.21)	(1.21)	(0.03)	(0.15)
	%	3.98	12.99	2.90	2.85
Urease activity			□mol NH_4^+-N g^{-1} h^{-1}		
	Total	12.7	5.6	2	2.7
		(1.3)	(0.09)	(0.3)	(0.19)
	Extractable	0.29	0.11	0.79	0.23
		(0.56)	(1.01)	(1.27)	(0.29)
	%	2.28	1.96	39.5	8.51
□-glucosidase activity			□mol p-NP g^{-1} h^{-1}		
	Total	3.2	2.5	2.7	4.3
		(0.17)	(0.158)	(0.179)	(0.62)
	Extractable	0.06	0.24	0.59	0.37
		(0.31)	(0.41)	(0.49)	(0.99)
	%	1.88	9.6	21.85	9.25
o-DPO activity			□moles ox. catechole g^{-1} 10 min^{-1}		
	Total	0.4	0.42	1.3	0.3
		(0.02)	(0.01)	(0.02)	(0.02)
	Extractable	1.21	0.89	1.42	2.87
		(0.47)	(0.46)	(0.28)	(1.35)
	%	302.5	211.9	109.2	956.67

*SS (sewage sludge), SSC (sewage sludge compost), MSW (municipal solid waste), MSWC (municipal solid waste compost) Numbers in parenthesis indicate standard deviation.

laccase purified from composted MSW.

Table 3. Total and extractable enzymatic activities in the studied organic wastes, and percentage of extractable with regards to total activity.

	β-Glucosidase activity			*o*-Diphenol oxidase activity			Urease activity			Alkaline fosfatase activity		
	Total enzyme			Total enzyme			Total enzyme			Total enzyme		
Source	temperature (A)	treatment (B)	AxB interaction	temperature (A)	treatment (B)	AxB interaction	temperature (A)	treatment (B)	AxB interaction	temperature (A)	treatment (B)	AxB interaction
F-ratio	95.1	106.8	15.1	33.3	836.0	110.3	174.4	39.0	26.0	203.8	428.6	52.3
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0	<0.001	<0.001	<0.001	<0.001
HSD*	10.5	8.8		8.6	7.3		11.5	9.6		5.1	4.3	
	Extractable enzyme			Extractable enzyme			Extractable enzyme			Extractable enzyme		
Source	temperature (A)	treatment (B)	AxB interaction	temperature (A)	treatment (B)	AxB interaction	temperature (A)	treatment (B)	AxB interaction	temperature (A)	treatment (B)	AxB interaction
F-ratio	177.9	5.4	16.5	931.7	113.7	113.7	365.6	6.63	5.33	370.9	179.3	20.1
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
HSD	13.0	10.9		6.7	5.7		8.6	7.2		6.7	5.7	

*SS (sewage sludge), SSC (sewage sludge compost), MSW (municipal solid waste), MSWC (municipal solid waste compost) Numbers in parenthesis indicate standar - 71 -d deviation.

However, the authors [18] found that the activity of *o*-DPO extracted from a thermophilic strain of bacteria was stimulated by keeping at 80 °C. In the pyrophosphate extract with a great amount of humic substances, the *o*-DPO activity was higher than that in organic wastes (Tables 2 and 3), but its thermostability was lower. Likely, *o*-DPO did not interact with the humic substance to form humic–enzyme complexes, and its presence in the pyrophosphate extract was especially in the form of free enzyme. Other authors observed that humic substances may alter the polyphenol oxidase stability [19]. As it is known that *o*-DPO is a one of the oxidoreductase enzymes, influenced by the function between oxidation and reduction, the thermal effect on its mechanism could attribute to the deformation of the linkage of complexes, which lead the enzyme to degrade easily.

In terms with UR in the extract, other authors [20] observed that the UR immobilized on montmorillonite or aluminum oxide showed a higher sensitivity to temperature than the free enzyme at 60 °C. Nonetheless, the residual activity at this temperature (60 °C) for the UR– clay complexes was 55% of the initial activity. In our experiment, only 5% of the UR activity detected initially in the humic extract remained at 60 °C. This suggests that UR is more efficiently protected against thermal denaturation in the complex with clay minerals than the complex with humic substance.

From an agronomic point of view, AP in the soil deserves special attention as it catalyzes the transformation of organic P into inorganic P available to plants [21]. It has been indicated that the immobilization of AP in the soil increases its thermal stability with regard to soluble enzyme [22]. However, lower thermostability was observed for AP measured in the humic extract of SS, SSC, and MSWC in comparison with that in the original organic materials (Table 3). The same tendency has been occurred to other authors that have studied.

AP with clay complexes [4]. They conclude that the drying effect led to a rapid and strong denaturation of absorbed protein. The immobilization of the enzyme on synthetic or natural humic materials could cause inhibitory effects to the enzyme [23–27]. However, other authors have showed a different perspective about the inhibitory effect of humic substance [28]: The incorporation of the enzyme into humic polymers reduced enzyme activity by phenolic compounds; however, this activity was more stable than the activity of free enzymes once added to the soil. Thus, it should be possible that the interaction with clay colloids changes the mechanism of humus–enzyme complexes in the soil, compared to the absence of the soil. Furthermore, collectively, the data indicate that the enzyme thermal stability is influenced by both the origin of the organic amendment and the degree of stabilization of its organic matter. The research about this topic should be completed with additional experiments consisting in the application of the organic wastes to a degraded soil as organic amendments to test the stability of the enzymatic activities with time. These studies would help us to understand the linkage between humus and enzymes and the possible use of enzymes as environmental tools for bioremediation.

From this study, it can be concluded that the enzymatic activities of SS and SSC are more thermoresistant than those of MSW or MSWC and that AP, o-DPO, and β-GL are more resistant than UR to temperature increase. In general, enzymes of SS were more thermoresistant than those of SSC. Concerning humus–enzyme complexes, in general, it can

be said that thermostability of enzymes in humic extracts was lower than the materials they were extracted from.

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Chapter 5:

Multi-factorial assessment of the effects of organic amendments on the microbial activity and the behavior of humus-enzyme complexes in a semi-arid soil

Multi-factorial assessment of the effects of organic amendments on the microbial activity and the behavior of humus-enzyme complexes in a semi-arid soil1

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ABSTRACT

The objectives of this study are to compare the biochemical responses of soil microorganisms to different organic amendments and doses and the dynamics of humic-enzyme complexes versus total enzyme activity. The experiment was carried out under laboratory conditions using soil microcosms (500 g) amended with two different doses (5 and 10g) of different materials during 360 days: sewage sludge from a wastewater treatment plant (SS), compost from that sludge (CSS), the organic fraction of municipal solid wastes (MSW), and compost from MSW (CMSW). The different carbon fractions, such as the total organic carbon (TOC), water-soluble carbon (WSC), and microbial biomass carbon (MBC), increased compared to the control soil without amendment, as well as dehydrogenase and hydrolytic enzymes (β-glucosidase and urease) activities. The immobilized enzymes in the soil humic extracts exhibited different behaviors compared to total activity, depending on the origin of the organic material which suggest neo-formation of humic-enzyme complexes.

Keywords: **semi-arid soil; organic wastes; microbial activity; humus-enzyme complexes; enzyme activity**

1. Introduction

Addition of organic amendments is a suitable strategy to achieve soil recuperation in semi-arid areas such as SE Spain, where the organic matter (OM) content and biological quality are low (Garcia et al., 1994; Bastida et al., 2008a). From a biochemical and microbial point of view, the status of a soil can be evaluated by assessing the state of its microbial community (Trasar-Cepeda et al., 1998; Bastida et al., 2006). Microorganisms are largely

Table 1. Characteristics of the control semi-arid soil from Santomera (SE-Spain).

	Soil	S.D.
pH	8.07	0.19
Electrical conductivity (dS m^{-1})	438.50	40.31
Total N (g $100g^{-1}$)	0.15	0.00
NH_4^+-N (mg kg^{-1})	1.95	0.49
Total P (mg kg^{-1})	805.60	66.04
Available P (mg kg^{-1})	58.30	0.14
Total K (g $100g^{-1}$)	0.81	0.04
Available K (meq $100g^{-1}$)	2.60	0.08
Cu (mg kg^{-1})	72.55	3.46
Zn (mg kg^{-1})	35.10	0.99
Cr (mg kg^{-1})	6.15	1.20
Ni (mg kg^{-1})	12.45	0.35
Pb (mg kg^{-1})	13.55	0.35

S.D.= Standard Deviation

responsible for the cycles of the elements within a soil and are involved in the decomposition of the OM at the ecosystem level via a variety of enzymes. In this sense, the addition of different organic amendments, such as solid organic wastes, sewage sludge, agricultural wastes, and animal manures, is a method of replenishing degraded soil quality through improvement of the biological status of the soil, which usually implies an increase in both microbial and enzyme activity (Masciandro et al., 2000; Albiach et al., 2000).

Soil hydrolases and oxidoreductases are considered the most-abundant classes among ten different enzyme types (Ruggiero et al., 1996; Nannipieri et al., 2002). As soon as they are released from the cell, they can be metabolized by soil microorganisms, unless stabilization takes place through adsorption and/or incorporation into clay and clay-humic complexes (Nielson et al., 2006). Enzyme persistence in the soil environment ranges from a few days to several years, depending on the location and soil conditions such as temperature, pH, soil fraction, and depth (Gianfreda, 1996; Kandeler et al., 1999; Ekenler et al., 2003). Nannipieri et al. (1996) suggested that extracellular enzymes in soil are firmly associated with humic OM in the complexed state, even though enzymatic activity and bioavailability decreases. These immobilized enzymes are surrounded by a network of humic molecules with pores large enough to permit the diffusion of substrates and products but not of proteolytic enzymes (Burns et al., 1972).

Inputs of fresh or matured organic amendments contribute at different levels to soil rehabilitation by supplying different substrates (Brookes et al 2008); fresh organic inputs are the most-readily utilized by microorganisms and provide more energy per unit, while matured inputs provide more-recalcitrant polymerized compounds, which are easily incorporated into soil humic substances. However, despite the huge variety of studies, including total enzyme activities, there is still scarce information on their influence on the formation and/or stabilization of humus-enzyme complexes (Ladd, 1975; Burns et al., 1982; Nannipieri et al., 1996). In particular, up-to-date findings in this topic are scarce (Bonmati et al., 2009; Adamcqzyk et al., 2009).

In addition, despite the fact that the effect of organic wastes from different origins or with differing stabilization effects is a commonly-addressed topic in soil science, studies which consider also the joint effect of other factors such as incubation time and dose are scarce (Prasad et al 2008; Cwalina-Ambroziak et al., 2010). It is desirable and challenging to cover these important factors in order to attain a better overall understanding of the soil response to organic amendments in ecological terms.

In this work, we developed a multi-factorial experiment in order to: 1) observe the comparative biochemical responses of microorganisms to different organic amendments and doses over a one-year period and 2) relate the response of the soil microbial activity to the dynamics of humic-enzyme complexes.

2. Materials and Methods

Soil sampling and experimental design

The proposed objectives were achieved by the incubation of soil with different organic materials under laboratory conditions. Four different organic residues were used in this study: a sewage sludge (SS) collected from a municipal wastewater treatment plant in El Raal-Murcia (SE Spain); the compost (CSS) produced from this sewage sludge; the organic fraction of a municipal solid waste (MSW) collected from the treatment plant of Mula (15 km from Murcia city), which receives all the household wastes produced in the metropolitan area of Murcia (300,000 inhabitants); and the compost produced from this organic material (CMSW). The MSW was obtained after manual and mechanical separation of most of the metallic, plastic, and paper materials from the waste. The composting (industrial scale) of SS and MSW was carried out in horizontal reactors, in which the material remained static but received mechanical ventilation. The maximum temperature reached (65° C) was maintained for a minimum of 48 hours, after which the temperature was maintained at 53-60° C during most of the process. The moisture level of the material was the optimum (60%) for increasing microbial activity. To improve oxygenation inside the sewage sludge pile during the composting process, a bulking agent (wood shavings) was added, on a volumetric basis, in the proportion of 1:2 (material:bulking agent). The composting process lasted 75 days for both SS and MSW. Three samples (each composed of 8-10 subsamples) of each organic material were collected and air-dried, then each sample was milled in order to homogenize the material. The total organic carbon (TOC) was 17.9% (SS), 20.6% (CSS), 17.0 (MSW), or 16.6% (CMSW), respectively. The total organic nitrogen (TON) was 4.6% (SS), 3.4% (CSS), 1.6% (MSW), or 2.6% (CMSW), respectively. The chemical and biochemical data of these materials are detailed by Moreno et al. (2007).

Soil is a Calcic kastanozem (FAO Soil survey staff, 1998), sampled in an experimental field located in Santomera (SE Spain). This area is affected by soil degradation processes, such as erosion, and deficiency of OM. Soil was sampled in the upper layer (0–15 cm), air-dried, and sieved to 2 mm. The main characteristics of the soil are shown in Table 1. The soil samples were stored at 4 °C for one week, prior to incubation with the organic amendments.

Triplicate mixtures of the soil and organic materials were incubated in hermetically-closed pots for 360 days at 28 °C, in the dark. The mixtures were prepared with 500 g of air-dried soil and 5 g (low dose) or 10 g (high dose) of dry, ground organic material. The microcosms were maintained at 60% of water-holding capacity during the incubation. Triplicate controls, without organic amendment but with water, were run during the incubation.

Chemical Analysis

The soil electrical conductivity and pH were measured in a 1/5 (w/v) aqueous extact, using a Crison mod.2001 conductivimeter and pH meter, respectively. The TOC was determined by oxidation with potassium dichromate in an acid medium and measurement of the excess dichromate using Mohr's salt (Yeomans and Bremner, 1989). The microbial biomass carbon was determined by a fumigation–extraction method, with extraction of organic C by K_2SO_4 (Vance et al. 1987) and determination

of the C content in the K_2SO_4 soil extracts with an organic C analyzer (Shimadzu TOC-5050A).

The water-extractable carbon (WSC) was obtained by shaking a mixture of soil and distilled water (1:10 soil:water ratio, w:v) for 2 h, followed by centrifugation and filtering through ashless filter paper (Albet 145 110). In this extract, the WSC was determined with a C analyzer for liquid samples (Shimazdu 5050A, Kyoto, Japan)

Enzymatic Assays

The soil dehydrogenase activity was determined as reported by Von Mersi and Schinner (1991), using p-iodonitrotetrazolium chloride as substrate and measuring the absorbance of the iodonitrotetrazolium formazam (INTF) produced in the enzymatic reaction. The soil urease activity was determined by the method of Kandeler and Gerber (1988), and the β-glucosidase activity was determined using the method of Eivazi and Tabatabai (1987).

Immobilized Enzymatic Assays and Infrared spectra

Immobilized enzymatic activities within humic extracts were determined using 1-ml pyrophosphate extracts obtained from the soil. Humic substances were extracted with 0.1M sodium pyrophosphate, pH 7.1, (w/v ratio=1:10) by mechanical shaking for 24 h. The centrifuged and filtered (0.2 μm Millipore membrane, Billerica, MA, USA) extracts were dialyzed against distilled water with a membrane of 12,000–14,000 Da molecular weight cut-off and a 25-Å pore diameter (Visking® dialysis tube, Serva GmbH, Heidelberg, Germany), to obtain a purified humic extract. After dialysis, the humic extracts were dehydrated and concentrated through polyethylene glycol to 1/10 of the original volume of the sodium pyrosphyate solution. Using 1 ml of this extract, the immobilized enzyme activities in the humic extract were determined using the procedures mentioned above.

Statistical analysis

All the results are reported as means. Four-way ANOVA with incubation time, origin of material, stabilization process (composting), and dose as factors, followed by Tukey's LSD test as a post-hoc test, was performed for the following parameters: TOC, WSC, MBC, and the dehydrogenase, urease, β-glucosidase, and phosphatase activities. Three-way ANOVA with incubation time, origin of material, and stabilization process (composting) as factors, followed by Tukey's LSD test as a post-hoc test, was performed for the activity of humic-enzyme complexes. In order to determine pair-wise

differences by post-hoc tests, the data were submitted to one-way ANOVA at each incubation time. The post-hoc test applied was Fisher's least significant difference (LSD) method, at the 95% confidence interval.

3. Results

3.1 Carbon fractions

Soils amended with the high dose (10 g) of

values of TOC and WSC. The TOC values of soils amended with sludge were significantly ($P<0.05$) higher at 360 days than for the other treatments, followed by

Table 2. The total organic carbon (TOC) and water-soluble carbon (WSC) contents of control and soils amended with different organic materials.

