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Dynamics of carbon in degraded semiarid soils: effects of organic amendments and associated soil microbial communities

Dinámica del carbono en suelos degradados de zonas semiáridas: implicación de enmiendas orgánicas y comunidades microbianas asociadas

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ABREVIATIONS

- **AWCD:** average well color development.
- **CLPP:** community level physiological profiles.
- **CVW:** composted vegetal waste.
- **DIC:** dissolved inorganic carbon.
- **DOC:** dissolved organic carbon.
- **EDTA:** Ethylenediaminetetraacetic acid.
- **EEA:** extracellular enzyme activity.
- **FAME:** fatty acid methyl ester.
- **GC-C-IRMS:** gas-chromatography-combustion-isotope ratio mass spectrometry.
- **H:** Shanon-Weaver index.
- **HD:** high dose.
- **HS:** humic substance.
- **HSD:** honestly significant difference.
- **INT:** p-iodonitrotetrazolium chloride.
- **INTF:** p-iodonitrotetrazolium formazan.
- **IRMS:** isotope ratio mass spectrometry.
- **LD:** low dose.
- **MUB:** modified universal buffer.
- **OM:** organic matter.
- **PCA:** principal components analysis.
- **PLFA:** phospholipid fatty acid analysis.
- **PLFA-SIP:** phospholipid fatty acid analysis-stable isotope probing.
- **SDS:** sodium dodecyl sulphate.
- **SDS-PAGE:** sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
- **SIP:** stable isotope probing.
- **SOM:** soil organic matter.
- **THAM:** tris (hydroxymethyl) aminomethane.
- **TOC:** total organic carbon.

- **V-PDB:** Vienna PeeDee Belemnite.

- **VW:** vegetal waste.

- **WSC:** water soluble carbon.

RESUMEN

El suelo desempeña funciones de gran importancia para el sustento de la vida en este planeta. Cabe destacar que es fuente de nutrientes, agua y aire para las plantas, el hábitat natural de miles de organismos, y además es un escenario fundamental para el desarrollo de ciclos biogeoquímicos. El suelo tiene la propiedad de poder retener sustancias mecánicamente o fijarlas por adsorción, así como de actuar como amortiguador. Ambas características dependen fuertemente del contenido de materia orgánica presente en el mismo y de su textura.

La desertificación del suelo es un problema muy extendido en todo el planeta, siendo las zonas áridas o semiáridas las más afectadas. En España, este fenómeno es muy importante debido a que toda la mitad sur se encuentra dentro de las categorías de tierras áridas, semiáridas y subhúmedas secas, y por lo tanto son muy susceptibles a sufrir un proceso de degradación de este tipo. Las comunidades más afectadas por la desertificación son: Murcia, Comunidad Valenciana y Canarias; donde la amenaza afecta casi al 100% del territorio. Las diferentes formas de degradación del suelo como consecuencia de la desertificación pueden incluir: i) la pérdida de nutrientes debido a la sobreexplotación agrícola; ii) la pérdida de superficie de tierra fértil debido a la erosión del viento y del agua, sobre todo debido a la pérdida de la vegetación; iii) deslizamientos provocados por la acción del agua y los efectos de la pérdida de vegetación; iv) el aumento de la salinidad y la acidificación de los suelos debido al riego inadecuado, y; v) la contaminación del suelo y del agua debido al uso excesivo de fertilizantes químicos. La disminución de la productividad agronómica de los suelos en zonas semiáridas está estrechamente relacionada con la pérdida de cobertura vegetal del suelo a consecuencia de la escasez de precipitaciones, de su propia litología, y de la degradación inducida por el hombre. Dicha pérdida de vegetación repercute notoriamente en una abundante pérdida de materia orgánica, y por tanto de carbono orgánico, la cual afecta negativamente a la calidad y la sostenibilidad del suelo. Además, en estas condiciones, la actividad de la comunidad microbiana está limitada por la escasez de nutrientes, lo cual también conlleva un impacto negativo para la sostenibilidad del suelo.

La recuperación del carbono orgánico del suelo hacia unos valores umbrales suficientes para poder gestionar sus funciones básicas es de vital importancia. Por este motivo, se han estado buscando métodos eficaces para la mejora de la calidad de los suelos semiáridos o áridos durante las últimas décadas. La restauración del suelo mediante enmiendas orgánicas puede afectar a los balances de carbono, aumentar las

reservas de carbono del suelo y sirve para luchar contra la desertificación en muchas regiones áridas y semiáridas.

La presente Tesis Doctoral tiene como Objetivo entender el ciclo del carbono en suelos semiáridos con un alto nivel de degradación y el desarrollo de estrategias para la restauración de estos suelos. Para ello nos hemos centrado particularmente en la capacidad que presenta este tipo de suelo para responder a fuentes de carbono exógenas derivadas de residuos vegetales, el papel que desempeña la comunidad microbiana en la degradación de estas fuentes de carbono, y su relación con los ciclos biogeoquímicos de los elementos importantes (C, N y P), con particular incidencia en el del carbono como elemento clave en la sostenibilidad de estos ecosistemas. Se maneja como Hipótesis general de trabajo de esta Tesis Doctoral el hecho de que las comunidades microbianas de suelos áridos y semiáridos tienen una limitada capacidad para el procesamiento de sustratos carbonados, a consecuencia del escaso contenido en materia orgánica que presentan habitualmente estos suelos.

La Tesis Doctoral se desarrolla en una zona semiárida del levante español (Región de Murcia), donde los suelos están bastante adaptados a condiciones severas, con una temperatura media anual de aproximadamente 18 ºC y precipitaciones anuales que se sitúan por debajo de los 300 mm. Los suelos de esta zona se encuentran en un estado avanzado de degradación, implicando frecuentemente un bajo contenido en materia orgánica, una escasa cobertura vegetal, y una baja biomasa microbiana. Además, los suelos empleados fueron abandonados después de haber sido sometidos a agricultura. Como ya hemos señalado anteriormente, existe una importante escasez de conocimiento sobre la dinámica del ciclo del carbono en suelos semiáridos y el papel que desempeñan los microorganismos en este ciclo. Para ampliar el conocimiento en este tema, en esta Tesis Doctoral se abordan experimentos en condiciones controladas de laboratorio y campo, con el objetivo de obtener una información completa sobre la dinámica del carbono orgánico y las enmiendas orgánicas de origen vegetal en relación con las poblaciones microbianas asociadas a su procesamiento.

La primera parte de la Tesis (Capítulos 1, 2 y 3) se realizó en condiciones controladas de laboratorio; mediante la utilización de tres moléculas de origen vegetal de distinta complejidad: glucosa, celulosa y lignina; dichas moléculas son parte fundamental de los propios restos vegetales que entran al suelo de forma natural. Dichas moléculas se utilizaron en su forma enriquecida en ^{13}C (isótopo estable del carbono) con el objetivo

de trazar a alta resolución la dinámica de estas materias orgánicas y las poblaciones microbianas implicadas en su procesamiento.