Days	0	15	30	60	90	180	270	360
				TOC (%)				
Control	**1.19**	**0.97**	**0.87**	**0.93**	**0.84**	**0.83**	**0.85**	**0.84**
	0.04	*0.06*	*0.05*	*0.04*	*0.00*	*0.02*	*0.04*	*0.02*
S5	**1.57**	**1.27**	**1.07**	**1.05**	**1.04**	**1.01**	**1.07**	**1.20**
	0.08	*0.07*	*0.05*	*0.02*	*0.02*	*0.05*	*0.06*	*0.06*
S10	**1.79**	**1.49**	**1.36**	**1.26**	**1.30**	**1.32**	**1.26**	**1.41**
	0.14	*0.18*	*0.01*	*0.05*	*0.07*	*0.10*	*0.02*	*0.01*
CS5	**1.31**	**1.07**	**1.00**	**1.02**	**1.13**	**0.92**	**0.95**	**0.98**
	0.09	*0.03*	*0.08*	*0.05*	*0.04*	*0.04*	*0.02*	*0.03*
CS10	**1.56**	**1.27**	**1.31**	**1.46**	**1.30**	**1.14**	**1.23**	**1.26**
	0.14	*0.09*	*0.08*	*0.15*	*0.07*	*0.08*	*0.05*	*0.03*
MSW5	**1.50**	**1.09**	**1.00**	**0.96**	**0.92**	**0.92**	**0.99**	**1.13**
	0.11	*0.08*	*0.08*	*0.02*	*0.03*	*0.01*	*0.08*	*0.01*
MSW10	**1.77**	**1.29**	**1.17**	**1.07**	**1.10**	**1.06**	**1.06**	**1.25**
	0.13	*0.09*	*0.09*	*0.04*	*0.01*	*0.01*	*0.06*	*0.01*
C-MSW5	**1.48**	**1.20**	**0.88**	**1.06**	**0.97**	**0.92**	**0.99**	**0.98**
	0.07	*0.07*	*0.02*	*0.05*	*0.04*	*0.03*	*0.04*	*0.03*
C-MSW10	**1.65**	**1.26**	**1.21**	**1.27**	**1.19**	**1.09**	**1.19**	**1.22**
	0.22	*0.06*	*0.05*	*0.01*	*0.01*	*0.08*	*0.09*	*0.07*
				WSC (mg C kg^{-1})				
Control	**71.39**	**57.05**	**44.68**	**39.66**	**30.72**	**30.07**	**29.30**	**23.35**
	6.22	*4.30*	*1.64*	*1.43*	*1.39*	*4.50*	*3.17*	*0.03*
S5	**167.18**	**58.07**	**45.71**	**41.99**	**46.13**	**40.34**	**42.51**	**38.14**
	5.98	*1.83*	*2.99*	*2.26*	*3.67*	*2.52*	*1.45*	*2.39*
S10	**221.13**	**65.19**	**65.68**	**47.05**	**52.55**	**50.56**	**45.51**	**42.49**
	4.18	*4.28*	*7.05*	*3.55*	*3.23*	*0.57*	*2.37*	*2.40*
CS5	**134.07**	**65.30**	**56.66**	**49.47**	**32.26**	**38.08**	**36.73**	**43.55**
	5.92	*2.97*	*3.17*	*3.88*	*3.98*	*2.08*	*0.39*	*1.54*
CS10	**176.26**	**125.59**	**63.99**	**55.35**	**55.36**	**48.66**	**47.05**	**42.60**
	6.18	*9.81*	*5.90*	*0.24*	*0.38*	*3.95*	*1.91*	*0.98*
MSW5	**488.60**	**65.81**	**56.35**	**57.77**	**44.80**	**39.89**	**42.25**	**37.92**
	13.01	*5.83*	*0.57*	*0.32*	*2.35*	*0.67*	*0.47*	*0.61*
MSW10	**635.38**	**78.90**	**61.82**	**65.17**	**68.61**	**51.71**	**44.40**	**38.53**
	16.08	*7.73*	*9.36*	*3.22*	*3.34*	*4.33*	*4.61*	*5.91*
C-MSW5	**149.67**	**85.56**	**66.60**	**56.34**	**57.74**	**45.00**	**40.92**	**44.35**
	0.79	*0.10*	*4.08*	*3.03*	*3.63*	*2.33*	*3.78*	*4.40*
C-MSW10	**210.34**	**152.04**	**80.28**	**76.42**	**82.14**	**74.52**	**60.10**	**56.00**
	10.59	*10.81*	*2.31*	*0.58*	*4.42*	*0.67*	*1.55*	*1.86*

The bold letters are the mean values and the italic letters underneath represent the standard deviation of the mean.

Table 3. One-way ANOVA of the different parameters analyzed in the control and amended soils during incubation. For each incubation time, data followed by the same letters are not significantly different according to the LSD test ($P<0.05$). βGla (β-glucosidase activity), Ure (Urease activity), DC (dehydrogenase activity), MBC (Microbial biomass C), TOC (Total organic C), WSC (Water-soluble C).

	βGla	Ure	DH	MBC	TOC	WSC		βGla	Ure	DH	MBC	TOC	WSC
0 days							***15 days***						
Control	a	a	a	a	a	a	Control	a	a	a	a	a	a
S5	a	cd	e	a	cd	cd	S5	ab	bc	c	a	c	a
S10	bc	e	h	g	e	e	S10	e	d	g	d	d	a
CS5	a	c	a	b	ab	b	CS5	ab	ab	b	b	ab	a
CS10	bc	de	b	f	cd	d	CS10	b	d	d	c	c	c
MSW5	cd	b	c	e	bc	f	MSW5	d	cd	e	b	ab	a
MSW10	d	b	g	e	de	g	MSW10	f	e	h	e	c	b
C-MSW5	b	b	d	c	bc	bc	C-MSW5	c	bc	c	b	bc	b
C-MSW10	cd	b	f	d	cde	e	C-MSW10	d	d	f	d	c	d
30 days							***60 days***						
Control	a	a	a	a	a	a	Control	a	a	a	b	a	a
S5	a	cd	b	a	bc	a	S5	c	d	b	a	bc	a
S10	cd	e	f	f	f	c	S10	c	de	de	d	e	b
CS5	bc	b	c	b	b	b	CS5	ab	c	bc	c	abc	b
CS10	bc	g	d	g	ef	bc	CS10	ab	e	c	f	f	c
MSW5	a	bc	e	d	b	b	MSW5	bc	bc	d	c	ab	c
MSW10	e	fg	g	g	cd	bc	MSW10	d	de	f	e	c	d
C-MSW5	ab	de	cd	e	a	c	C-MSW5	bc	b	bc	b	bc	c
C-MSW10	d	f	e	f	de	d	C-MSW10	c	de	e	d	e	e
90 days							***180 days***						
Control	ab	a	a	a	a	a	Control	a	a	a	a	a	a
S5	abcd	cd	bc	b	cd	bc	S5	ab	f	b	b	bc	bc
S10	bcd	e	cd	e	g	cd	S10	ab	g	d	f	e	e
CS5	abc	cd	b	de	ef	a	CS5	c	cd	b	de	ab	b
CS10	d	f	d	f	g	d	CS10	d	e	e	g	d	de
MSW5	cd	b	e	cd	b	b	MSW5	c	cde	c	c	ab	b
MSW10	d	e	f	e	de	e	MSW10	d	de	g	g	cd	e
C-MSW5	abc	bc	b	c	bc	d	C-MSW5	b	b	c	cd	ab	cd
C-MSW10	a	de	g	c	f	f	C-MSW10	c	bc	g	ef	cd	f
270 days							***360 days***						
Control	a	a	a	a	a	a	Control	a	a	a	a	a	a
S5	cde	b	d	b	c	cd	S5	b	b	bc	a	d	b
S10	cde	b	e	e	d	de	S10	bc	d	e	de	e	c
CS5	de	b	d	d	b	b	CS5	b	c	b	f	b	c
CS10	e	c	f	d	d	e	CS10	bc	c	cd	g	d	c
MSW5	cd	b	b	a	b	cd	MSW5	bc	b	b	cd	c	b
MSW10	cde	c	h	f	c	cde	MSW10	c	d	d	ef	d	b
C-MSW5	b	a	c	b	b	bc	C-MSW5	b	c	cd	b	b	c
C-MSW10	c	a	g	c	d	f	C-MSW10	bc	c	e	bc	d	d

Immobilized activity	**0 days**		**180 days**		**360 days**	
	βGla	**Ure**	**βGla**	**Ure**	**βGla**	**Ure**
Control	a	c	a	a	b	a
S10	a	a	b	d	d	a
CS10	b	bc	c	c	c	b
MSW10	a	b	a	a	a	a
CMSW10	c	a	d	b	c	b

the high doses of MSW and C-MSW (Table 2). The TOC content of the control soil decreased through the incubation time, from 1.19 to 0.84 mg kg^{-1} (a 30% loss) (Table 2). The losses of TOC during the incubation of the soil with the different materials from T0 to 360 days were: 24% (S5), 21% (S10), 25% (CS5), 19% (CS10), 25% (MSW5), 29% (MSW10), 34% (C-MSW5), and 26% (C-MSW10). The natural TOC losses during the incubation of the control soil were in the range of those of the other treatments (30%), indicating that soil OM can be degraded even in ecosystems with low but stabilized OM contents, such as semi-arid areas.

The WSC showed a fast decrease during the first months, followed by stabilization during the rest of the incubation (Table 2). The soil amended with a high dose of C-MSW showed the highest value of WSC ($P<0.05$) after 360 days of incubation. From 0 to 360 days of incubation, the WSC losses were: 67% (control), 77% (S5), 81% (S10), 67% (CS5), 75% (CS10), 92% (MSW5), 94% (MSW10), 70% (C-MSW5), and 73% (C-MSW10).

Statistically, the TOC, WSC, and MBC (Table 3) were affected significantly ($P<0.01$) by incubation time, origin of material, stabilization process (composting), and dose. At the beginning of the incubation, all applications of the organic amendments significantly ($P<0.05$) increased the carbon fractions, compared to the control soil without amendment (Table 3).

Addition of the organic materials to soil significantly increased the contents of humic substances, humic acids, and

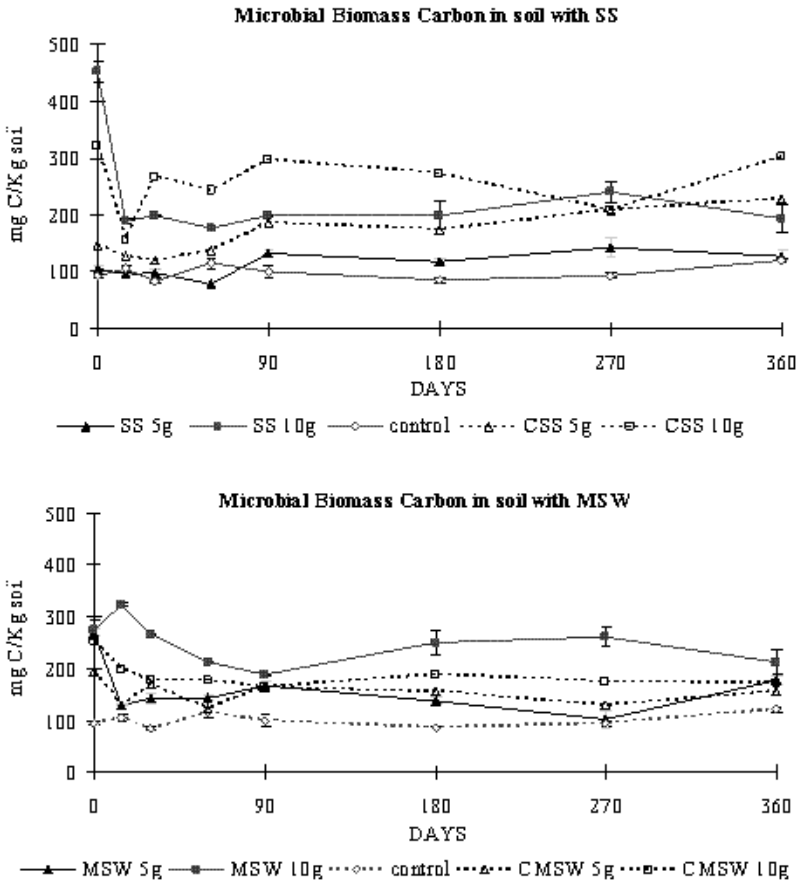

Figure 1.- The microbial biomass carbon in control and soils amended with different organic materials, during a one-year incubation.

fulvic acids, comparing to the control

soil, at the end of the incubation (Table

4). However, the trend of each chemical

Table 4. The humic substances (HS), fulvic acid (FA), and humic acid (HA) contents of control and amended soils after 360 days of incubation.

Soil Treatment	HS	FA	HA
Control	18353.9 a	735.6 a	1095.6 a
SS	2080.1 b	880.7 b	1175.4 b
CSS	2681.4 e	911.0 b	1744.0 d
MSW	2169.6 c	974.7 c	1173.9 b
CMSW	2559.0 d	1042.9 d	14.66.1 c

For each incubation time, data followed by the same lower-case letters are not significantly different according to the LSD test

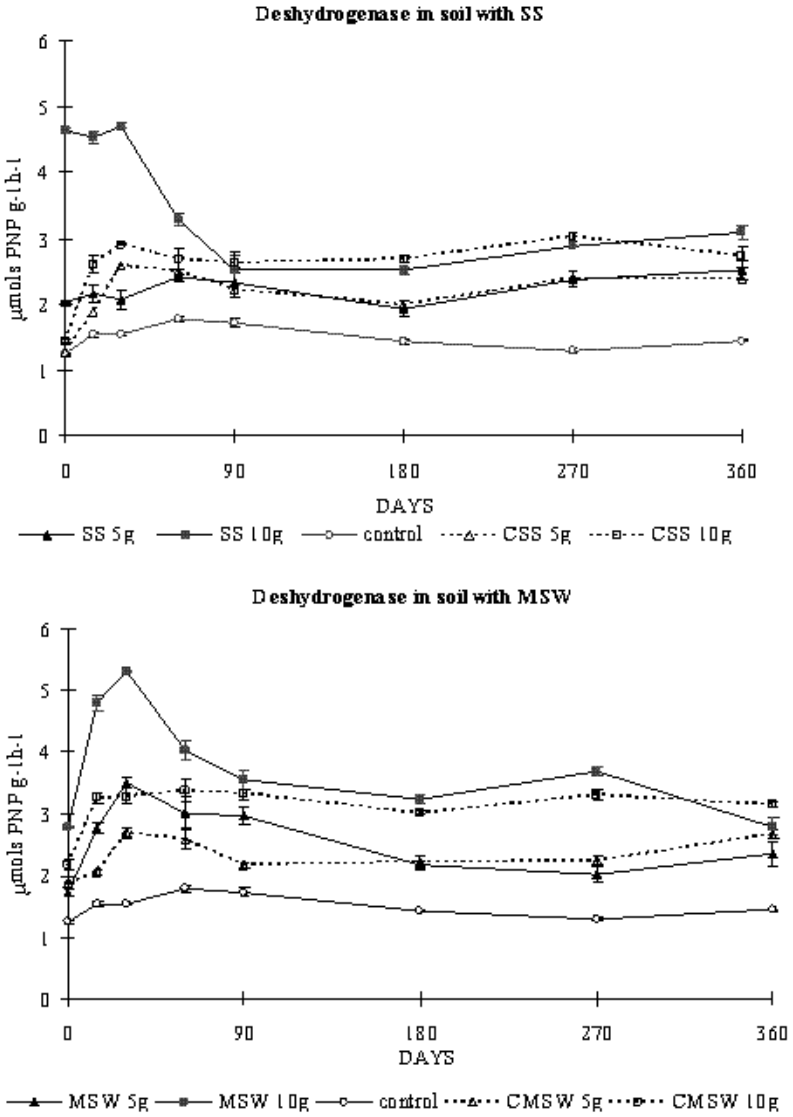

Figure 2.- The dehydrogenase activity in control and soils amended with different organic materials, during a one-year incubation.

addition of composted materials produced the highest values of fulvic acids in soil, while the behavior of humic acids was more related to the origin of the material, and the addition of MSW, fresh or composted, gave the highest values in the soil (P<005).

3.2 Microbial biomass and total enzyme activity

Statistically, the MBC (Table 3) was affected significantly (P<0.01) by incubation time, origin of material, stabilization process (composting), and dose. At the beginning of the incubation, application of the different organic amendments significantly (P<0.05) increased the MBC, except in the case of the low SS dose (5 g) which did not produce significant differences from the control soil without amendment (Figure 1). At 180 days, all amended soils had significantly-higher

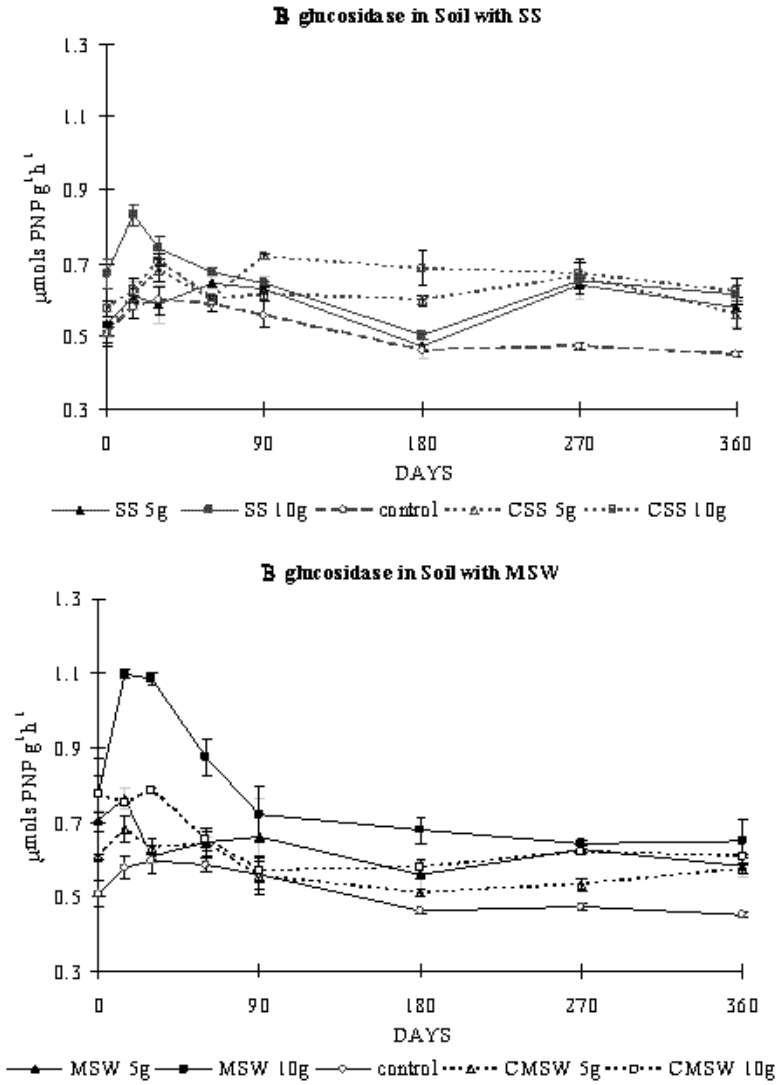

Figure 3.- The β-glucosidase activity in control and soils amended with different organic materials, during a one-year incubation.

values than control soil. At this time, the high doses of CSS and MSW produced the highest values and this trend was quite conserved until the end of the incubation (360 days).

Statistically, the time, dose, origin, and stabilization significantly affected ($P<0.01$) all the studied enzyme activities (Table 3). Dehydrogenase activity was higher in soil amended with fresh organic materials than in soil receiving composted materials (Figure 2).

At 0 days, the soil amended with the high dose of sludge showed a dehydrogenase activity which was significantly ($P<0.05$) the highest, followed by the soil receiving the high dose of MSW. However, at 60 days, dehydrogenase activity was

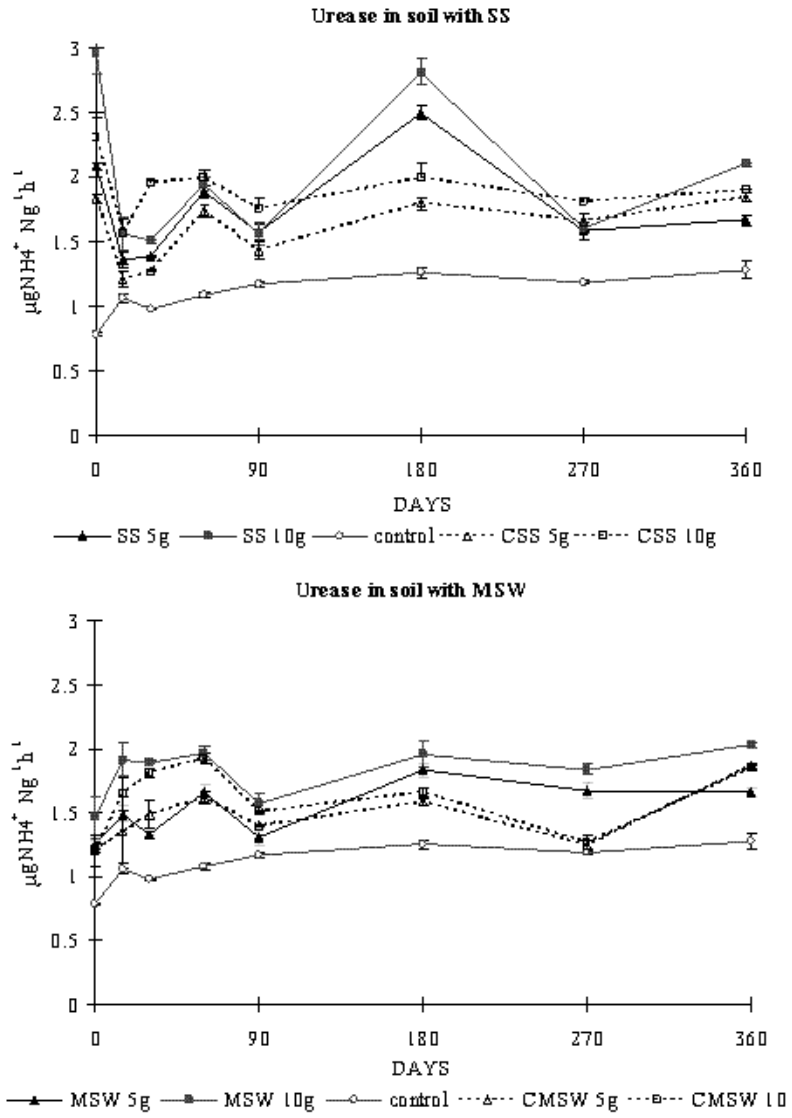

Figure 4.- The urease activity in control and soils amended with different organic materials, during a one-year incubation.

significantly higher (P <0.05) in soil amended with the high dose of MSW or C-MSW than in soil amended with sludge. At 360 days, it was significantly higher (P <0.05) in soil amended with the high dose of SS or C-MSW than in the other soils (Figure 2).

The soils amended with fresh materials (SS and MSW) showed higher values of β-glucosidase activity at 15 days than soils amended with composted materials (Figure 3). This trend was followed by a decrease during the incubation of such fresh materials with soil. At the end of the incubation (360 days), the soil amended with the high dose of fresh MSW showed the highest β-glucosidase activity, in comparison with soils amended with SS or composts.

At the beginning of the incubation (0 days), the soils amended with fresh or composted sludge showed the highest urease activities (P<0.05) (Figure 4). Soils amended with fresh or composted MSW showed significantly-lower (P<0.05) values of this activity than soils amended with SS. However, at the end of the incubation, soils amended with the high dose of fresh sludge or MSW showed the highest (P<0.05) urease activity (Figure 4).

3.3. Immobilized enzyme activity in the humic substances Statistically, the activities of all the immobilized enzymes were affected significantly (P<0.01) by incubation time, origin of material, stabilization process, and dose (Table 3). Generally, the activity of the humus-enzyme complexes extracted from soil amended with MSW was not significantly different (P<0.05) from that of the control soil (Table 3). Initially (0 days), the C-MSW (high dose) gave higher activity of immobilized β-glucosidase than the rest of the treatments, followed by amendment with the compost derived from sludge (Table 5). However, the same result was not found in the case of urease extracted in the humus complexes. The control soil showed the highest urease activity immobilized in humic substances at the beginning of the incubation. Noteworthy, at the end of the incubation, the immobilized β-glucosidase activity was significantly (P<0.05) higher in soils amended with the high dose of sludge than for the compost treatments. However, in the case of immobilized urease activity, the result was the opposite: higher humus-enzyme activity was found in compost-treated soil than for the sludge treatment and control soil (Table 5). In general,

Table 5. The immobilized hydrolytic activity of soil incubated with different organic materials. Nd: non-detected.

	β-glucosidase (nmols PNP* g^{-1} h^{-1})			Urease (μmols NH_4^+-N g^{-1} h^{-1})		
Days	**0**	**180**	**360**	**0**	**180**	**360**
Control	**16.11**	**Nd**	**Nd**	**46.57**	**Nd**	**Nd**
	10.34	--	--	*0*	--	--
S10	**Nd**	**41.45**	**150.96**	**21.92**	**107.60**	**Nd**
	--	*6.03*	*3.56*	*2.62*	*5.04*	--
CS10	**27.64**	**57.39**	**112.12**	**42.20**	**67.85**	**75.66**
	1.58	*1.03*	*1.09*	*4.30*	*6.25*	*0*
MSW10	**13.07**	**7.11**	**Nd**	**36.65**	**Nd**	**Nd**
	0.43	*1.78*	--	*8.65*	--	--
C-MSW10	**34.54**	**117.04**	**104.37**	**21.10**	**41.98**	**71.53**
	0.84	*7.96*	*2.56*	*0*	*0*	*8.41*

The bold letters are the mean values and the italic letters underneath represent the standard deviation of the mean. * PNP, p-nitro-phenyl phosphate.

immobilized urease activity increased during incubation of soil with composted materials but decreased in soils treated with fresh materials and in control soil.

The percentages of immobilized β-glucosidase and urease activity relative to the total activity in soil amended with SS were 24.5% and 0%, respectively. In the case of soil amended with C-MSW, these percentages were 17.1% and 3.86%, respectively, for β-glucosidase and urease.

4. Discussion

In general, the dose, origin, stabilization process, and incubation time influenced all the measured parameters: carbon fractions, total enzyme activity, and immobilized enzyme activity. Nevertheless, the trends are complex and will be analyzed further in this discussion.

It is important to note that 30% of the TOC was lost naturally in the control soil during its incubation. This result suggests that even the stabilized OM in semi-arid areas is able to be degraded when the soil moisture (along the incubation) is adequate. Hence, organic amendments in these areas make even more sense since natural OM can be lost during the precipitation events that improve soil moisture conditions. Additions of high doses of fresh materials were responsible for the highest increments of TOC in the soil, with no influence of the stabilization process at the beginning of the experiment. These changes in TOC at the beginning of the experiment were related to an increase in the size of the microbial community (MBC) and to the highest values of WSC, which provides labile carbon sources for the microbial community (Bastida et al., 2008b; Belete et al 2001). The TOC losses during the incubation were within the range of the control soil values and indicate that mineralization processes are controlled mostly by soil conditions rather than the type of organic material added.

As in the case of MBC, all the organic amendments increased the WSC content at the initial stage of incubation, and soils amended with fresh material showed higher WSC than with composted materials. This could be due to the presence of higher amounts of labile OM in fresh materials than in composted ones, since it is degraded during the composting process - as shown in our previous study (Moreno et al., 2007). In contrast to the general and slow decrease of TOC during the incubation, the WSC showed a fast decline, especially in the first stages of incubation. This result suggests a change in the OM pool of amended soils, as suggested by Mondini et al. (2003), which mainly affects labile fractions. Hence, the differences in the WSC losses followed a clear trend depending on the origin of the material and its stabilization. The WSC losses of the soil amended with MSW were higher than 90% and those of soil amended with fresh sludge up to 81%. In contrast, the losses of WSC in the soil amended with composted materials were lower than in those amended with fresh ones.

The development of microbial biomass is sustained by the biosynthesis of enzymes that provide energy and nutrients for microbial development.

Dehydrogenase activity in soil has been considered as a general index for evaluating soil microbial activity (Beyer et al., 1993; Brookes et al., 2008). Compared to compost, the application of fresh materials led to a fast increase of dehydrogenase activity followed by a concomitant reduction, which is related to the decrease of easily-degradable substrates (Sierra Wittling et al. 1996: Saviozzi et al.2002) - as indicated by the WSC. Higher values of dehydrogenase activity after 360 days of incubation were found in soils amended with high dose of sludge or C-MSW: these results do not entirely fit with the MBC values. Although some authors have found a relationship between these two parameters (Rossel et al., 1997), the existence of a non-active (i.e. sporulated) microbial community might be the reason for this mismatch in a semi-arid climate (Bastida et al., 2006).

Soil is a mosaic of metabolic processes (Nannipieri et al., 1990) and enzyme activity is governed by a high number of biotic and abiotic factors (Schloter et al., 2003). Upon their release from the cell, extracellular enzymes can be denatured or maintain their activity. The activities of β-glucosidase and urease play a vital role in the dynamics of carbon and nitrogen in soil (Nannipieri et al., 1979), and many diverse organisms produce these enzymes: they can be derived from microorganisms such as bacteria, fungi, or protozoa and also from animals and plant residues (Criquet et al., 2004). In our study, the amended soils showed significantly-higher total activities than the control soil, in agreement with other reports on the enhancement of hydrolytic enzymes by organic amendments (Jordan et al., 1995; Kremer and Li, 2003). The highest peaks of all three hydrolytic activities appeared in SS-amended soil (high dose) in the early stage of incubation. Later, a reduction of the activity in soil amended with fresh materials was found, due to the scarcity of easily-degradable compounds in the water-soluble fraction (Benítez et al., 2004). By contrast, during the whole incubation time, more-steady values of enzyme activities occurred with the composted soil amendments, caused by the existence of a more-stable OM fraction (García et al., 1993; Jiménez et al., 2007).

The values of total enzyme activity may be related to the amount of enzyme originally present in the materials or to “de novo” biosynthesis by soil microorganisms, which are stimulated by organic compounds in the added materials. In the case of β-glucosidase, C-MSW had higher activity than the other materials themselves (Moreno et al., 2007). However, this difference had disappeared already after 15 days of incubation of this material with soil, and at the end of the experiment no dissimilarities were found among the different treated soils.

Enzyme activity linked to humic substances may act as a reservoir of microbial activity in soil (Burns, 1982). Bastida et al. (2008b) found that the humus-linked enzyme activity in semi-arid soils was higher after long-term organic amendment with municipal solid wastes. These results contrast with those of the current work, where MSW addition did not increase the

immobilized enzyme activity measured after one year of incubation, whereas the rest of the treatments did. This difference between the two sets of results might be explained by the time-scales involved. In the present work, the activity of the complexes was analysed for up to one year of incubation, while Bastida et al. (2008b) analyzed the immobilized activity after almost 20 years of incubation under field conditions, where plants may promote enzyme production and further immobilization into the humic complex. Nevertheless, it is difficult to understand these dynamics in the MSW-amended soils, since the general activity and microbial biomass were increased significantly after incubation of the soil with MSW, relative to the control. So, we can discard an inhibitory effect of MSW on the microbial community.

The behavior of this complexed activity at the end of the incubation was different to that of the activity of the total hydrolases. As indicated above, the total β-glucosidase activity was stimulated by high doses of the four materials with no significant difference among them (except that addition of MSW generated the highest β-glucosidase) and total urease activity had increased more in response to fresh organic amendment than to composted amendment addition at 360 days. However, in the case of the immobilized enzymes, the highest β-glucosidase activities were in soil treated with sludge (fresh or composted) or C-MSW, and the immobilized urease activity was higher in soil treated with composted materials than with fresh ones (in contrast to the trend observed for the total activity). So, it is clear that the patterns of the total and immobilized enzyme activities differed.

The differing behaviors of the total and immobilized enzymes at the end of the incubation could be due mostly to a preferential "de novo" linkage of enzymes within the humic compounds. Nevertheless, a chemical change in the OM of soil amended with different organic materials has been described (Jindo et al., in preparation) and chemical differences in organic materials of distinct origin (García et al., 1992) might influence enzyme linkage to the humic matrix.

Sewage sludge contains easily-degradable compounds that could stimulate the synthesis of hydrolytic enzymes and their release from intracellular media into the soil. This might be the reason why, after 15 days of incubation, the addition of such fresh material had produced a high level of total β-glucosidase activity able to degrade organic compounds. Subsequently, these protein molecules could have been entrapped into the humic matrix, leading to higher values of immobilized activity than in compost-treated soils. In fact, the activities of these complexes increased with the time of incubation in the SS-amended soil. For instance, β-glucosidase reached its highest degree of immobilization, as a percentage of the total enzyme activity, in soil amended with SS (24.5%). However, SS did not contain the highest amount of β-glucosidase, neither total nor in immobilized complexes (Moreno et al., 2007). This fact supports our hypothesis of biosynthesis and complexation of

new enzymes released by microbial cells.