El primer experimento (Capítulo 1) consistió en la adición de una molécula de baja complejidad (glucosa) a un suelo semiárido en dos dosis diferentes (75 y 300 μgC g^{-1}), y su incubación a 28 ºC y una capacidad de retención hídrica controlada en un 60% durante dos meses; mientras que en el segundo experimento (Capítulo 2) fueron adicionadas moléculas de mayor complejidad (celulosa y lignina) en una dosis de 75 μg C g^{-1} a dos suelos semiáridos con distinto nivel de degradación y diferente textura. El experimento fue realizado en las mismas condiciones de laboratorio que el experimento anterior y tuvo una duración de cuatro meses. El primer suelo empleado fue procedente de Abanilla, cuya composición es: 34.6% arcilla; 7.7% limo, y 57.7% arena. El pH de este suelo es de 7.8, y su conductividad eléctrica de 2.6 dS m^{-1}. El segundo suelo fue recogido en Santomera, y su composición es: 18.8% arcilla, 9.5% limo y 71.7% arena. Su pH es de 7.7 y la conductividad eléctrica de 0.3 dS m^{-1}. En estos dos experimentos se aplicaron, entre otras, técnicas basadas en el análisis de la composición isotópica del CO_2, fracciones hidrosolubles de C, y de sustancias húmicas, así como la composición isotópica de ácidos grasos (PLFA-SIP). Mediante este conjunto de técnicas se pudo trazar exhaustivamente el ciclo del C, evaluando su persistencia, mineralización y las poblaciones microbianas asociadas con la dinámica de estas moléculas.

Posteriormente, en el Capítulo 3, se atendió a una perspectiva bioquímica para entender el ciclo del C, observando el efecto de la adición de celulosa y lignina en la actividad y estructura funcional de la comunidad microbiana mediante diversas actividades enzimáticas y perfiles fisiológicos en microplaca (Biolog). Gracias a estos experimentos se pudo concluir que, mientras que la adición de moléculas de baja complejidad provocó un aumento de la biomasa microbiana, la adición de moléculas complejas no estimuló significativamente el desarrollo microbiano. Sin embargo, estas últimas indujeron un reciclaje de la biomasa microbiana y/o un almacenamiento del carbono derivado de estas moléculas en la biomasa microbiana. La adición al suelo de cualquiera de estas moléculas no supuso la completa mineralización de las mismas, si no que al contrario, manifestaron una persistencia en el suelo proporcional a la complejidad química de la molécula. Su permanencia en el suelo indicó que estas moléculas son capaces de aumentar la cantidad de carbono orgánico del suelo, ya sea por entrampamiento físico o químico en el mismo o por su conversión en sustancias húmicas. Por otro lado, la textura y el nivel de degradación del suelo influyeron

notablemente en el tiempo de respuesta de la comunidad microbiana a la adición de moléculas orgánicas de origen vegetal; así como en su mineralización y en los diferentes grupos microbianos que contribuyen a su degradación.

Como parte de las conclusiones de estos Capítulos (1, 2 y 3) es importante resaltar que los suelos semiáridos no pierden su potencial para responder a entradas de materia orgánica, independientemente de su complejidad, a pesar de no haber recibido materia orgánica durante un largo período de tiempo. Además, ese potencial aumenta con el nivel de degradación del suelo. Aunque los suelos semiáridos respondan de manera rápida a la adición de moléculas complejas, la respuesta de la comunidad microbiana a estos sustratos no tiene un efecto a largo plazo a nivel enzimático, pero sí en la estructura funcional de dicha comunidad.

Una vez adquirido un conocimiento fundamental y exhaustivo en relación con el ciclado de carbono en condiciones controladas mediante el uso de tecnologías innovadoras, se evaluó el efecto a largo plazo de la adición de enmiendas orgánicas a nivel de campo, lo cual representa un escenario real para el estudio de la restauración de suelos (Capítulos 4 y 5).

Para este propósito, en el Capítulo 4, se evaluó el efecto a largo plazo de enmiendas orgánicas de origen vegetal y de su dosis en las propiedades químicas, bioquímicas y microbiológicas de un suelo semiárido. Para ello, se realizó un estudio en parcelas enmendadas 5 años atrás con residuo de poda con diferentes niveles de estabilización (fresco y compostado) y en distinta dosis (150 y 300 t ha^{-1}). Se emplearon diferentes técnicas para medir los cambios en la actividad microbiana después de la adición de la materia orgánica, así como el análisis de ácidos grasos fosfolípidos y el perfil fisiológico de las comunidades microbianas para estudiar los cambios en la biomasa y estructura de la comunidad. Como resultado, se pudo observar que la adición de residuos de poda, y especialmente, el residuo de poda fresco, provocó un aumento en la biomasa microbiana y además tuvo un efecto en la actividad, estructura y estructura funcional de la comunidad microbiana presente en el suelo, comparado con un suelo control al que no se aplicó ninguna enmienda. De este modo, podemos concluir que la adición de residuos de poda a un suelo semiárido tiene un efecto beneficioso para el mismo y que no sería necesario proceder a un proceso de compostaje para mejorar la calidad microbiológica del suelo a largo plazo.

En cuanto a la dosis de la materia orgánica adicionada al suelo, se puede decir que no tuvo un efecto notable a largo plazo en la actividad microbiana debido a la existencia

de una dosis umbral. Se puede concluir con este estudio que una dosis demasiado elevada de enmienda orgánica puede inducir una inhibición de las actividades enzimáticas. De este modo, teniendo en cuenta las actividades enzimáticas, la dosis óptima para la adición del residuo de poda a un suelo semiárido sería 150 t ha^{-1}. Sin embargo, la dosis sí que conllevó un efecto notable en la estructura funcional de la comunidad microbiana presente en el suelo, siendo esta muy diferente dependiendo de la cantidad de materia orgánica añadida 5 años atrás.

Finalmente, en la última parte de esta Tesis Doctoral, se estudió el efecto que ejerce la adición enmiendas orgánicas a largo plazo (24 años) en las enzimas involucradas en el ciclo del carbono bajo condiciones semiáridas, además de su influencia en la biomasa y la estructura de la comunidad microbiana (Capítulo 5). Además, se pretendió completar el conocimiento del ciclo del carbono en este tipo de suelos mediante el estudio de la diversidad de isoenzimas que presentan la celulasa y β-glucosidasa. Para este propósito, se empleó un suelo que fue enmendado con la fracción orgánica de basura doméstica en dos dosis, 24 años atrás. Las dosis aplicadas se eligieron de modo que inicialmente se aumentara el contenido en carbono orgánico del suelo en 0.5 y 1.5% con respecto a su contenido en carbono orgánico total. En este experimento se utilizó la zimografía como técnica para el estudio de la diversidad de isoformas de la celulasa y la β-glucosidasa. La zimografía está basada en la extracción de las proteínas del suelo, su separación por electroforesis en gel y su posterior visualización en gel mediante una tinción específica para cada enzima. Además de esta técnica, también se empleó el análisis de los ácidos grasos fosfolípidos para estudiar los cambios provocados en el tamaño de la biomasa microbiana y la estructura de la comunidad microbiana por la adición de las enmiendas. Como resultado de este estudio se pudo observar que tanto las actividades como la biomasa microbiana aumentaron con la adición de enmiendas orgánicas a largo plazo, además de incrementarse el número de isoenzimas en relación a la cantidad de materia orgánica añadida. Sin embargo, tal y como ya se había descrito en el Capítulo 4, el aumento de las actividades enzimáticas no fue proporcional a la dosis aplicada.