Addition of composted materials to soil produced higher values of humic substances and humic acids at 360 days than the addition of fresh materials. In principal, this could lead us to assume higher immobilized activities in compost-treated soil. This happened for immobilized urease, but not for β-glucosidase: soil amended with SS showed the highest immobilized activity, suggesting that the immobilization process might also be partially related to the molecular weight of each enzyme (Nannipieri, 2006). In fact, the percentage immobilized urease activity was much lower than for β-glucosidase.

Conclusions

Organic amendment had positive biochemical effects on the quality of a semi-arid soil in a one-year period. The applied doses significantly influenced the microbial activity as well as the carbon fractions in the first stages. However, little difference between the two doses was found at the end, reflecting a sensitive response of this semi-arid soil to low inputs of OM. In the same way, the stabilization of the organic materials had a short-term effect but did not alter the total microbial activity measured after one year of incubation.

Immobilized enzymes do not only represent a soil biological reservoir for substrate hydrolysis, but may also enhance the biological capacity of soil to carry out certain processes related to the cycling of elements. This capacity increased with the addition of compost prepared from MSW or SS, in the case of β-glucosidase. For hydrolases, the dynamics of enzymes immobilized into humic substances were different to those of the total enzyme activity. A preferential capacity of particular enzymes to be entrapped into humic compounds after their synthesis has been found. Although some authors have claimed toxic effects of SS, we were not able to detect such effects in our semi-arid, low-OM soil. Furthermore, the application of such material benefitted the formation of humic-enzyme complexes, to an even-higher degree than compost amendments, during the one–year incubation.

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Chapter 6:

Influence of stability and origin of organic amendments on humification in semi-arid soils

Influence of stability and origin of organic amendments on humification in semi-arid soils

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ABSTRACT
The application of soil organic amendments is a widely accepted strategy to keep soil fertility by maintaining and increasing the levels of soil organic matter. The objective of this study was to evaluate the effect of land application of organic wastes of different sources and stabilization degrees on the soil organic matter humification, measured by changes in the chemical and structural characteristics of humic and fulvic acids (by CPMAS 13C-NMR and FT-IR). A microcosm experiment lasting 360 days was carried out with 500 g of a semi-arid soil amended with different materials: sewage sludge from a wastewater treatment plant, composted sewage sludge, the organic fraction of municipal solid wastes and composted municipal solid wastes. The addition of the organic amendments increased the amount of humic acids in the soil in all cases, whereas the amount of fulvic acids remained very similar to that of the control soil. The humic acids of the amended soils were characterized by an enrichment of N and aromatic C compounds, along with carboxylic groups which increased their aromatic and hydrophobic characteristics. After the one-year incubation, some of the labile compounds added with the organic amendments had been incorporated into the soil humic pool and protected from degradation, contributing to the build-up of soil OM. Land application of organic wastes represents a key waste management option in semi-arid regions where these materials can be used as an exogenous source of OM for soil rehabilitation.
Keywords: **sewage sludge; municipal solid wastes; composting; humic acids; fulvic acids; FT-IR; thermogravimetric analysis; CPMAS ^{13}C-NMR**

1. Introduction

Soil quality is a key element sustaining the productivity of agro-ecosystems and there is increasing interest in its relationship to other environmental issues, such as land protection and soil carbon sequestration (Lal, 2001). Intensive agricultural systems deteriorate soil quality and fertility by diminishing the levels of soil organic matter (SOM). The loss of soil quality is aggravated by the negative effects of climate change such as decreased rainfall and enhanced soil erosion, which are especially important in semi-arid areas such as the Mediterranean region (Spaccini et al., 2002; Bastida et al., 2007). Under these conditions, where semi-arid soils are characterized by low levels of organic matter (OM), exogenous organic C inputs represent an effective strategy to

improve soil quality and maintain ecosystem sustainability. The increasing world population is leading to the production of large amounts of organic wastes from urban activities, which could be used as exogenous OM inputs into the soil (Albaladejo et al., 2008; Singh and Agarwal, 2008). Senesi et al. (2007) reviewed the availability of different organic materials generated from urban activities, such as sewage sludge (SS), municipal solid waste (MSW) and other agro-industrial wastes. These authors highlighted the importance of these wastes as soil organic amendments and as a valuable source of humic material for the soil.

Humic substances, mainly composed of humic (HA) and fulvic (FA) acids, are an essential part of SOM since they are closely related to soil C and N cycles (Stevenson, 1994). Soil humic substances are derived mostly from the decomposition of animal and plant litter. In particular, plant cell wall compounds such as lignin and structural polysaccharides together with lipids and proteinaceous materials, contribute to the structure and composition of the humic substances through different humification processes (Lu et al., 2000). For this reason, the addition of organic wastes to soil is expected to increase the soil humic substance pool and also to alter its chemical properties and functions, depending on the nature of the organic amendments and localized environmental conditions (Ouédraogo et al., 2001; Adani et al., 2007).

The addition of "fresh" organic amendments, such as SS, MSW or other untreated wastes, provides labile OM which improves the quality and nutritional status of the soil. This is especially important in degraded soils, where the labile compounds serve as an important source of energy and C for soil microorganisms (Sánchez-Monedero et al., 2004; Bastida et al., 2008). However, fresh organic wastes may cause negative effects in soil due to the presence of phytotoxic substances (ammonium, phenolics, salts, ect) and unbalanced nutritional composition. Composting is a suitable strategy to overcome these negative effects and, at the same time, generates a well-stabilized OM (Mondini et al., 2003). The newly-formed humic-like substances resemble native soil humic substances and are more resistant to degradation than "fresh" organic amendments, thereby enhancing their role in soil fertility (Senesi and Plaza, 2007). Besides their chemical recalcitrance, other physicochemical characteristics of the humic substances of mature composts, such as hydrophobicity and sorptive interaction with minerals, also protect SOM from microbial degradation, acting as a sink for SOM (Piccolo et al., 2004).

Our understanding of the changes in soil humic substances induced by different organic treatments is still quite limited (Adani et al., 2007), especially in the case of semi-arid soils. Under these semi-arid conditions, which are subject to intense mineralization processes, low precipitation and high temperatures also restrict the production of biomass and the input of organic residues to the soil, hampering SOM humification. The aim of this work was to provide information on the chemical transformations undergone by soil HAs and FAs, using spectroscopic techniques (13C CPMAS- NMR and

FT-IR), after a one-year incubation of soil amended with different organic amendments, in order to evaluate the effect of their origin (SS and MSW) and their stabilization degree ("fresh" and "compost").

2. Materials and Methods

Organic materials

Four different organic wastes of urban origin were used in this study: sewage sludge (SS), composted sewage sludge (CSS), municipal solid waste (MSW) and composted municipal solid waste (CMSW). The physical and chemical characterization of of these organic wastes is shown in Table 1. The SS was obtained from a municipal-wastewater treatment plant located in El Raal-Murcia (SE Spain), which treats wastewater of urban origin with an activated-sludge biological process. The sludge was stabilized aerobically and then dehydrated by centrifugation. The MSW was obtained after manual and mechanical separation of most of the metallic, plastic and paper materials from a treatment plant which receives the source-separated organic fraction of all the household waste produced in the metropolitan area of Murcia (SE Spain). The composting of the SS and MSW was carried out in a full-scale plant, using the static pile system with forced aeration. The CSS was obtained by mixing the SS with woodchips, used as bulking agent at a ratio of 1:2 (volume) in order to improve oxygenation inside the pile. The CMSW was obtained from the MSW described previously. In both cases, maximum temperatures above 65 °C were maintained for a minimum of 48 h (to guarantee disinfection of the material), after which the temperature was maintained at 53-60 °C during most of the process. The moisture level of the material was the optimum (60%) for sustaining microbial activity. The composting process lasted 90 days for both CSS and CMSW. A representative sample of each organic material (composed of 8-10 subsamples) was collected, air-dried and ground to 0.5 mm.

Soil incubation experiment

The proposed objectives were achieved by the incubation of soil with different organic materials under laboratory conditions. The soil was a Calcic kastanozem (FAO, 1998), sampled in an experimental field located in Santomera (SE Spain) and characterized by a sandy loam texture. This area is affected by soil degradation processes such as desertification as a consequence of human activity and aggravated by climatic conditions (annual rainfall of 300mm and mean temperature of 18°C) (Bastida et al., 2006), leading to low contents of soil OM (Table 1).

Soil was sampled in the upper layer (0–10 cm), air-dried and sieved to 2 mm. Ten grams of the organic materials were added to 500 g of soil (roughly equivalent to 40 Mg ha-1) and the water content of the amended soils was adjusted to 60% of the soil water-holding capacity with distilled water. The mixtures of soil and organic materials were prepared in triplicate and incubated in pots under aerobic conditions for 360 days, at 28 ± 1 °C in the dark. Control incubations were run with non-amended soil.

Extraction and purification of humic substances

The quantification of the different humic fractions in the amended and non-amended soils was performed after 360 days of incubation by measuring the organic C in the sodium pyrophosphate (0.1 M Na4P2O7) extractable organic fraction (EXC) and in the fulvic acid (FAC), after precipitation of the humic-like acids (HAC) at pH 2.0 (García et al., 1994). Organic C was measured in the extracts by oxidation with potassium dichromate (K2Cr2O7) and spectrophotometric determination of Cr3+ at 590 nm (Sims and Haby, 1971).

The extraction of the humic substances was carried out with 10 g of soil or organic material and 100 mL of 0.1 N NaOH, in N2 atmosphere. This was repeated several times until colourless supernatants were obtained. The suspensions were centrifuged at 5000 g for 15 min and the filtered extracts were acidified with H2SO4 to pH 2.0 and kept for 24 h at 4°C; they were then centrifuged to separate the precipitated humic acids (HA) from the supernatant fulvic acids (FA). The FA were purified by eluting the supernatant solution through a glass column filled with an XAD-8 resin; they were adsorbed on the resin whereas non-humic material passed through the column. The adsorbed fulvic acids were eluted using one bed volume of 0.1M NaOH. The Na-fulvates were passed through a strongly-acidic cation-exchange resin (Amberlite 120+) in order to obtain H+ saturated FA. Finally, the FA samples were freeze-dried, to keep the material stable until use. The humic acids were purified by treatment with 10 volumes of a dilute 0.5 % hydrofluoric acid (HF) and hydrochloric (HCl) solution (5 mL L-1 HCl + 5 mL L-1 HF). This procedure was repeated three times. After centrifugation at 4000 g for 15 min, the sample was washed repeatedly with water, followed by dialysis against deionized water using a 12- to 14-kD

	Soil	SS	CSS	MSW	CMSW
TOC (g kg^{-1})	6.8 (0.8)	179 (15)	206 (6)	170 (12)	166 (10)
Kjeldhal N (g kg^{-1})	1.0 (0.1)	45.6 (0.2)	33.9 (0.3)	16.3 (0.2)	25.6 (0.3)
pH	8.07 (0.19)	6.82 (0.09)	7.05 (0.04)	6.73 (0.05)	7.90 (0.08)
EC (dS m^{-1})	0.44 (0.04)	6.82 (0.06)	3.00 (0.03)	6.79 (0.02)	3.93 (0.02)
Total P (mg kg^{-1})	805 (66)	12.4 (0.2)	10.9 (0.3)	2.0 (0.4)	2.5 (0.5)
Olsen P (mg kg^{-1})	58.3 (0.1)	-	-	-	-
Cu (mg kg^{-1})	73 (3)	177 (1)	219 (1)	283 (1)	336 (1)
Cr (mg kg^{-1})	6 (1)	8 (1)	15 (1)	107 (3)	48 (10)
Ni (mg kg^{-1})	12 (1)	16 (1)	22 (2)	120 (7)	77 (10)
Pb (mg kg^{-1})	14 (1)	28 (2)	55 (5)	125 (1)	233 (18)
$CaCO_3$	49.3 (1.9)	-	-	-	-
Clay	21.0 (0.0)	-	-	-	-
Silt	31.8 (1.8)	-	-	-	-
Sand	47.2 (1.8)	-	-	-	-

cut-off membrane. The dialysate was freeze-dried for chemical characterization.

Humic substance characterizationChemical Analysis

The elemental composition was determined using a CHN Perkin-Elmer autoanalyzer (Perkin-Elmer, Foster City, CA). The oxygen (O) content was calculated from the difference (i.e. O % = 100 − C % − H % − N %) and can therefore include trace fractions of S and/or P.

Thermal analysis of the solid, freeze-dried HAs and FAs was performed using an SDT-2960 simultaneous DSC-TGA thermal analyzer (TA instruments, New Castle, DE, US). Thermal analyses were performed under a static-air atmosphere with the following experimental conditions: a temperature equilibrating at 30 °C followed by a linear heating rate of 5 °C min-1 from 30 to 105 °C (used for calculating the moisture content of the HA). At this point, an isotherm was maintained for 10 min and then ramping continued at 5 °C min-1 from 105 to 680 °C. The ash content in the solid HAs was calculated from the inorganic residue remaining at the end of the ramp. The main weight losses occurred in the 110-350 °C and 350-550 °C ranges. The ratio mass loss at 350-550 °C (W2) / mass loss at 110-350 °C (W1) was used as an index of the thermal lability of humic structures (Liefield et al., 2007)

Infrared spectra were recorded on a Perkin-Elmer 16F PC FT-IR spectrophotometer using the pellet technique, by mixing 1 mg of dried HA or FA with 300 mg of pre-dried and pulverized spectroscopic grade KBr (from Merck Co). The following broad band assignation was used (Inbar et al.,

l and chemical characteristics of the soil and soil amendments: posted sewage sludge (CSS), municipal solid waste (MSW) and d waste (CMSW). Standard deviation in brackets.

1990; Amir et al., 2005; Mao et al., 2008): 3400-3410 cm− 1 (H-bonded O-H stretching vibrations of hydroxyl groups from alcohols, phenols and organic acids, as well as H-bonded N-H groups); 2925-2930 cm− 1 (C-H stretching of alkyl structures); 1630-50 cm− 1 (aromatic and olefinic C=C vibrations, C=O in amide (I), ketone and quinone groups); around 1120 cm− 1 (C-O band stretching of lignins); and 1020-1100 cm− 1 (C-O band stretching of polysaccharides).

Cross-polarization magic angle spinning (CPMAS) ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were acquired in the solid samples with a Varian 300 (Varian Inc, CA, US), equipped with a 4-mm-wide-bore MAS probe, operating at a 13C resonating frequency of 75.47 MHz. The spectra were integrated in the chemical shift (ppm) resonance intervals of 0-46 ppm (alkyl C, mainly CH2 and CH3 sp3 carbons), 46-65 ppm (methoxy and N alkyl C from OCH3, C-N and complex aliphatic carbons), 65-90 ppm (O-alkyl C, such as alcohols and ethers), 90-108 ppm (anomeric carbons in carbohydrate-like structures), 108-145 ppm (phenolic carbons), 145-160 ppm (aromatic and olefinic sp2 carbons), 160-185 ppm (carboxyl, amides and esters) and 185-225 ppm (carbonyls) (Kogel-Knabner, 2002). The relative areas of alkyl carbons (0-46 ppm) and sp2 (108-160 ppm) carbon components were summed to represent the proportion of hydrophobic carbons in the humic samples (degree of hydrophobicity [HB]). Similarly, the summation of relative areas in intervals related to polar groups such as carbonyls of ketones, quinones, aldehydes, and carboxyls (46-65, 65-90 and 160-185 ppm) indicated the degree of carbon hydrophilicity (HI). The HB and HI values were used to calculate the HB/HI ratio (Canellas et al., 2010).

3. Results and Discussion

Humic substance yield

The addition of the organic amendments caused a marked increase in the amount of both the HAC and FAC fractions of the soil humic substances, compared to the native soil, after the one-year incubation (Table 2). The increase of soil humic substances was affected mainly by the degree of stabilization of the organic materials (fresh vs composted) rather than their origin (sludge *vs* MSW). The mature composts gave rise to a higher amount of soil humic substances than did the fresh organic wastes, especially in the case of the HAC fraction - which increased by 80 and 68% after the addition of CSS and CMSW, respectively. The normalization of these alkaline extractable fractions to soil total organic C (TOC) at the end of the incubation (Table 2) showed a similar humification degree of SOM in soils amended with both composts

ctable organic fraction (EXC) and the humic soil (HAC) and fulvic acid
l (Control) and in soil amended with sewage sludge (SS), composted
pal solid waste (MSW) or composted municipal solid waste (CMSW)

XC (g kg^{-1})	HAC (g kg^{-1})	EXC (g kg^{-1})	HAC (g kg^{-1})	
3 a*	1.10 a	0.73 a	21.7 d	13.1 d
06 b	1.18 b	0.88 b	14.6 a	8.4 a
55 e	1.74 d	0.91 b	21.0 c	13.8 e
15 c	1.18 b	0.97 c	17.2 b	9.4 b
51 d	1.47 c	1.04 d	20.6 c	12.1 c

ring the same letters do not differ significantly according to mean
ple range test at probability level P < 0.05. TOC: total organic C.

than the soil control, whereas soils amended with raw SS and MSW had a lower degree of humification. This is in agreement with the humification of the organic materials during composting, where there is generally a decrease of the fulvic acid fraction as the decomposition proceeds and a relative increase of the humic acid fraction (Chen et al 1993).

In the case of the soil fulvic acids, which underwent a significantly-lower increase than the HA fraction, the addition of MSW or CMSW increased in higher amount of soil fulvic acids (32% for fresh and 40% for compost) than SS or CSS (19% and 23%, respectively). The stability of the organic amendments did not affect significantly the amount of soil FA, probably due to the degradation of the FA fraction during incubation or its polymerization into more-complex structures such as HA - as a consequence of the humification processes taking place in the soil (Jouraiphy et al., 2005). Thus, the major increase in the soil HA fraction after incubation may be due to the new formation of soil humic substances or the incorporation of those originally present in the organic amendments, whereas there was no increase in the fulvic acid fraction because of the ongoing degradation processes that this fraction underwent during incubation.

Elemental and thermogravimetric analysis of HA and FA.

The elemental compositions of the HA and FA extracted from the organic materials and soils are shown in Table 3. Despite the significant increase observed in the amount of these fractions after one year of incubation, elemental analysis (C, H, O, N) of the extracted soil HAs and FAs revealed only a minor alteration of their chemical composition. The main changes in the elemental composition of the soil humic acid were observed in the concentration of N and consequently in the N/C atomic ratio. The addition of SS increased the N concentration in the soil HA, as a consequence of the proteinaceous composition of this material (11.3% N in the HA from SS), and its incorporation into the HA-like substances. The contribution of the N input to the soil was clearly shown in

Table 3. The elemental composition and thermogravimetric ratio of humic acids and fulvic acids in control soil and in soil amended with sewage sludge (SS), composted sewage sludge (CSS), municipal solid waste (MSW) or composted municipal solid waste

Origin	Humic acids								Fulvic acids							
	Mass (%)				Atomic ratios			W2/W1[a]	Mass (%)				Atomic ratios			W2/W1
	C	H	O	N	H/C	N/C	O/C		C	H	O	N	H/C	N/C	O/C	
SS	54.0	9.2	25.5	11.3	2.0	17.9	0.4	0.8	39.9	5.9	45.0	9.3	1.8	20.0	0.9	1.2
CSS	52.2	7.9	34.7	5.2	1.8	8.5	0.5	1.1	46.1	5.1	43.6	5.1	1.3	9.5	0.7	0.9
MSW	51.5	7.1	32.5	8.9	1.7	14.8	0.5	1.2	48.5	5.8	40.0	5.7	1.4	10.1	0.6	0.8
CMSW	53.9	7.6	29.4	9.1	1.7	14.5	0.4	1.1	49.7	4.7	39.7	5.9	1.1	10.1	0.6	0.6
SE[b]	*0.13*	*0.18*	*0.27*	*0.04*	*0.04*	*0.08*	*0.01*	--	*1.06*	*0.17*	*1.43*	*0.32*	*0.04*	*0.21*	*0.05*	–
T0[c]																
Soil	59.4	7.2	28.1	5.3	1.4	7.7	0.4	1.1	42.1	3.2	49.6	5.1	0.9	10.5	0.9	1.0
Soil-SS	56.5	7.6	26.1	9.8	1.6	14.8	0.3	1.0	42.1	6.2	38.1	13.7	1.8	29.0	0.7	0.4
Soil-CSS	59.0	5.8	30.3	4.8	1.2	7.0	0.4	0.8	47.7	6.1	38.2	8.0	1.5	14.4	0.6	1.0
Soil-MSW	55.5	7.1	32.6	4.9	1.5	7.6	0.4	1.1	53.7	5.2	34.0	7.1	1.2	11.3	0.5	0.8
Soil-CMSW	59.7	7.6	24.8	7.9	1.5	11.4	0.3	0.9	53.6	5.1	33.1	8.2	1.1	13.1	0.5	0.6
SE[b]	*0.35*	*0.12*	*0.45*	*0.09*	*0.02*	*0.08*	*0.01*	--	*2.00*	*0.42*	*2.48*	*0.58*	*0.09*	*1.01*	*0.08*	–
T360[c]																
Soil	60.8	7.3	25.2	6.7	1.4	9.5	0.4	1.0	41.9	4.1	49.6	4.5	1.2	9.2	0.9	0.9
Soil-SS	56.2	7.2	28.8	7.8	1.5	11.8	0.4	1.1	45.3	4.1	46.5	4.2	1.1	7.9	0.8	0.6
Soil-CSS	55.7	6.4	30.9	6.9	1.4	10.7	0.4	1.1	46.3	3.9	45.4	4.5	1.0	8.3	0.7	0.7
Soil-MSW	56.0	6.6	30.4	7.0	1.4	10.8	0.4	1.4	51.0	4.7	39.6	4.6	1.1	7.8	0.6	0.6
Soil-CMSW	56.4	6.3	29.8	7.5	1.3	11.4	0.4	1.1	52.5	5.0	37.2	5.2	1.1	8.5	0.5	0.6
SE[b]	*0.35*	*0.18*	*0.49*	*0.09*	*0.03*	*0.16*	*0.01*	--	*0.79*	*0.29*	*1.03*	*0.08*	*0.06*	*0.24*	*0.03*	–

the soil amended with SS at the beginning of the incubation, mostly affected by the presence of exogenous humic materials (Table 3). However, after one year, the N content of the HA in SS-amended soil was reduced from 9.8 to 7.8%, probably due to its microbial degradation during the incubation and/or the release of N compounds from the humic structure due to a weak association. In contrast to fresh SS, the N concentration of all other HAs extracted from the amended soils increased over the year. This could occur through incorporation of N into the humic polymer through typical humification processes such as the reaction of N-containing compounds (peptides) with quinones, generated by oxidation of polyphenols in lignin-building units of organic materials, to form the humic substance polymers in soil (Stevenson, 1994). In the case of the FA fraction, the N content was reduced markedly in all samples after one year of incubation. This could be due to the incorporation of part of the FA into the HA structure, because an increase of N was also observed in HA. However, since the FA polymer is dominated by relatively-low-weight compounds, the linkage with N is weaker than in HA and, consequently, they are more readily available to soil microorganisms.

Other important features of the soil humic substances after one year incubation are the concentration of H and the H/C atomic ratio, which is commonly used to indicate the maturity of humic substances (Belzile et al., 1997; Lu et al., 2000). A low H/C ratio is characteristic of a complex humic substance structure with aromatic character, as a consequence of polymerization (humification) reactions undergone by the OM during composting (Sánchez-Monedero et al., 2002). In our study, the decrease of the H/C atomic ratio can be seen clearly in the FA from the mature composts (Table 3): it was due to probably the large amount of biodegradable compounds, such as simple peptides and carbohydrates, with a relatively-high H/C ratio in FA compared with HA. During the composting process, the hydrogen content was generally reduced in both SS and MSW, due to the loss of aliphatic compounds, which are more readily biodegradable. Hence, before the incubation started, there were already different features of each FA depending on the origin and the degree of stabilization. After the incorporation of the organic amendments into the soil, the H content and H/C ratio of the HA were not significantly affected during incubation, whereas in the case of the FA, the H/C ratios were reduced further in most cases down to values between 1.0 and 1.2, indicating that aromatic compounds accumulated gradually while the aliphatic compounds decreased.

Similarly to the H/C atomic ratio, an increase in the W2/W1 ratio obtained from thermal analysis can also reflect an increase of the aromatic character of the soil humic substances. However, this ratio did not show a clear trend in soil HA and FA after one year of incubation (Table 3).

FT-IR characterization of HA and FA from soils and organic materials

The FT-IR spectra of the HAs extracted from the amended soils are similar to those of typical soil HA (Stevenson,

1994), dominated by the 3400 cm-1 broad band characteristic of OH and NH bonded groups and the 1600-1640 cm-1 band characteristic of aromatic and olefinic C=C and C=O groups (Figure 1). As already observed in the elemental composition, the FT-IR spectra of the HAs extracted from the amended soils did not differ noticeably among the amendments. Only minor structural differences existed between the soil HA from the control and the amended soils. First of all, a small increase

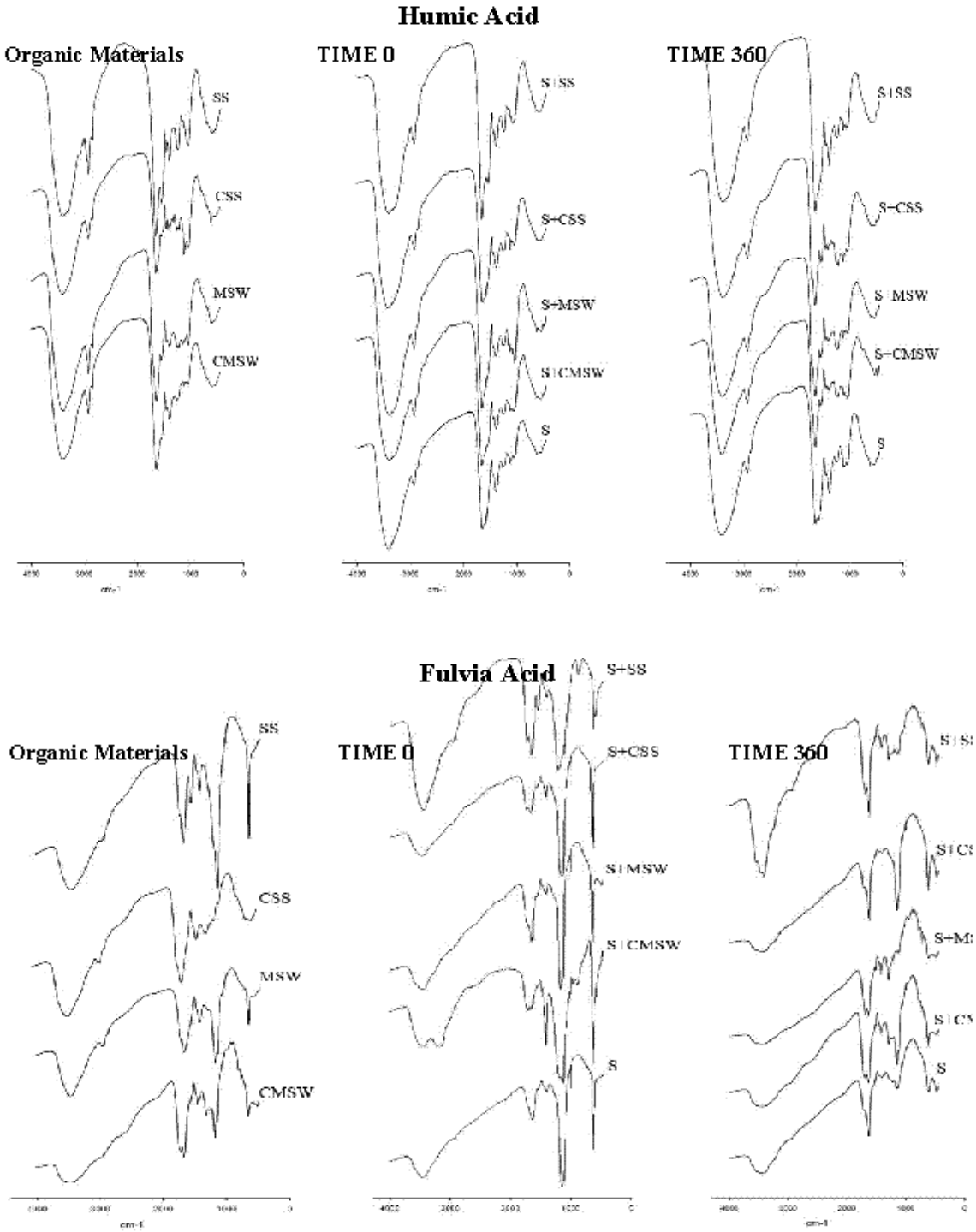

Figure ¬1 – FT-IR of humic and fulvic acids isolated from organic amendments and amended soils: S (soil), SS (sewage sludge), CSS (compost from sewage sludge), MSW (municipal solid wastes) and CMSW (compost from municipal solid wastes).

of the band at 2925-2930 cm− 1, characteristic of the C-H stretching of alkyl structures, was observed. The HA from amended soils also also showed a new shoulder at 1540 cm− 1, due to the incorporation of C=O groups, probably from amides II, judging from the increase in the N concentration. This shoulder was already present in the IR spectra of the HA extracted directly from the organic materials (Figure 1). In the case of the SS spectra this signal appeared as a small but well-defined band due to the presence of proteinaceous compounds in the structure. Another structural difference in the HA from the amended soils is a new, sharp appearance around 1230-1260cm− 1, along with the absorbance in the region 1100-1140 cm-1, which could be due to the C–O stretching and O–H deformation - characteristic of carbohydrates - and also to native lignin (Adani et al., 2007). Adani et al. (2007) found an increase in the absorbance in this region due to the lignin parent material from MSW, even after four years of incubation. During composting, there is an increase in the carboxylic group content due to oxidation of easily-degradable C, mainly of aliphatic origin. Hence, the incorporation of mature composts into the soil increased the proportion of carboxylic groups in the humic structure from the beginning of the incubation. The high amount of polar C=O and O-H groups in the humic molecules may be responsible for additional bonds, principally H-bonding and van der Waals forces (Senesi and Testini, 1980), which would be related to a greater complexation of N and other relatively-labile C compounds, such as polysaccharides and proteins, and also lignin fragments. These bands were also appreciable after one year of incubation, reflecting the rearrangement of humic acid polymers and the incorporation of nitrogen into the HA structure (Lobartini and Tan, 1988); 1380 cm− 1 (N-O stretching), 1410-20 cm− 1 (amide I), 1530-45 cm− 1 (amide II).

The FA spectra of the soils generally showed bands similar to those observed in the soil HA and, as mentioned already in the case of the HA, the FA spectra did not show a marked difference among the different organic amendments after one year of incubation (Figure 1). In contrast to the HA, the IR spectra of the FA extracted from the organic materials were dominated by a sharp band at 1000-1260 cm-1, characteristic of carbohydrates and amino groups, which could reflect the presence of labile C compounds, such as carbohydrates from hemicelluloses and celluloses characteristic of MSW or amide and amino groups characteristic of sewage sludge (Hafidi et al., 2005). This peak was still appreciable in the soils amended with fresh organic materials (SS or MSW) but it was reduced markedly during incubation, reflecting the degradation/release of these labile compounds. However, this band was appreciable after the one-year incubation of the soils amended with composts (CSS and CMSW), reflecting the protection of these compounds in the humic polymer - probably by hydrophobic forces. Since the FA is expected to contain more labile and hydrosoluble compounds than HA, originating mainly from polysaccharides, proteins and alcohols, these compounds may have been partially protected inside the fulvic polymer during the one-year incubation and incorporated largely into the FA moiety (Gigliotti et al., 2003; Amir et al., 2004; Plaza et al., 2008).