Haciendo una visión global de esta Tesis Doctoral, llegamos a la conclusión de que los suelos semiáridos, a pesar de su avanzado estado de degradación al tener frecuentemente una escasa cubierta vegetal (derivado de las propias condiciones climáticas y un frecuente abandono agrícola) y no recibir abundantes entradas de C orgánico, presentan una alta disponibilidad biótica para gestionar materia orgánica de

origen vegetal de distinto nivel de complejidad y estabilidad. Se ha observado que sólo una pequeña fracción de la comunidad microbiana es capaz de degradar las formas más complejas de carbono, pero esta fracción microbiana es fundamental para el inicio del ciclo del carbono en suelos semiáridos y, por tanto, para la sostenibilidad de estas áreas. Además, las entradas de materia orgánica al suelo mejoran la calidad de los suelos semiáridos a largo plazo, aumentando el contenido en carbono orgánico en los mismos, favoreciendo el crecimiento de la biomasa microbiana y variando la estructura funcional de la comunidad microbiana, como una adaptación ecológica a la presencia de nuevos metabolitos en el suelo derivados de dichas materias orgánicas. Por otro lado, la adición de moléculas compuestas únicamente por carbono, no sólo ejerce un efecto sobre el ciclo del carbono si no que también influye sobre otros ciclos biogeoquímicos como el del nitrógeno o el fósforo como hemos podido ver por los cambios producidos en la actividad enzimática relacionada con estos elementos. Este hecho implica que estos ciclos no pueden considerarse independientes si no que actúan de forma conjunta para condicionar la calidad y sostenibilidad de un suelo.

SUMMARY

Soil plays a highly important role in sustaining life on earth. Among its fundamental functions, soil is a source of water, nutrients and air for plants. It is also the natural habitat for thousands of organisms and is an important backdrop for biogeochemical cycling. Soil has the ability to mechanically retain substances or to fix them by adsorption. It is also able to act as a shock absorber. Both characteristics are highly dependent on the content of organic matter in the soil and the texture.

Soil desertification is a widespread problem around the world and negatively impacts on soil functions, yet it is particularly acute in arid and semiarid areas. In Spain, this phenomenon is of particular significance. Indeed, the entire southern half of the country falls within the categories of arid, semiarid and dry sub-humid lands, which are highly susceptible to degradation processes like desertification. The following Spanish Autonomous Communities are those most affected by desertification: Murcia, Valencia and the Canary Islands, where the threat affects almost 100% of the territory. The different forms of soil degradation resulting from desertification may include: i) the loss of nutrients due to the overexploitation of agricultural land; ii) the loss of fertile soil due to erosion from wind and water, and, especially, due to vegetation loss; iii) landslides caused by the action of water and the effects of vegetation loss; iv) increased salinity and acidification in the soil due to inadequate irrigation; and v) the contamination of the soil and water due to excessive use of chemical fertilisers.

The decline in agricultural productivity in soils in semiarid areas is closely related to the loss of ground cover resulting from low rainfall, the soil lithology and human-induced degradation. This loss of vegetation most notably leads to a significant loss of organic matter, and therefore of organic carbon, which negatively affects the quality and sustainability of the soil. In these conditions, the activity of the microbial community is limited by the scarce nutrients and hence there is a negative impact on soil sustainability.

In such degraded soils, it is of vital importance to restore levels of soil organic carbon to sufficient thresholds to maintain its basic functions. For this reason, there has been an ongoing search for effective methods to improve soil quality in semiarid and arid areas in recent decades. Soil restoration by organic amendments can affect carbon balances, increase soil carbon stocks and contribute to the fight against desertification in many arid and semiarid regions.

The aim of this Doctoral Dissertation is to understand the carbon cycling of semiarid soils showing a high level of degradation and the development of strategies for soil

restoration. To reach this goal we have focused on the following topics in particular: the ability of this type of soil to respond to exogenous sources of carbon derived from plants; the role of the microbial community in the degradation of these carbon sources; and the link between these carbon sources and the biogeochemical cycling of the important elements (C, N and P), with a particular focus on carbon as a key element in the sustainability of these ecosystems. The general working Hypothesis of this Doctoral Thesis is that the microbial communities in arid and semiarid soils have a limited capacity for processing carbonaceous substrates as a result of the low content of organic matter usually found in these soils.

This Doctoral Thesis was carried out in the Region of Murcia (south-east Spain) under semiarid climate where the soils are well-adapted to harsh conditions. This zone has an average annual temperature of about 18°C and an annual rainfall of less than 300 mm. The soils are in an advanced state of degradation, which typically means they have a low content of organic matter, minimal ground cover and low microbial biomass. Furthermore, the soils used had been abandoned after being used for agricultural purposes.

As we mentioned earlier, there is a significant lack of knowledge about the dynamics of the carbon cycle in semiarid soils and the role of microorganisms in this cycle. In this Doctoral Thesis we conducted experiments in both controlled laboratory and field conditions in order to contribute to knowledge of this issue. The goal of these experiments was to obtain complete information concerning the dynamics of organic carbon and plant-derived organic amendments with respect to the microbial populations associated with processing of this organic matter.

The first part of the thesis (Chapters 1, 2 and 3) was carried out in controlled laboratory conditions using three molecules of varying complexity: glucose, cellulose and lignin. These molecules are an essential part of the natural plant inputs to soil. We used glucose, cellulose and lignin molecules enriched with ^{13}C (a stable isotope of carbon) in order to track the dynamics of these organic materials and the microbial populations involved in their processing in high resolution. The first experiment (Chapter 1) consisted of adding a low complexity molecule (glucose) to a semiarid soil in two different doses (75 and 300 μg C g^{-1}) followed by incubation at 28ºC and at 60% of the soil water retention capacity for two months. In the second experiment (Chapter 2), more complex molecules (cellulose and lignin) were added in a dose of 75 μg C g^{-1} to two semiarid soils with different levels of degradation and different textures. The

experiment was conducted in the same laboratory conditions as the previous experiment and lasted for four months. The first soil used was collected from the municipality of Abanilla in the Region of Murcia (SE Spain). The composition of this soil is as follows: 34.6% clay, 7.7% silt and 57.7% sand. This soil has a pH of 7.8 and an electrical conductivity of 2.6 dS m^{-1}. The second soil was collected from the municipality of Santomera, also in the Region of Murcia (SE Spain). The composition of this soil is as follows: 18.8% clay, 9.5% silt and 71.7% sand. Santomera soil has a pH of 7.7 and an electrical conductivity of 0.3 dS m^{-1}.

In these two experiments we applied, among other techniques, those based on the analysis of the isotopic composition of CO_2, water-soluble fractions of C and of humic substances and the isotopic composition of fatty acids (PLFA-SIP). These techniques made it possible to thoroughly track the C cycle, evaluating the persistence and mineralisation of C in addition to the microbial populations associated with the dynamics of the added molecules.