CPMAS ^{13}C NMR characterization of HA and FA from soils and organic materials

The ^{13}C-NMR spectra of the HA extracted from soils and organic amendments are shown in Figure 2. Compared to the control, the HA extracted from the amended soils at the beginning of incubation presented two distinctive peaks, around 55 ppm and 73 ppm, assigned to methoxy and O-alkyl groups characteristic of the relatively easily biodegradable compounds such as cellulose, hemicellulose and some fractions of lignin (Gonzalez-Vila et al., 1999;

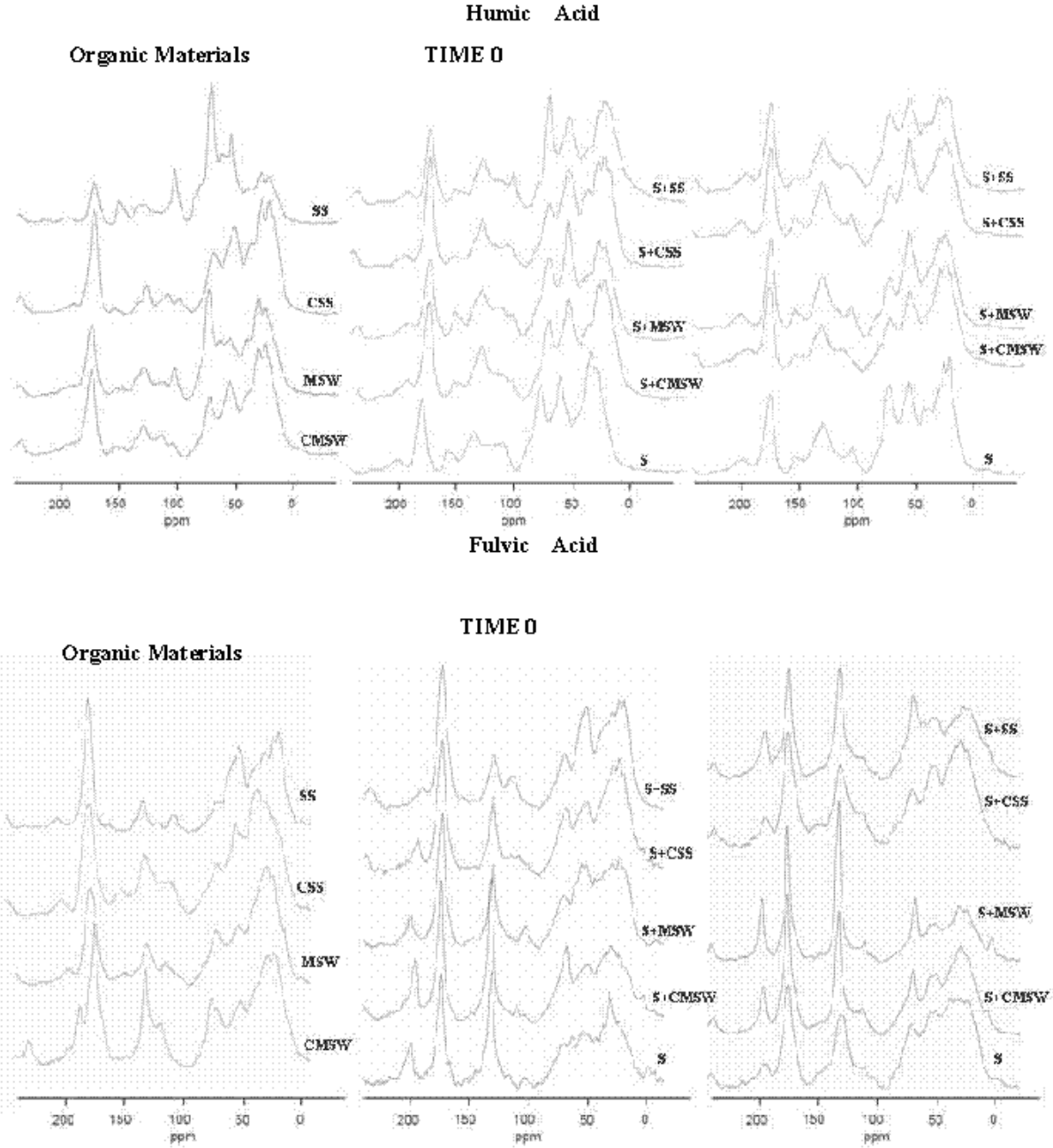

Figure 2 - ^{13}C CPMAS-NMR of humic and fulvic acids isolated from organic amendments and amended soils: S (soil), SS (sewage sludge), CSS (compost from sewage sludge), MSW (municipal solid wastes) and CMSW (compost from municipal solid wastes).

Spaccini and Piccolo, 2009). These bands dominated the spectra of both SS and MSW which exhibited intense bands at 73 ppm.

In the case of SS, an intense band at 55 ppm was also observed presumably due to the presence of proteins. Despite the difficulty in clearly identifying the presence of proteins using ^{13}C-NMR (Almendros et al., 2000), there are several regions of the spectra which may be useful to monitor the changes of this fraction during incubation. Hence, proteins can contribute to the intensity of different bands, such as alkyl (C-H) and carbonyl (C=O from peptide bond) signals and especially the N alkyl region (45-60 ppm). After one year of incubation these peaks still remained, but their intensities had decreased markedly. This implies that these labile organic compounds had been protected in the soil HA presumably through polymerization reactions.

In contrast to the soils amended with fresh organic materials, the HA extracted from the compost-amended soils exhibited a dominant signal at 174 ppm. This peak reflects the presence of carboxyl groups, from aliphatic acids of plant and microbial origin, and also the N-amide groups in amino acid moieties, which may be absorbed into the humic polymer during composting as a consequence of oxidation and humification processes. The abundant N of the SS-amended soil, shown by the elemental analysis, was incorporated into humification, increasing the signals of carboxyl and amide groups (Kogel-Knabner, 1997). The addition of SS and CSS increased the proportion of N-alkyl and/or methoxyl groups. Since the N-alkyl C may include both amine and amide functional groups (Adani et al., 2007), the N preservation by peptide bonds inside the humic polymer could have been maintained even after one year, as

Table 4. The Alkyl C/O-alkyl C ratio, Alkyl C/N-alkyl C ratio, Aromaticity Index and Hydrophobicity (HB/HI) of humic acids and fulvic acids in control soil and in soil amended with sewage sludge (SS), composted sewage sludge (CSS), municipal solid waste (MSW) and composted municipal solid waste (CMSW)

Origin	Humic acids						Fulvic acids					
	Alkyl C/ *O*-alkyl C		Alkyl C/ *N*-alkyl C		HB/HI[a]		Alkyl C/ *O*-alkyl C		Alkyl C/ *N*-alkyl C		HB/HI[a]	
	T0[b]	T360[b]	T0	T360	T0	T360	T0	T360	T0	T360	T0	T360
Soil	2.3	2.4	2.0	2.0	1.1	1.1	3.0	2.5	2.2	2.0	1.2	1.2
Soil-SS	2.7	2.2	2.2	1.8	1.2	1.0	4.2	2.5	2.0	2.3	1.1	1.2
Soil-CSS	3.2	3.4	1.9	1.9	1.1	1.2	3.6	3.4	2.6	2.4	1.2	1.3
Soil-MSW	2.1	2.8	1.7	2.0	1.0	1.2	3.3	2.8	1.9	2.4	1.0	1.3
Soil-CMSW	3.0	3.6	2.1	2.4	1.2	1.4	2.5	3.0	2.3	2.1	1.2	1.3

[a]HB/HI = (alkyl C + aromatic C + phenolic C) / (N-alkyl + O-alkyl C + carboxyl,amides, esters).
[b]T0: beginning of incubation; T360: after 360 days of incubation

suggested by the elemental analysis and IR.

The NMR spectra of the FA (Figure 2) also reflected the transformation of N compounds during incubation. The soil FA fraction typically includes a rather high percentage of N in labile forms such as amino acids and simple peptides (Stevenson, 1994). At the beginning of incubation, a high input of N was incorporated into the fulvic polymer through peptide bonds, reflected in the enlarged broad range of carboxyl (C=O) and/or amide groups (CO-N) (160-185ppm) and also as an intense band at 55 ppm (N-alkyl). After one year, the presence of this broad range, and of the aromatic groups range (95-108ppm), continued in almost all samples. In contrast to the fresh organic amendments (SS and MSW), soil-CSS and soil-CMSW maintained the intensity of the peaks at 50 and 30 ppm, presumably due to lignin-derived phenolic compounds in the case of CMSW, and mostly due to lipids and protein in CSS (Schnitzer and Preston, 1983; Albrecht et al., 2008). Even though lipids and proteins can be considered as easily-degradable compounds, these fractions remained in the soil probably incorporated into the humic substances through strong bonds. This association was also supported by the presence of peak 1423-1426 cm-1 in the IR spectra of soil FA samples, which reflects the presence of carbonyls associated with OH from phenols and alcohols (Riberio et al., 2001).

In general, the ^{13}C NMR spectra of the HA extracted from the amended soils after one year of incubation resembled those of the native soil HA. The main change was a shift of the carbon fractions, which moved to the aromatic (95-108 ppm), phenolic (145-160 ppm) and carboxyl and/or amide groups (160-185 ppm). This reflects the incorporation phenolic compounds into the structure of soil HA during the incubation, probably derived from lignin. Even though the OM of the composted materials (CMSW and CSS) is relatively mature and stable, the HA isolated from the compost-amended soils showed the presence of some labile compounds, which could be degraded further in the soil, as observed by Leifeld et al. (2002), who reported continued humification of the compost after addition to the soil.

All these structural transformations were reflected clearly by the changes in the alkyl C/O-Alkyl C ratio, represented in Table 4. This index has been proposed as an indicator of the capacity for decomposition of OM (Baldock et al., 1997; de Nobili et al., 2008), and also as a sensitive indicator of humification during the early stages of leaf material decomposition (Almendros et al., 2000). A high ratio indicates a highly-decomposed OM, which consequently is more resistant to rapid C loss (Webster et al., 2001). In our study, soil HA and FA from both compost-amended soils (CSS and CMW) showed higher Alkyl C/O-Alkyl C ratios than those of "fresh" amended soil (SS and MSW) after one year incubation, meaning that the humic fraction formed in compost-amended soils was more stable. This trend was not so clear in the case of the alkyl C/N-alkyl C ratios. These ratios were only slightly higher in the HA extracted from compost-amended soils than in the HA from soils amended with fresh materials (Table 4). Hence, the use of composts as organic amendments not only generated the greatest quantity of humic substances over a one-year period (Table 2), but also more recalcitrant humic substances.

The hydrophobicity index (HB/HI) is an important characteristic of HA and FA (Zandonadi et al., 2007; Muscolo et al., 2007; Nardi et al., 2007; Dobbs et al., 2010). These authors suggested that the hydrophobic domain can preserve bioactive molecules, such as auxins, in the humic matter and also enhance the interaction with microorganisms and root growth in the rhizosphere. For this reason, the HB/HI index reflects the proportion of hydrophobic humic components, derived from microbial debris and plant residues, which are able to randomly incorporate more polar molecules, protecting labile compounds against degradation (Piccolo et al., 2004).

The HA and FA extracted from soils amended with compost (CSS and CMSW) showed HB/HI ratios (Table 4) that were slightly higher than those in soil amended with fresh materials (SS and MSW). The hydrophobicity was enhanced by transformations occurring during the composting process and even after the addition to the soil, where ongoing humification took place. A process of hydrophobic protection prevents rapid microbial decomposition of the labile OM entering the soil with litter or plant residues. This may have been responsible for the higher yield of soil humic substances in soil amended with composts, compared to fresh organic amendments, and also for the different protective capacity of the soil HA and FA, driven by the original characteristics of the organic amendments.

Conclusions

Land application of either SS or MSW to a semi-arid soil significantly increased the soil humic pool after one year of incubation. CPMAS ^{13}C NMR was the most-sensitive technique, compared with FT-IR and elemental and thermal analyses, with respect to observing the changes in the soil humic substances during incubation. The soil humic substances extracted after one year incubation, formed by newly-formed and exogenous humic material, were characterized by N incorporation into the humic polymer and the enrichment of aromatic C compounds. The chemical characteristics of the soil humic substances increased the hydrophobic character of the structure, which may have been responsible for the enhanced protection of labile compounds from the fresh amendments and the incorporation of these compounds in the soil humic pool. The addition of composted material to the soil significantly increased the amount of soil HA and also its aromatic and hydrophobic properties. However, the origin of the organic amendments did not have an important effect on the characteristics of the soil humic substances determined after the one-year incubation. Land application of organic wastes to a semi-arid soil can increase the amount of soil humic substances and also their quality, which could be a key element regarding the problem of soil degradation.

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Chapter 7:

Root growth promotion by humic acids from composted and non-composted urban organic wastes

Root growth promotion by humic acids from composted and non-composted urban organic wastes

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ABSTRACT

Background and Aims: Besides general effect of organic residues on soil quality and plant crop, hormonal direct effect on plant growth by extracted humic acids of organic materials is interesting and profitable theme. In the present work, we studied on direct interaction between humic acid and root growth, depending on different origin of organic materials.

Methods: All extracted humic acids of four organic materials (sewage sludge, compost sewage sludge, municipal solid waste, compost municipal solid waste) were characterized chemically by elemental analyses, ion pair chromatography (ICP), size exclusion chromatography (HPSEC), solid-state nuclear magnetic resonance (13C-CPMAS-NMR) and quantification of IAA. Later, different morphological effects on maize (principal root growth, lateral root growth, root area, root mitosic site, root dry weight and H+-ATPase activity of plasma membrane) were analyzed.

Results: All humic acids samples promoted root growth and proton pump activity in maize vesicles, especially those composted samples, which contained more carboxylic groups and had a more-hydrophobic character, produced preferentially morphological and biochemical effects.

Conclusion: The conformational dynamics of humic hydrophobic associations in the rhizosphere may release auxin-like plant growth promoters and enhance plant biochemical activities. These organic wastes represent a renewable source of humic acid for use as plant root promoter.

Keywords: **humic-acids; auxin-like; sewage sludge; municipal solid wastes; composting**

1. Introduction

Utilisation of organic wastes originating from anthropogenic activities has been recognised as a promising alternative for solid waste management. The maturity of compost is one of the essential criteria in recycling of organic wastes, as well as its marketing and utilisation in agriculture (Campitelli and Ceppi, 2008). The composting process provides more stable and mature organic material and, in consequence, an enrichment in "real" humic substances (Provenzano et al., 2001). During this biological degradation process, rearrangement of the molecular characteristics of humic acid (HA)-like substances takes place: the labile carbon content is reduced, while the aromatic fraction is enriched (Chefetz et al 1996). In addition, the selective preservation of hydrophobic alkyl molecules takes place: these are represented by long-chain fatty acids, aliphatic alcohols, linear hydrocarbons and plant polyester derivatives, such as long-chain alkyl dicarboxylic acids and □-hydroxy acids (Spaccini and Piccolo, 2009). In this way, the stabilised organic matter is newly created.

The organic material has indirect beneficial effects on plant growth, improving soil structure, increasing microbial populations, behaving as a pH buffer and serving as a micronutrient carrier (Magdoff and Weil 2004). On the other hand, direct interactions with plants are those that require uptake of organic macromolecules, such as humic substances, into plant tissues, resulting in various biochemical effects at the cell wall or cell membrane, or in the cytoplasm (Chen et al, 2004). Moreover, hormonal effects on plant development are one of the ways in which humic substances trigger these direct effects. It is reported that in different parts of plant (root, shoot, leaves) can be observed these effects by increasing micro-nutrient uptake (Adani et al 1998; Eyheraguibel et al., 2008), and even under unfavourable conditions as nutrient deficiency and high salinity (Bidegain et al., 2000; Khaled and Fawy, 2011), the direct effects of humic substances on plant growth still last strongly.

Indole-3-acetic acid (IAA) is a well-known natural auxin which promotes cell elongation, apical dominance and rooting. Since the beginning of the last century until now, humic subtances have been studied as agents endowed with auxin-like activities (Bottomley 1917; Guminski 1968;, Nardi et al., 2002). In general, the effect of humic acid (HA) on plant physiology is recognized with regard to enhancement of root growth (O'Donnell RW, 1973; Vaughan et al., 1985; Eyheraguibel et al., 2008) and nutrient uptake (Tan and Nopamornbodi, 1979; Chen et al., 2004; Pinton et al., 2007). Auxins induce plasma membrane (PM) H+-ATPase activities in cell roots, which couple adenosine triphosphate (ATP) hydrolysis to H+ transport across cell membranes (Sze et al., 1999). Consequently, the apoplast is further acidified, the cell walls are loosened and cells eventually elongate (Hager et al., 1991), thereby favouring an increase in acid root growth. At the same time, the activation of PM H+-ATPase improves the uptake of plant nutrients, by enhancing the electrochemical proton gradient that drives ion transport

across cell membranes via secondary transport systems (Morsomme and Boutry, 2000). Humic acid-like auxins induce PM H+-ATPase synthesis and it activity (Pinton et al., 1992; Canellas et al., 2002; Quaggiotti et al., 2004). Due to the important role of this ATPase in energetic metabolism, its analysis has been a useful physiological indicator of HA bioactivities (Canellas et al., 2006). In previous work, a putative relationship between the chemical nature of humic substances and their effects on plant growth and the PM H+-ATPase was observed (Canellas et al., 2008; 2009). In this work, we used urban wastes at different degrees of stabilisation as sources of humic substances to be used as a plant growth promoter.

2. Materials and Methods

Organic materials of urban origin

Four different organic wastes of urban origin were used in this study: sewage sludge - SS (TOC = 179 g kg-1; N = 45.6g kg-1 ; EC= 6.82mS cm-1; pH= 6.82; P total = 12.4%; K total = 0.35%), composted sewage sludge - CSS (TOC = 206 g kg-1; N = 33.9g kg-1 ; EC= 3.00mS cm-1; pH= 7.05; P total = 10.9%; K total = 0.38%), municipal solid waste - MSW (TOC = 170 g kg-1; N = 16.3 g kg-1 ; EC= 6.79mS cm-1; pH= 6.73; P = 2.04%; K total = 0.71%) and composted municipal solid waste - CMSW (TOC = 166 g kg-1; N = 25.6 g kg-1; EC= 3.93mS cm-1; pH= 7.90; P total = 2.46%; K total = 0.51%). More detailed information on their characteristics is described in previous work (Moreno et al., 2007). The SS was obtained from a municipal wastewater treatment plant located in El Raal-Murcia (SE Spain), which treats wastewater of urban origin by an activated biological process. The sludge was stabilised aerobically and then dehydrated by centrifugation. The MSW was obtained after manual and mechanical separation of most of the metallic, plastic and paper materials from a treatment plant which receives the source-separated organic fraction of all the household wastes produced in the metropolitan area of Murcia (SE Spain). The composting of the SS and MSW was carried out in a full-scale plant, using the static pile system with forced aeration. The CSS was obtained by mixing SS with woodchips, used as bulking agent at a ratio of 1:2 to improve oxygenation inside the pile. The CMSW was obtained from the MSW described previously. In both cases, maximum temperatures above 65 °C were maintained for a minimum of 48 h (to guarantee disinfection of the material), after which the temperature was maintained in the range 53-60 °C during most of the process. The moisture level of the material was the optimum (60%) for sustaining microbial activity. The composting process lasted 90 days for both CSS and CMSW. A representative sample of each organic material (composed of 8-10 sub-samples) was collected, air-dried and ground to 0.5mm in order to homogenise the material.

HA extraction

The extraction and isolation of the humic substances were carried out, adopting the methology described by Stevenson (1994) and Sánchez-Monedero (et al., 2002a), with 10g of organic material and 100 mL of 0.1 N

NaOH, under an N2 atmosphere. The extraction was repeated several times until colourless supernatants were obtained. The extract was centrifuged at 5000 g for 15 min and the filtered solutions were then acidified with H2SO4 to pH 2 and kept for 24 h at 4°C, before being centrifuged to separate the precipitated humic acids (HA) from the supernatant. The HA were purified by solubilisation with 10 volumes of a diluted HF-HCl solution (5 mL L-1 HCl [12 M] + 5 mL L-1 HF [48%, v/v]). This procedure was repeated three times. After centrifugation at 4000g for 15 min, the sample was repeatedly washed with water, followed by dialysis against deionised water using a 12- to 14-kD cut-off membrane. The dialysed sample was freeze-dried and lyophilised for chemical characterisation.

Elemental composition and nutrient contents

The elemental composition was determined using a CHN Perkin-Elmer autoanalyzer (Perkin-Elmer, Foster City, CA). The oxygen (O) content was calculated from the difference (i.e. O % = 100 − C % − H % − N %). The macro and micro-nutrients and heavy metals were determined by ICP-DES (iCAP 6500 series).

Solid-State Nuclear Magnetic Resonance Spectroscopy

Cross-polarisation magic angle spinning (CPMAS) ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were acquired in the solid samples with a Varian 300, equipped with a 4-mm-wide bore MAS probe, operating at a ^{13}C resonating frequency of 75.47 MHz. The spectra were integrated in the chemical shift (ppm) resonance intervals of 0-46 ppm (alkyl C, mainly CH2 and CH3 sp3 carbons), 46-65 ppm (methoxy and N alkyl C from OCH3, C-N and complex aliphatic carbons), 65-90 ppm (O-alkyl C, such as alcohols and ethers), 90-108 ppm (anomeric carbons in carbohydrate-like structures), 108-145 ppm (phenolic carbon), 145-160 ppm (aromatic and olefinic sp2 carbons), 160-185 ppm (carboxyl, amides and ester) and 185-225 ppm (carbonyls) (Kogel-Knabner, 2002). The relative areas of the alkyl (0-46 ppm) and sp2 (108-160 ppm) carbon components were summed to represent the proportion of hydrophobic carbons in humic samples (degree of hydrophobicity [HB]). Similarly, the summation of relative areas in intervals related to polar groups such as carbonyls of ketones, quinones, aldehydes and carboxyls (46-65, 65-90 and 160-185 ppm) indicated the degree of carbon hydrophilicity (HI). The HB and HI values were used to calculate the HB/HI ratio (Canellas et al., 2010).

Hormone extraction and analysis

Indole-3-acetic acid was extracted and purified according to the method of Dobrev and Kaminek (2002) and Aguirre et al (2009), from 1 gram of homogenised humic acid, and then analyzed to quantify as described previously by Albacete et al., 2008. Dry-frozen humic acid was homogenised in liquid nitrogen and placed in 5 ml of cold (-20°C) extraction mixture of methanol/water/formic acid (15/4/1 by vol., pH 2.5). After overnight extraction at -20°C solids were separated by

centrifugation (20 000 g, 15 min) and re-extracted for 30 min in an additional 5 ml of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus tC18 cartridge (SepPak Plus, Waters, USA). The residue was dissolved in 5 ml of 1 M formic acid and loaded on an Oasis MCX mixed mode (cation-exchange-reverse phase) column (150 mg, waters USA), preconditioned with 5 ml of methanol followed by 5 ml of 1 M formic acid. To separate different CK forms (nucleotides, bases, ribosides, and glucosisides) from IAA, the column was washed and eluted stepwise with different appropriate solutions indicated in Dobrev and Kaminek (2002). IAA was analysed in the same fraction. After each solvent was passed through the columns, they were vacuum, and solvents were evaporated at 40 °C under vacuum. Samples then dissolved in a water/acetonenitrile/formic acid (94.9:5:0.1 by vol.) mixture for HPLC/MS analysis. The analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA,

Table 1.
Elemental composition, micro-elements and heavy metals of humic acids from sewage sludge (SS), composted sewage sludge (CSS), municipal solid waste (MSW) and composted municipal solid waste (CMSW) (dry weight basis).

	Mass / %				Atomic ratios			ppm									%					
	C	H	O	N	H/C	N/C	O/C	Al	Cd	Cu	Fe	Mn	Ni	Pb	Zn	B	Ca	K	Mg	Na	P	S
SS	54.0	9.2	25.5	11.3	2.0	17.9	0.4	103.8	0.4	58.1	477.6	2.2	<2	<2	26.0	25.8	0.1	0.1	0.0	1.5	0.22	1.27
CSS	52.2	7.9	34.7	5.2	1.8	8.5	0.5	375.7	1.7	170.8	899.6	4.2	10.3	8.6	62.3	20.0	0.2	0.1	0.1	1.4	0.13	1.29
MSW	51.5	7.1	32.5	8.9	1.7	14.8	0.5	917.0	0.9	1756.1	1963.3	10.2	140.7	67.7	102.0	37.4	0.8	0.2	0.1	2.8	0.02	1.82
CMSW	53.9	7.6	29.4	9.1	1.7	14.5	0.4	101.8	0.9	1070.2	2192.9	7.7	76.7	23.8	67.4	25.8	0.5	0.1	0.1	2.1	0.09	0.89
SE[a]	0.13	0.18	0.27	0.04	0.04	0.08	0.01	0.31	0.02	0.05	0.14	0.04	0.01	0.01	0.24	0.20	0.18	0.14	0.26	0.24	0.04	0.15

S.D.= Standard Deviation

USA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray interface. Previous to injection, 100 µl of each fraction was filtered through 13-mm-diameter Millex filters having a nylon membrane of 0.22µm pore size (Millipore, Bedford, MA). 8 µl of each sample, dissolved in mobile phase A, was injected onto a Zorbax SB-C18 HPLC column (5µm, 150×0.5 mm, Agilent Technologies) at 40 °C and eluted at a flow rate of 10 µl min-1. Mobile phase A, consisting of water/acetonitrile/formic acid (94.9:5:0.1 by vol.), were used for the chromatographic separation. The elution consisted of maintaining 100% A for 5 min, and then a linear gradaient from 0 to 6% B in 10 min, followed by another linear gradient from 6 to 100% B in 5 min, and finally 100% B maintained for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analytical run. The UV chromatograms were recorded at 280 nm with the DAD (diode-array detector) module (Agilent Technologies). The mass spectrometer was operated in the positive emode with a capillary spray oltage of 3500V and a scan speed of 22,000 (m/z) s-1 from 50 to 500 m/z. The nebulizer (He) pressure was set to 30 psi, whereas the drying gas was set to a flow of 6.01 min-1 at 350 °C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). For quantification of IAA, calibration curves were constructed for each component analysed (0.05, 0.075, 0.1, 0.2, and 0.5 mg L-1) using internal standards: [13C6] indole-3-acetic acid (Cambridge Isotope Laboratories Inc., Andover, MA, USA). Recovery percentages ranges were 92% and 95%. All samples were run in triplicate.

High-Performance Size Exclusion Chromatography

The high-performance size exclusion chromatography (HPSEC) system consisted of a YoungLin 900 Avance solvent pump and two detectors in a series: a UV-Vis variable wavelength detector (Perkin-Elmer LC-295) set at 280 nm and a Refractive Index (RI) detector (Fisons Instruments, Refractomonitor IV). A rheodyne rotary injector, equipped with a 100-KL sample loop, was used to load the calibration standards and humic solutions. Size exclusion separation occurred through a Polysep-GFC-P 3000 column (Phenomenex), preceded by a Polysep-GFC-Guard column and by a 0.2-KL stainless-steel inlet filter. Both columns were packed with rigid, spherical silica gels, chemically bonded with hydrophilic compounds. Phosphate buffer (NaH2PO4, 0.0625 M, pH 7, ionic strength 0.104 M) was used to dissolve HA (0.6 g L-1) and as chromatographic eluent at 0.6 mL min-1 flow rate. The void volume (V0 = 10.96 mL) and the total permeation volume (Vt = 25.88 mL) of the column were determined with Blue dextran (2000 kD) and water (18 D), respectively. The HPSEC system was calibrated using sodium polystyrenesulphonate standards (Polymer Standard Service, Mainz, Germany) with molecular weight ranging from 1100 to 130,000 D. Size

exclusion chromatograms for both th
UV and RI detectors were evaluate
using Origin 6.1.

Maize

Maize seeds (var UENF 506) provide
by the Plant Science Department o
UENF were surface-sterilised b
soaking in 0.5% NaCl for 30 mir
followed by rinsing and then soaking i

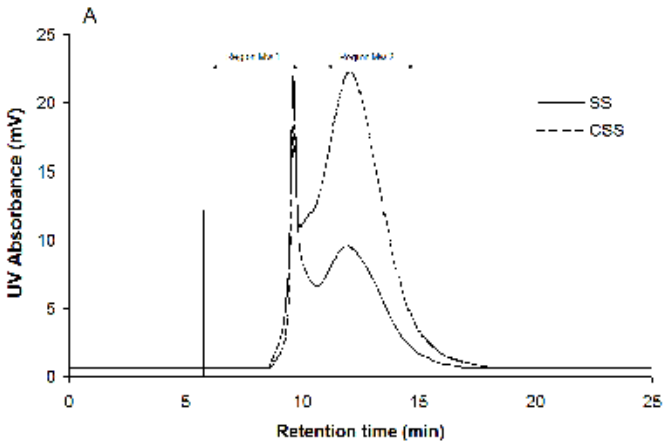

Fig. 1.A. HPSEC chromatograms of the humic acid of SS (Sewage Sludg
Sludge).

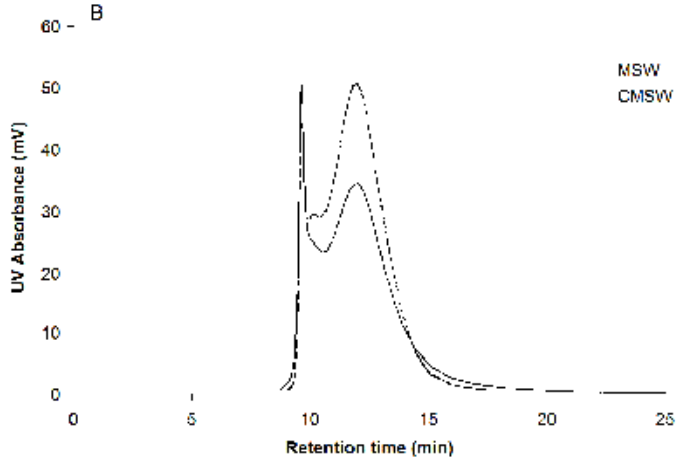

Fig. 1.B. HPSEC chromatograms of the humic acid of MSW (Municipal So
Municipal Solid Waste).

water for 6 h. The seeds were then sown

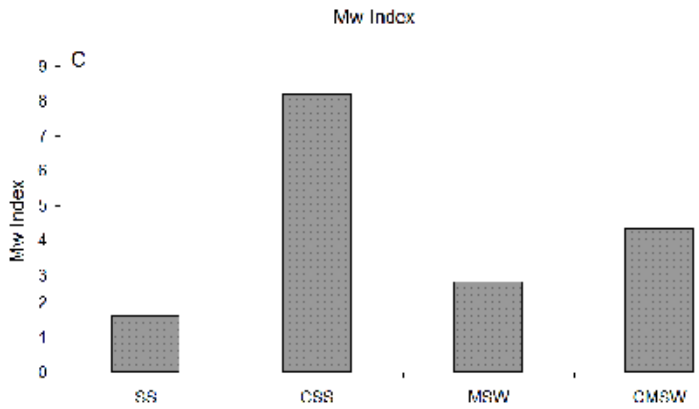

Fig. 1.C. Molecular weight index of 4 humic acids extracted from SS (Sewage Sludge), CSS (Composted Sewage Sludge), MSW (Municipal Solid Waste) and CMSW (Composted Municipal Solid Waste).

seedlings were treated with increasing concentrations (0, 0.25, 0.5, 1.0, 2.0 and 5.0 mM C L-1) of HA. After regression analysis, a new experiment was carried out using 2 mM C L-1of each HA. Four-day-old maize seedlings with roots approximately 0.5 cm long were transferred into a solution containing 2 mM CaCl2 and either 0 or 2 mM C L-1 of HA. A minimal medium (2 mM CaCl2) was used to avoid any interference from nutrient constituents that could interact synergistically with HA regarding plant growth and metabolism (Pinton et al. 1999). Roots were collected on the seventh day and scanned at 300 dpi to estimate their length and area using Delta-T Scan image analysis software (Cambridge, UK) (Bouma et al., 2000). Additional samples of seedling roots were collected for further experiments.

Frequency of Sites of Lateral Root Emergence

Seeds of maize were germinated for 4 d in wet filter paper and rooted in a medium containing 0 or 2 mM C L-1 of each HA. The whole root systems (three replicates) of both treatments were harvested every day during a period of 7 d to evaluate the number of mitotic sites, as follows: the entire root system was washed in water and cleared by heating at 75°C for 20 min in KOH (0.5%, w/v). Afterward, root samples were rinsed in water and stained for 14 h in the dark in hematoxylin staining solution. Then, they were rinsed in water and destained in 80% (w/v) lactic acid at 75°C for 30 to 90 s. Individual specimens were transferred to Petri plates containing water and observed using stereoscopic microscopy to evaluate the number of mitotic sites, visible as red dots on a pink to white background of root tissue. Hematoxylin stock solution consisted of 1 g hematoxylin, 0.5 g ferric ammonium sulphate and 50 mL of 45% (w/v) acetic acid, and was stored in the dark at temperature. The stain was prepared by diluting the stock solution 40-fold in water.

Biochemical assays

PM vesicles were isolated from maize roots grown with or without 2 mM C L-1 of bulk HA and each chemical derivative by a differential centrifugation method (Canellas et al. 2002). The vesicles were either used immediately or frozen under liquid N2 and stored at -70°C until use. The protein concentration was determined by the Lowry method. The ATPase activity in PM vesicles was determined by colorimetrically measuring the release of Pi. Between 80 and 95% of the PM vesicle ATPase activity measured at pH 6.5 was inhibited by vanadate (0.1 mM), an effective inhibitor of the P-type H+-ATPases. In all experiments, ATPase activity was measured at 30°C, with and without vanadate, and the difference between these two activities was attributed to the PM H+- ATPase.