Subsequently, in Chapter 3, we sought to better understand the C cycle from a biochemical perspective, observing the effect of the addition of cellulose and lignin on the activity and functional structure of the microbial community through various enzyme activities and physiological profiles on microplates (Biolog). Thanks to these experiments we were able to conclude that, while the addition of low complexity molecules caused an increase in microbial biomass, the addition of complex molecules did not significantly stimulate microbial growth. Nevertheless, the complex molecules (cellulose and lignin) induced microbial biomass recycling and/or storage of the carbon derived from the molecules in the microbial biomass. Neither lignin nor cellulose was completely mineralised after being added to the soil. To the contrary, both showed a level of persistence in the soil proportional to the chemical complexity of the molecule. The permanence of these molecules in the soil indicated that they are capable of increasing the amount of soil organic carbon, either by physical or chemical entrapment in the soil or via their conversion into humic substances. Furthermore, the texture and level of degradation of the soil considerably influenced the response time of the microbial community to the addition of plant-derived organic molecules, the mineralisation of these molecules, and the different microbial groups contributing to breaking the molecules down.

Among the conclusions of these Chapters (1, 2 and 3) it is important to note that semiarid soils do not lose their potential to respond to organic input, regardless of the

complexity of the carbon compound, despite not having received organic matter for a long period of time. Furthermore, this potential to respond to organic input increases with the level of degradation of the soil. However, although semiarid soils respond quickly to the addition of complex molecules, the response of the microbial comunity to these substrates does not have a long-term effect at the enzyme level. Nevertheless, there is a long-term effect on the functional structure of the microbial community.

Once we had acquired a detailed fundamental and comprehensive knowledge regarding the cycling of carbon in controlled conditions through the use of innovative technologies, we evaluated the long-term effect of adding organic amendments to the soil in the field, which represents a real setting for the study of soil restoration (Chapters 4 and 5).

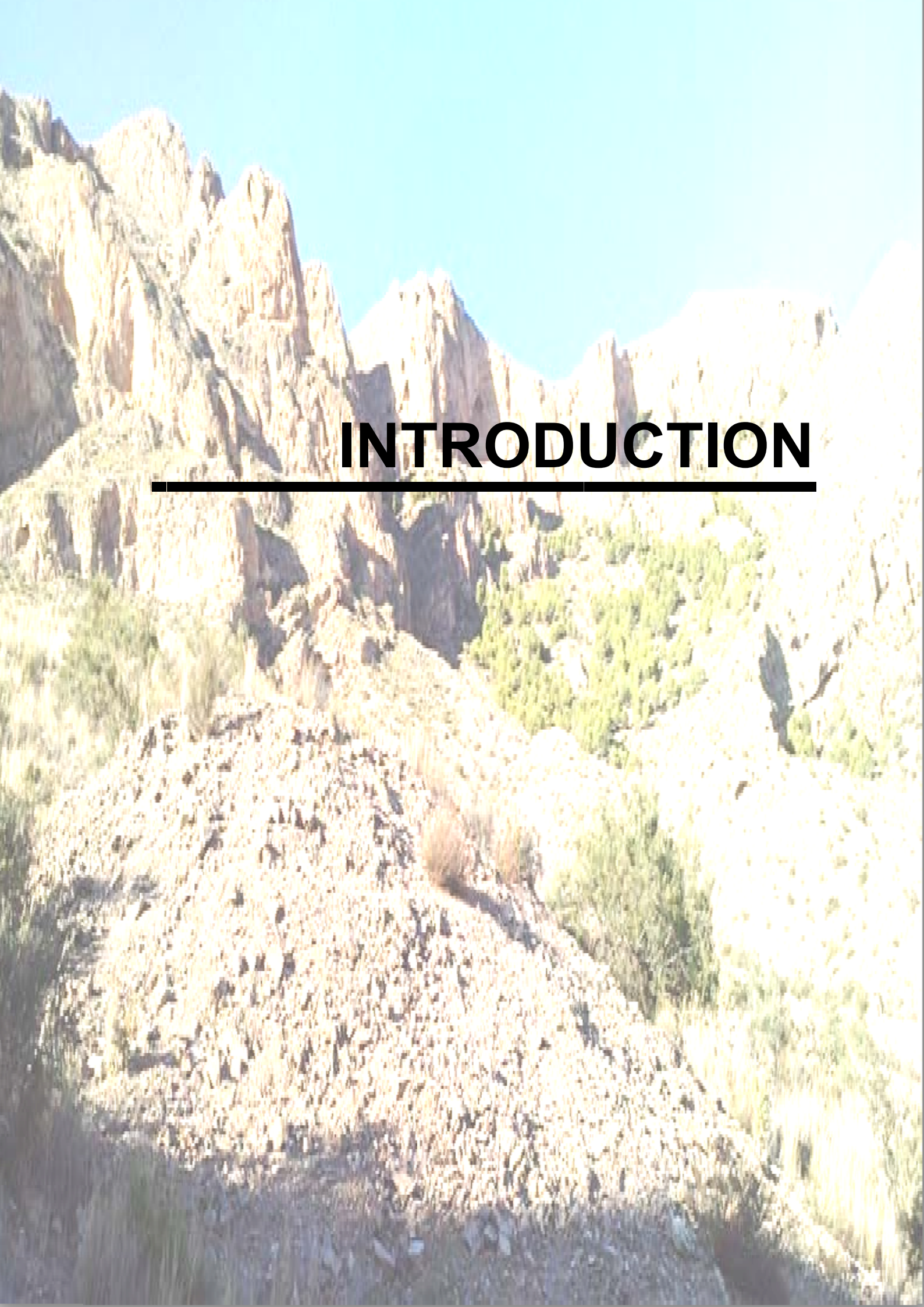
In Chapter 4**,** we evaluated the long-term effects of plant-derived organic amendments and of the applied dose on the chemical, biochemical and microbiological properties of a semiarid soil. For this purpose, a study was conducted in plots amended 5 years earlier with pruning waste at different levels of stabilisation (fresh and composted) and at different doses (150 and 300 t ha^{-1}). Different techniques were used to measure the changes in microbial activity after the addition of organic matter. Furthermore, phospholipid fatty acid analysis and the microbial community-level physiological profile were used to study changes in the biomass and the structure of the microbial community. As a result, we were able to observe that the addition of pruning waste, particularly fresh, caused an increase in microbial biomass and also had an effect on the activity, structure and functional structure of the microbial community in the soil with respect to a control soil to which no amendments were added. Regarding the stabilisation of pruning wastes, we can conclude that the addition of pruning waste to semiarid soils has a beneficial effect and that it is not necessary to compost the waste to improve the microbiological quality of the soil at long-term.

Regarding the amount of organic matter added to the soil, the dose applied had no noticeable long-term effect on microbial activity due to the existence of a threshold dose. In fact, it can be concluded from this study that too high a dose of an organic amendment can actually lead to the inhibition of the enzyme activities. Therefore, taking enzyme activity into account, the optimal dose of pruning waste to be added to semiarid soils would be 150 t ha^{-1}. Nevertheless, the dose did have a noticeable effect on the functional structure of the microbial community in the soil, which was found to be very different depending on the amount of organic matter added 5 years earlier.

Finally, in the Chapter 5, we studied the long-term (24 years) effect of organic amendments on the enzymes involved in the carbon cycle in semiarid conditions. In this chapter, we also evaluated the influence of organic amendments on microbial biomass and community structure. Furthermore, we aimed to complete our knowledge of the carbon cycle in semiarid soils by studying the diversity of isoenzymes exhibiting cellulase and β-glucosidase activity. For this purpose, a soil was used that had been amended 24 years before with the organic fraction of household waste in two doses. The doses applied were chosen so that the organic carbon content of the soil would initially increase by 0.5 and 1.5% with respect to the total organic carbon content. Zymography was used in this experiment for studying the diversity of isoforms of cellulase and β-glucosidase. Zymography is based on extracting soil proteins, separating them by gel electrophoresis and then observing them in gel by using a specific stain for each enzyme. Besides this technique, we also used phospholipid fatty acid analysis to study changes caused by the amendments in the biomass and the structure of the microbial community. As a result of this study we were able to observe that both the enzyme activities and the microbial biomass increased in the long term with the addition of organic amendments. Furthermore the number of isozymes increased as a function of the amount of organic matter added. Nevertheless, as we describe in Chapter 4, the increase in enzyme activity was not proportional to the dose applied.

Taking a **global picture of this Doctoral Thesis**, we have reached the ***Conclusion*** that semiarid soils, despite their advanced state of degradation due to frequently minimal ground cover (resulting from climatic conditions and frequent agricultural abandonment) and the fact they have not received much organic C input, nevertheless have a high level of biotic availability for handling plant-derived organic matter of different levels of complexity and stability. We observed that only a small fraction of the microbial community is able to degrade the more complex forms of carbon, but this microbial fraction is crucial for starting the carbon cycle in semiarid soils and therefore for the sustainability of semiarid areas. Moreover, adding organic matter to the soil improves the quality of semiarid soils in the long term. Indeed, organic amendments increase the content of organic carbon in such soils at long-term, favour the growth of the microbial biomass and change the functional structure of the microbial community as an ecological adaptation to the presence of new soil metabolites derived from the organic matter added. Furthermore, the addition of molecules composed solely of carbon not only has an effect on the carbon cycle but also influences other

biogeochemical cycles such as the nitrogen and phosphorus cycles, which we were able to observe through changes in the enzyme activity related to these elements. This implies that these cycles cannot be considered as independent from one another but that they in fact act together to determine the quality and sustainability of a soil.



INTRODUCTION

1- THE SOIL: FUNCTIONS AND QUALITY

Soil is a non-renewable resource on a human timescale, which means that we must preserve it and, if possible, improve its quality and production capacity by applying measures to prevent degradation and desertification. Soil performs essential functions on the planet including the following: i) it provides a suitable environment for plant growth, providing a physical support and serving as a reservoir of moisture and essential nutrients; ii) it regulates water flows; and iii) it acts as a system capable of reducing the harmful effects of contaminants via physical, chemical and biological processes.

In recent decades efforts have been made to increase food production in order to alleviate existing needs in our society. Rapid population growth and increased demand due to the standards of contemporary life have driven this increase in production. Soil plays a key role in food production, but if we are not careful and do not use sustainable production methods, we can risk the sustainability of the soil itself. In fact, when we over-exploit our natural resources, in part because of increasing poverty, this results in unsustainable ecological damage, thus jeopardising the future for the sake of unrestrained exploitation in the present.

Soil is a natural resource that performs crucial functions in the environment. Indeed, its role is essential in both the survival of the ecosystems we live in and in the development of human activities. The soil is considered a fertile layer ranging in thickness from a few centimetres to several metres that serves as a support for stable vegetation. Soil formation is a very slow process that varies from region to region and according to the specific ecosystem. The length of this process depends of course on factors such as the type of soil in question, the climate conditions, and the topography, temperature and humidity. Nevertheless, while it takes a very long time for soils to form, the degradation of soils due to actions that in most cases are produced by humans (changes in soil use, processes of pollution, etc.) is unfortunately a much faster process.

There are many definitions of soil in the literature, but we believe that the most adequate is that of Doran et al. (1999), according to whom “soil is a non-renewable and dynamic living resource, whose status and functioning is critical for food production and the maintenance of environmental quality at the local, regional and global levels”. Furthermore, soil is not only important because it is the basis for agriculture and for different ecosystems, but also because all life on the planet depends on it. Because of the functions performed by the soil, it is a crucial natural resource for both the

environment and the development of human activities (Blum and Santelises, 1994; Larson and Pierce, 1994; Soil Society of America, 1995; Warkentin, 1995; Doran and Safley, 1997; Blum, 2005). A healthy soil should therefore be able to carry out the following functions:

i) Ecological functions

- Provide physical support for plant growth, giving plants the water, air and nutrients they need to grow.
- Be able to filter, absorb and transform organic matter to protect the environment and provide water of adequate quality.
- Be the habitat and gene pool for plants, animals and organisms that need to be protected from extinction.
- Serve as the main factor in controlling the flow of water in the hydrological cycle and the flow of chemical species in biogeochemical cycles.

ii) Functions linked to human activities

- Provide the physical environment that supports technical and industrial structures and socio-economic activities such as housing, industrial development, transport systems, etc.
- Serve as a source of raw materials: water, clay, sand, gravel, minerals, etc.
- Be the main support for agriculture and therefore a motor for the economies of predominantly agricultural regions.
- Form part of cultural heritage in that the soil contains significant archaeological and paleontological remains that preserve the history of the earth and humanity.

Given all of the functions carried out by the soil, this natural resource clearly plays a fundamental role in the overall balance of the earth. Furthermore, soil makes life possible on this planet, allowing plants to grow by providing them with anchorage, water and nutrients. Nevertheless, several harmful human activities coupled with negative environmental factors like the climate have been contributing to the gradual degradation of the soil in many regions of the planet. Soils are resilient, however, and they are able to adapt to and diminish the impact of a range of stresses. This means that despite the fact that soils have lost certain functions, they have nevertheless been able to maintain an acceptable level of qualities. In fact, the slow rate at which soils have lost their functions has led to a certain amount of “carelessness” on the part of the

institutions that should be ensuring the conservation of our natural resources, and even on the part of some soil scientists (Ochoa et al., 2007). However, due to global change and human factors resulting from population growth, pressures on the soil are likely to become even more pronounced in the coming years. Hence, there is a need to estimate sensitively the quality of soils.

The quality of the soil can be understood as its capacity to maintain its biological productivity and environmental quality, while at the same time promoting the health of animals, plants and humans themselves (Doran and Parkin, 1994). Due to the fact that the soil includes a multitude of biological and geochemical processes with a high level of spatial and temporal heterogeneity, there are three types of indicators for quantifying soil quality (Syers et al., 1995): chemical, physical and biological.

Different authors agree that biochemical and biological properties are best for estimating soil quality due to their high sensitivity to changes that occur in the soil (Dick and Tabatabai, 1992; Visser and Parkinson, 1992; Bastida et al., 2008c; Zornoza et al., 2015). Among these indicators, the enzyme activities related to the cycling of soil nutrients such as N, P, C and S stand out in particular. These enzyme activities provide us with information on the microbiological status of the soil and its physicochemical properties and quickly reflect changes in soil quality (Aon and Colaneri, 2001).

2. SOIL ORGANIC MATTER AND ORGANIC CARBON

Soil is the largest pool of organic C in the biosphere, storing more C than plants and the atmosphere combined. Moreover, this carbon in the soil can be found in both its organic form (the main type discussed in this Doctoral Dissertation) and inorganic form (Allison, 1965; Jobbagy and Jackson, 2000). Both types of carbon should be considered when estimating the carbon pools and fluxes in the soil. This is because, despite the importance of organic carbon (as discussed below), the interactions between atmospheric CO_2 and the inorganic components of carbon in the soil, through the decomposition, precipitation and dissolution of carbonates, are also important regulators of atmospheric CO_2 in the very long term (Berner et al., 1983).