3. Results

Characterisation of humic acid-like fractions (elemental composition and nutrient content)

The carbon contents of the HA-like fractions from the four different materials were similar (Table 1). The highest N concentration was shown by SS-HA, which was related to the dominant proteinaceous composition of

this material (11.3% N in the HA from SS). A low H/C ratio is characteristic of a complex humic substance structure with aromatic character, as a consequence of polymerisation of the OM during composting (Sánchez-Monedero et al., 2002b). In our study, the slight decrease of the H/C atomic ratio in both composted materials can be seen. The micronutrient contents varied, depending on the origin of the material and the composting process.

HPSEC

The molecular sizes of the HA were evaluated by HPSEC (Figure 1). Commercially-available size calibration standards, such as the polystyrene sulphonates used in this study, differ from humic substances in hydrodynamic radius and interaction with the column stationary phase (Piccolo et al., 2001). Hence, our focus regarding this parameter is not on the molecular sizes from size exclusion chromatograms, but on only HA behaviour, so that we can see the structural transformation before and after the composting process. As a result, the apparent molecular distribution changes pronouncedly with the progress of humification, including a relative decrease in weight during the composting process (Figure 1).

CP-MAS ^{13}C NMR

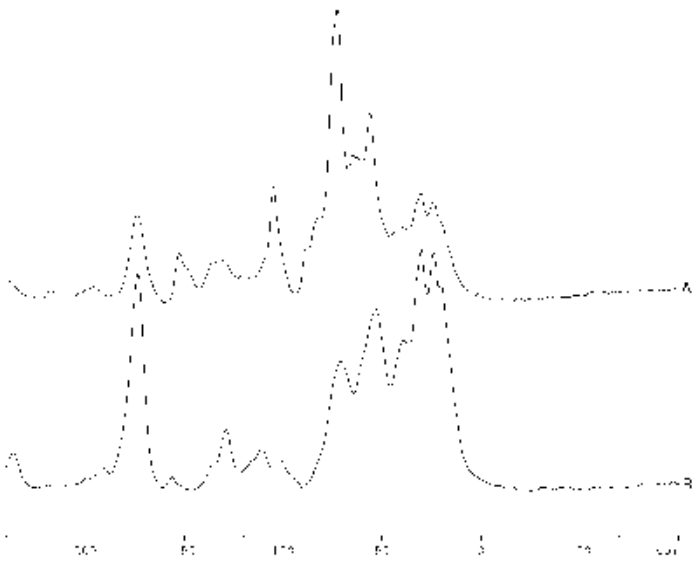

Fig. 2.(A). ^{13}C CPMAS-NMR of the humic acid of (A) Sewage Sludge and (B) Composted Sewage Sludge.

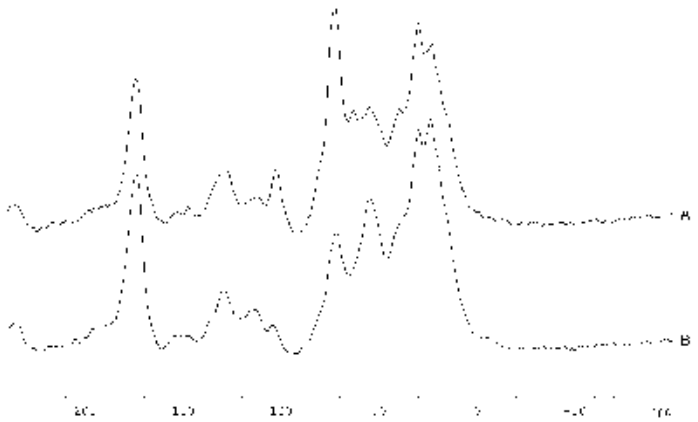

Fig. 2.B. ^{13}C CPMAS-NMR of the humic acid of (A) Municipal Solid Waste and (B) Composted Municipal Solid Waste.

The strong signals in the alkyl-C (0–40 ppm) and O-alkyl-C (50–110 ppm) regions of the NMR spectra of all HA revealed an initial composition dominated by both alkyl and sugar components (Fig. 1). The broad peak, centred at around 30 ppm, indicated a large content of methylenic chains or CH2 groups deriving from various lipid compounds, plant waxes and plant biopolyester. The HA extracted from the fresh organic materials (SS and MSW) presented more sharply two distinct peaks, around 55 ppm and 73 ppm, assigned to methoxy and O-alkyl groups characteristic of the relatively-easily-biodegradable compounds such as cellulose, hemicellulose and some fractions of lignin (González-Vila et al., 1999; Spaccini and Piccolo, 2009). The signal at 102 ppm is assigned to the anomeric C1 carbon of cellulose and was more intense in SS HA. The broad band around 129 ppm may be related to alkyl substitutions in the p-hydroxy phenyl ring of cinnamic and p-coumaric units of both lignin and suberin biopolymers, as well as to both partially-degraded lignin structures and condensed aromatic and olefinic carbons (Hatcher et al., 1995). The small shoulder in the phenolic aromatic region (140–160 ppm) indicates a low content of O-substituted ring carbons. The prominent signal around 174 ppm indicates a large content of carboxyl groups in aliphatic acids of plant and microbial origin and/or amide groups in amino acid moieties (Spaccini and Piccolo. 2009), and this signal increased at the end of composting process (CSS and CMSW), meaning to the HA becoming more stable and reactive (Plaza et al., 2008). In term of hydrophobicity, the relative amount of apolar compounds (0-45 + 110-165 ppm) increased and the hydrophobic index changed from 1.09 to 1.17 and from 1.02 to 1.20 for MWS-CMWS and SS-CSS, respectively.

Hormone extraction and analysis

Indole-3-acetic-acid was detected in all extracts of HA (Figure 3), and the highest value was shown in HA from CSS. The abundance of this hormone was increased after composting, by 47% in CSS and 21% in CMSW.

The Effect of HA on Root Growth

The effects of different HA on root growth are reported in Figure 4. The HA-like materials from the organic residues were seen to have an optimum bioactive concentration of 20 mg C L-1, at which the largest plant response among different HA concentrations was demonstrated in previous work also (Canellas et al., 2008 and 2009). Even though all HA treatments stimulated root growth, the intensity of the plant response varied extensively among HA samples. All HA treatments increased root area, by between 30 and 70%. The HA produced through the composting process enhanced the principal root length, by 30 to 40% with respect to the control, while HA present at the initial stage only produced a slight positive effect on the principal roots. In addition, root dry weight in seedlings treated with HA from composted material was increased generally by 25 to 30% with respect to control seedlings. Lateral root emergence plays a vital role in plant nutrient and water uptake and HA treatment favours cell differentiation and new lateral root induction. The

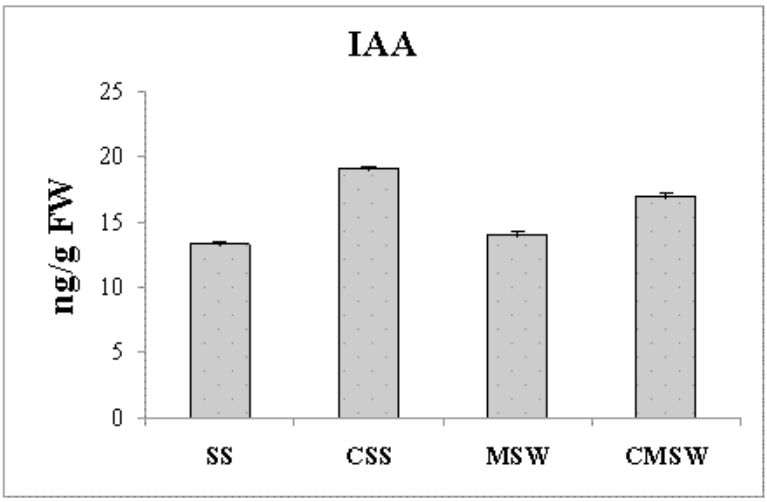

Fig. 3 Determination of IAA in the humic acid from sewage sludge (SS), composted sewage sludge (CSS), municipal solid waste (MSW) and composted municipal solid waste (CMSW), by HPLC/MS.

number of lateral roots increased by 22 to 111% in maize treated with different HA; the greatest effects were observed for the treatments with HA from composted material. The HA treatment clearly induced the proliferation of root mitotic sites with respect to control plants (by 37 to 128%). This marked effect on the root morphology was mainly observed with both mature HA. Maize seedlings treated with HA exhibited a clear stimulation of vanadate-sensitive ATPase activity (Figure. 4). Treatment with MWS, CMWS, SS or CSS stimulated PM H+-ATPase activity, by 236 (MSW) to 638% (CSS). The HA isolated from mature compost promoted the largest increases, as observed also in the number of emerging lateral roots and mitotic sites.

4. Discussion

Composting is a biodegradable process taken place by a succession of different microbial communities and produces more humified organic matter as mature and stabilized product. The main chemical modifications during humification of organic residues by composting were a decrease of carbohydrate moieties and increases of the levels of alkyl and aromatic C, due to selective preservation (Figure 2), as previously observed by Inbar et al., (1990). And as a consequence, the modification of HA characterization is archived after composting process, which are reflected by more functional groups with increasing hydrophobicity. The change in humic structure was also observed in the molecular size that the size-fraction of humic aggregates in HA solution decreased with compost maturation (Figure 1) and at the end of composting the HA showed aggregates of relatively-small size.

The HA chemical descriptors obtained by NMR were related to the root growth promotion (Figure 2) and PM H+-ATPase induction in vesicles isolated from maize seedlings (Figure 4). The HA isolated at the end of composting were more bioactive than those isolated initially. The presence of phytotoxic components was unfound in MWS and

SS (both HA increased plant growth relative to control plants, but to a lesser extent than CMWS and CSS). In general, the amount of micronutrients and trace elements supplied to the growth solution by the HA was not significant (Table 1) except copper and iron in MSW and CMSW. The changes in root morphology can be attributed to possible changes in cell energetic metabolism. The

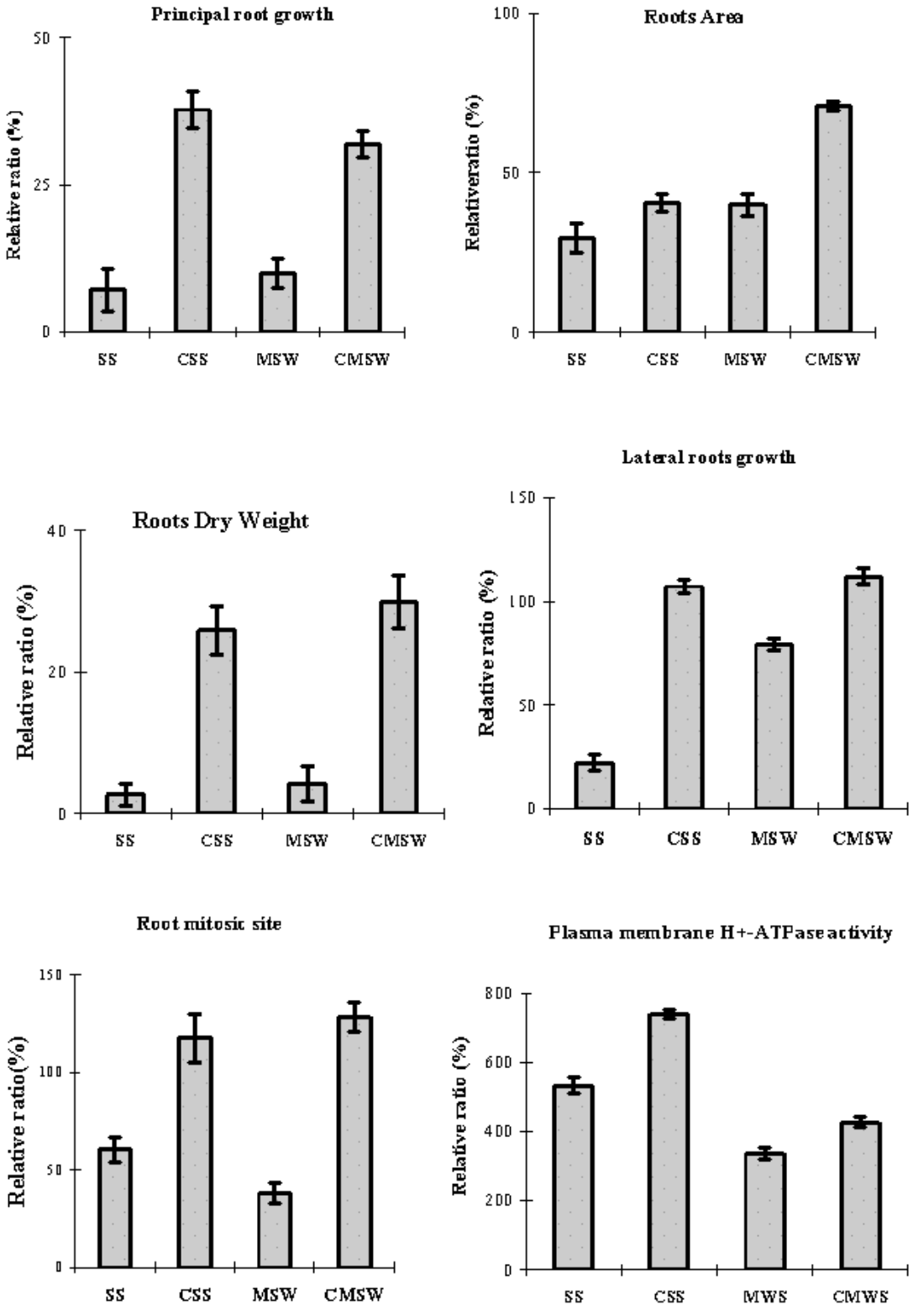

Fig. 4. Effect on plant growth of the humic acid from sewage sludge (SS), composted sewage sludge (CSS), municipal solid waste (MSW) and composted municipal solid waste (CMSW): percentage values, with respect to the control.

HA isolated from materials of urban origin nduced the proliferation of sites of lateralroot emergence in maize roots (Figure 4). These differentiation sites are precursors of lateral roots and are formed by meristematic cells that have a PM enriched with H+-ATPases (Jahn et al., 1998). Canellas et al. (2002) reported previously that HA isolated from vermicompost induced the synthesis of PM H+-ATPases in a similar way; the phytohormone auxin is required to regulate the initiation and emergence phases of lateral root development (Casimiro et al., 2001). It has been reported that auxin can induce de novo synthesis of the PM H+-ATPase in plant tissues (Hager et al., 1991) and the major isoform (MHA2) expressed in maize was induced by humic substances (Quaggiotti et al., 2004). The main function of the PM H+-ATPase is to generate a proton electrochemical gradient, thereby providing the driving force for the uptake and efflux of ions and metabolites across the PM (Sze et al., 1999). However, it has been shown that the HA-induced H+ pump activity could also play a key role in the acid growth mechanism, related to the HA effects on root growth and morphology (Canellas et al., 2002; Zandonadi et al., 2007).
07).

The presence of auxins in the humic structure has been well demonstrated by a number of methods, such as gas chromatography-mass spectrometry and DR5::GUS gene reporter (Muscolo et al., 1998; Canellas et al., 2002; Trevisan et al., 2010; Dobbss et al., 2010). According to the findings of Muscolo (et al., 1996, 2007a and 2007b), even though two humic acids with different fractions (low relative molecular mass<3,500 D and high relative molecular mass>3,500 D) had the same amount of IAA, they did not induce the same biological activity. Therefore, the amounts of IAA in HAs of different origin were measured in our study, as well as the molecular size by HPSEC, to observe the influence on biological activity of different factors. The conclusion that can be drawn from the present work is in accordance with other reports (Trevisan et al 2009; Canellas et al 2010), namely that the induction of biological activity by humic acids is influenced more by functional groups and chemical composition than by the IAA content. It is seemingly attributable to the presence of carboxylic groups in the hydrophobic pocket, where the auxin binds with its receptor, being an important key to trigger the biological activity (Rubery, 1981; Napier, 2001, 2004). In addition, previous reports associated the hydrophobic character of humic acids with their enhancement of PM H+-ATPase expression (Canellas et al., 2008, 2009). It is possible that hydrophobic humic components deriving from plant degradation and microbial activity are able to randomly incorporate more-polar molecules and hence protect them against microbial degradation (Spaccini et al. 2000).

Conclusion

The present work confirms the great efficiency of HA-like substances from organic wastes with respect to improving plant growth. The more hydrophobic the character of the HA, the larger the potential incorporation of bioactive molecules. Organic residues from urban sources can be considered appropriate materials for HA extraction and use as a plant growth promoter. Their increases in bioactivity due to composting were related to chemical transformations during the composting process, when strong hydrophobicity is generated. Furthermore, the earlier and more-profuse rooting provoked by composted HA may be useful in agriculture, considering the obvious economic benefits of more-plentiful harvests in a shorter time.

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