In arid and semiarid areas, the amount of inorganic carbon stored in the soil is approximately two to ten times greater than the amount of stored organic carbon (Eswaran et al., 2000; Batjes, 2004). Furthermore, the accumulation ratio of inorganic carbon is generally higher than that of organic carbon (Landi et al., 2003). Inorganic carbon can be classified as lithogenic or pedogenic (West et al., 1988). Lithogenic carbonate is inherited from the parent material of the soil without changes in content.

Pedogenic carbonate, on the other hand, results from the dissolution and precipitation of both carbonate ions originating from plant roots and microbial respiration and of calcium and magnesium ions produced by erosion.

Despite the large amount of inorganic carbon present in the soil, it is the soil organic matter that is considered to be the fundamental component in the sustainability and proper functioning of the ecosystem-soil. This is because the processes of degradation and mineralisation of organic matter that occur in the soil play an important role in maintaining the balance between the production and consumption of CO_2 from the biosphere, both by recycling the atmospheric CO_2 and by forming organic matter, which is not only a reservoir of atmospheric carbon but also of other elements. The essential steps in the global cycles of water, C, N, P and S, occur in the soil. In fact, the speed of the cycles and the availability of these elements are continually being altered by different forms of life and the constant search for sources of food and energy (Doran et al., 1999) as well as by human actions and climate change.

The organic matter in the soil mostly comes from animal waste (carcasses and excrement) and plant remains (roots, aerial organs, excretions at the rhizosphere level, soluble substances from the aerial organs transferred to the soil by rain water or dew, etc.) in different stages of decomposition as well as from the microbial biomass. Of these sources, plant remains contribute the greatest amount of organic matter to the soil. Organic matter is the bottleneck in the sustainability of the soil-plant system, and it thus plays an essential role in soil functions, determining soil quality, water-holding capacity and susceptibility to degradation (Giller and Cadisch, 1997; Albiach et al., 2001; Feller et al., 2001). Moreover, organic matter fosters soil biological activity and is essential for the development of life in the soil (Aguilera et al., 1999). This type of matter provides energy resources to the organisms living in the soil, which are mostly heterotrophic, in the form of labile carbon (carbohydrates or low molecular weight organic compounds) (Borie et al., 1999). These organisms, in turn, break down the organic matter and actively participate in the cycling of many elements used by plants, aiding in the formation and stabilisation of soil structure and porosity (Singer and Munns, 1996; Krull et al., 2002).

Considering the origin of organic matter in the soil, we can define this matter as “a complex system of substances in a permanent dynamic state produced in the soil by the addition of organic waste, mainly of plant origin, and to a lesser extent of animal origin, in different stages of decomposition and evolution” (Moreno, 1997). Organic matter also contains living organisms, the biota, which are involved in the breakdown

and transformation of organic waste (Aguilera, 2000). Soil organic matter mainly consists of organic carbon, which is found in the form of minimally altered organic waste from plants, animals and microorganisms; in the form of humus; and in very condensed forms similar in composition to elemental carbon (Jackson, 1964) (Fig. 1). In this organic matter we thus find a labile fraction, which is available as an energy source that maintains the chemical characteristics of its source material (carbohydrates, lignins, proteins, tannins, fatty acids), and a more stable humic fraction, which consists of fulvic acids, humic acids and humin (Galantini, 2002; Aguilera, 2000) (Fig. 1).

Humic substances, or humus, are the main components of soil organic matter, making up at least 50% of the total. In fact, humus is the most abundant organic material in the terrestrial environment (Dickinson, 1974; Hayes and Clapp, 2001; Simpson et al., 2007). Humus can be defined as a fraction of heterogeneous organic matter with a high molecular weight that ranges from brown to black in colour and is formed through a series of secondary synthesis reactions (Stevenson, 1982). Within the humic fraction we can find humic acids, fulvic acids and humin. The organic carbon that is retained in humic substances is not as bioavailable for microorganisms and soil enzymes as the labile carbon (Kelleher and Simpson, 2006; Bachmann et al., 2008). This type of stabilisation of organic carbon depends on the soil type, vegetation, soil management practices and environmental conditions (Jastrow et al., 2007).

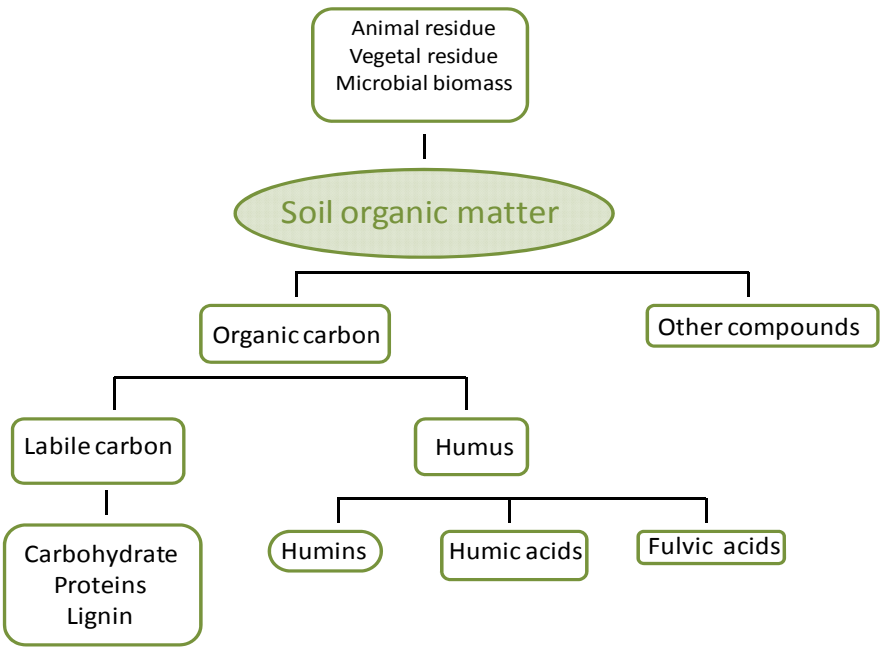


Fig. 1. Organic matter composition

Organic matter affects most of the physical, chemical and biological properties of the soil related to the soil's quality, sustainability, and productive capacity. Soil management practices should thus either maintain or increase levels of soil organic matter (Bauer and Black, 1994; Carter, 2002; Wander et al., 2002; Acevedo and Martínez, 2003; Sánchez et al., 2004). According to Graetz (1997), the following soil properties are affected by organic matter:

i) Physical properties: Organic matter forms aggregates and provides structural stability by combining with clay and forming the exchange complex. This favours the penetration and retention of water, reducing erosion and promoting gas exchange.

ii) Chemical properties: Organic matter increases the exchange capacity of the soil, the store of nutrients for plant life and the buffer capacity of the soil, which enhances the effect of mineral fertilisers and facilitates the absorption of these fertilisers through the cell membrane of the roots.

iii) Biological properties: Organic matter encourages mineralisation processes and the development of ground cover plants, provides food for microorganisms and stimulates plant growth in a balanced ecological system.

Changes in soil organic matter are very slow, and it takes many years to observe considerable variations. Nevertheless, there is evidence that the biological properties of a soil have the potential to be sensitive indicators of ecological stress or ecosystem restoration (Dick and Tabatabai, 1993; Rice et al., 1996; Bastida et al., 2006b; Bastida et al., 2008b,c; Zornoza et al., 2015).

2.1. Dynamic of soil organic carbon

As mentioned above, organic carbon is the main component of soil organic matter. Organic carbon is initially delivered to the soil by the fixation of CO_2 via photosynthesis by plants and certain microorganisms. In this process, carbon dioxide and water react to form carbohydrates and simultaneously release oxygen, which passes to the atmosphere. A portion of the carbohydrates are consumed directly to provide the plant and microorganisms with energy, and the carbon dioxide formed in the process is released through the leaves or roots. When the plant dies, its organic matter is incorporated into the soil. The activity of the soil fauna, especially of the microbial communities, metabolises or mineralises some of these substrates and transforms others into more resistant organic compounds (humus). These two processes occur in parallel, with one or the other dominating depending on the soil characteristics and the

nature of the plant litter provided. Organic matter thus includes organic compounds in various stages of decomposition, from fresh plant litter and microbial cells to humic materials stabilised in mineral surfaces. Soil organic matter is principally lost through mineralisation (decomposition into simple inorganic products: CO_2, NH_3, H_20, etc.), although leaching and erosion also have a significant long-term impact in some areas (Trumbore and Torn, 2003). Decomposition into CO_2 can be produced by both soil microorganisms (heterotrophic respiration) and plant roots (autotrophic respiration). Furthermore mineralisation can occur in both fresh organic matter, considered as labile carbon that microorganisms can easily break down, and in humified organic matter, where the mineralisation process is much slower (Fig. 2).

The role of microorganisms is crucial in the transformation of organic matter, both in the processes of mineralisation and humification. Microorganisms need carbon as an energy source (they oxidise it and return it to the atmosphere as CO_2), and they take the carbon they need from the remains of plants and microbial material. Furthermore, changes in the dynamics of organic carbon in the soil are closely related to changes in microbial activities (Pascual et al., 1997; Marcote et al., 2001; Xue and Huang, 2013). Humification is the process of transformation of organic matter into humus and is largely responsible for the accumulation of organic matter in the soil (Fig. 2).

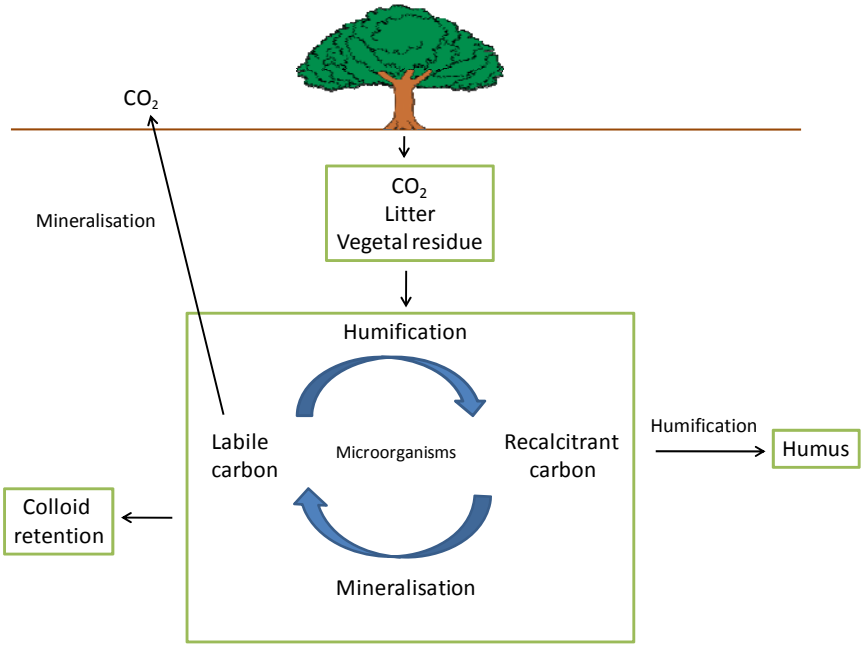


Fig. 2. Dynamics of carbon in the soil

As noted earlier, vegetation controls the storage of carbon in the soil in two ways. Firstly, the amount of vegetation in a soil determines the amount of carbon input. Secondly, the vegetation controls the structure and complexity of the organic matter added to the soil (Grayston and Prescott, 2005). This means that in different soils with the same parent material, the same development time and the same climatic conditions, the amount of carbon stored in the soil will vary depending on the type of vegetation (Izquierdo et al., 2005). The decomposition of organic matter is also known to be affected by climate, the quality of the carbon substrates provided and the physical environment. Moreover, organic matter is a complex mixture of compounds that decompose on very different time scales. For this reason it is very important to study the relative decomposition of organic substrates to understand the carbon dynamics.

The quality of the carbon in soil organic matter is very important because it provides the energy for microorganism growth and activity (Fontaine et al., 2003). It thus seems clear that adding carbon via different types of organic amendments is necessary in order to maintain or increase the amount of carbon in the soil, especially in arid and semiarid areas. Even with such additions, however, the organic matter content in the soil changes very slowly (Campbell et al., 1991).

3. THE SOIL MICROBIAL COMMUNITY

The living part of the soil is largely made up of microorganisms, which are responsible for the dynamics and transformation of soil organic matter. Microbial populations are thus an integral part of a framework of interactions that affect plant development (by transforming compounds into elements that can be assimilated by the roots) and soil quality. As a result, soil microorganisms thus ensure the stability and productivity of both agricultural systems and natural ecosystems (Bastida et al., 2008c). The microorganisms in the rhizosphere contribute to plant growth by increasing the availability of limiting nutrients such as phosphorus and nitrogen. In turn, the composition and activity of the bacterial community is strongly influenced by the type of vegetation in the soil (Thomson et al., 2010; Semmartin et al., 2010; Lucas-Borja et al., 2012).

Table 1 presents some of the most important functions performed by the microorganisms in the soil (Hendrix et al., 1990; Kennedy and Papendick. 1995; Paul and Clark, 1996).

Table 1. Soil microorganism functions

Concept	Function
Nutrient availability	Biological nitrogen fixation Association with forming mycorrhizae fungi Production of organic chelates Redox reactions Waste decomposition Phosphate solubilisation Mineralisation of nutrients
Biological control	Control of plant diseases Antagonistic reactions, parasite and pathogen control
Biodegradation of pesticides and contaminants	Reduction of toxic metals Use of pesticides as a source of nutrients Inactivation of pesticides
Aggregate formation	Formation of humus Production of substances that cement particles Production of hyphae that join particles

The soil microbial community can be studied from different points of view (Fig. 3). Firstly, it is essential to know how the microorganisms operate using various indicators of microbial activity. Secondly, it is important to study the "size" of the microbial community, i.e., the microbial biomass. Thirdly, it is not only important to focus on the microbial biomass, but also on indicators related to the structure and microbial composition of the community (Fig. 3). This makes it possible to know which microbial groups are more closely related to a particular process (such as soil restoration). Each of these perspectives is described in detail in the following sections.

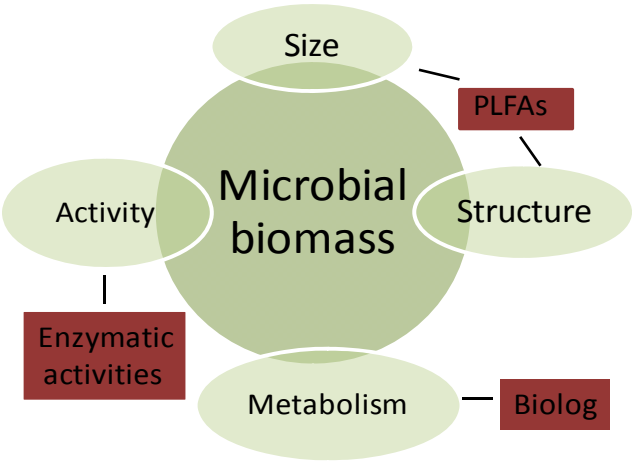


Fig. 3. Ways of studying soil microbial communities

3.1. Microbial activity: mineralisation and enzymes

As described above, microorganisms are involved in the mineralisation of soil organic matter. In this process, the microorganisms break down organic matter into simple inorganic compounds that can be used by plants for growth. In addition, mineralisation is key in the overall functioning of the carbon cycle. Enzymes make this process possible. These are proteins whose main role is to catalyse chemical reactions in living systems, acting on specific substrates and transforming them into products necessary for biological cycles (Skujins, 1967). Microorganisms release enzymes into the soil through secretion and cell lysis. These enzymes are necessary for the decomposition of organic matter and nutrient cycling in terrestrial ecosystems (Taylor, 1989; Zhang et al., 2005). Once extracellular enzymes have left the shelter of the cell, they are exposed to an inhospitable environment in that non-biological denaturalisation, adsorption, inactivation, and degradation by proteolytic microorganisms all conspire to damage the enzymes unless they survive by mineral and/or humic association, which makes them more resistant to proteolysis than the free enzymes. Generally, the enzymes immobilised in mineral and/or organic colloids experience an alteration in their status, properties and natures (such as the kinetics, stability and mobility of the enzymes), since they are physically and chemically linked to other surrounding chemical compounds (Ceccanti et al., 1978; Pascual et al., 2002; Nannipieri, 2006). This suggests that the soil can act as a temporary reservoir, and implies that at a given point in time enzyme activity may not be necessarily linked to the overall microbial activity (Alef and Nannipieri, 1995; Joinville et al., 2004; Paul and Clark, 2007, Stursova

and Sinsabaugh, 2008). Furthermore, extracellular enzymes are more resistant than intracellular enzymes since they have undergone structural changes that have given them thermostability, the ability to be active over a wide range of pH values and resistance to proteases.

In 1978, Burns preformed the first review of soil enzymes, compiling their origins, ranks, kinetics, history and interactions, in addition to some methodological approaches. Enzymes are relatively resistant to denaturation processes (Ladd, 1978), so it is therefore difficult to extract them from the soil. As a result, they are studied indirectly by measuring their activity. Traditionally, enzymes have been widely used as bioindicators of soil quality as they contain information on both the status and physicochemical conditions of the soil (Aon and Colaneri, 2001; Bastida et al., 2006a). For instance, enzymes have been used to examine soil microbial functional responses to factors such as increased nutrient deposition (Saiya-Cork et al. 2002; Sinsabaugh et al. 2005); heavy metal contamination (Moreno et al., 1999); organic amendment (Bastida et al., 2008b; Bastida et al., 2007; Pascual et al., 1997; Tejada et al., 2007); soil management (Madejon et al., 2007; Melero et al., 2009; Moreno et al., 2008); plant diversity (Gonzalez-Polo and Austin, 2009; Zhang et al., 2011); agro-ecosystems (Bandick and Dick, 1999; Masciandaro and Ceccanti, 1999; Acosta-Martínez et al., 2008; Moreno et al., 2009); and climate change (Henry, 2012). In short, studying the enzyme activities of the soil microbial community is very important when investigating the biogeochemical cycles. Furthermore, the soil enzymes provide information on the functional role of the soil microbial community.

It has been widely demonstrated that organic amendments increase the enzyme activity in the soil (Martens et al., 1992; Crecchio et al., 2004; Bastida et al., 2008a). Ceccanti and Garcia (1994) indicated that the importance of understanding enzyme activities in soils could mainly be due to the role enzymes play in the breakdown and evolution of organic matter. Furthermore, processes like the mineralisation and humification of organic matter are largely governed by oxidation, reduction and hydrolysis, hence the importance of understanding oxidoreductases and hydrolases (Pascual, 1995). Nevertheless it is important to bear in mind that it is not feasible to determine all of the enzymes present in the soil, considering that more than 500 different enzymes are involved in the C and N cycles alone (Schloter et al., 2003). It is thus necessary to make a selection, limiting analyses to a number that will work at the methodological level, but that will also provide the information necessary to determine the conditions of the soil. The enzymes used in this thesis are described below. They

are mainly related to the carbon cycle (cellulase, β-glucosidase, lipase, polyphenol oxidase, invertase); phosphorus (phosphatase); and nitrogen (urease). We have also studied dehydrogenase and catalase, which are related to the overall activity of the microorganisms in the soil.

Dehydrogenases are linked to the oxidation of organic compounds and are therefore indicators of overall soil microbial activity (Skujins, 1976). These enzymes are generally considered to be substrate-specific, but they nevertheless play an important role in the breakdown of organic matter, especially for bacteria, which are usually involved in the final stages of the consumption and mineralisation of aromatic compounds (Bugg et al., 2011). This is a complex group of intracellular enzymes that have been linked to the microbial biomass (Aon et al., 2001) and can be used as indicators of the redox potential and oxidative capacity of the soil. Dehydrogenase activity normally increases with the addition of organic amendments due to the increased availability of carbon for the soil microorganisms. Furthermore, the activity of this enzyme is indicative of the improved performance and fertility of amended soils (Pascual et al., 2002; Tejada et al., 2006).

Catalase is an intracellular enzyme that is present in all aerobic bacteria and in most facultative anaerobic bacteria. There is no catalase, however, in obligate anaerobic bacteria (Trevors, 1984). This enzyme acts on the toxic H_2O_2 produced by the electron transport chain and various hydroxylation and oxidation reactions, breaking this compound down to form oxygen and water. Catalase was the first enzyme studied in soils (Woods, 1899) and is considered to be an indicator of the overall activity of the soil microorganisms. Furthermore, this enzyme is associated with soil fertility (García and Hernández, 1997).

The enzyme **cellulase** catalyses the hydrolysis of cellulose, which is insoluble and cannot be directly assimilated by microorganisms. This hydrolysis occurs outside the cell. At least three enzymes participate synergistically in the microbial degradation of cellulose. The first two stages involve the participation of enzymes in the cellulase complex: endo-1,4-β-glucanases (EC 3.1.2.4), which hydrolyse the β-1,4 linkage of the cellulose molecule, and exo- β-1,4 glucanases or cellobiohidrolasas (EC 3.1.2.91), which attack the free cellulose chain ends to produce cellobiose. In the third stage, cellobiose is degraded by β-glucosidase, producing glucose. In soil enzymology it is complicated to determine cellulase activity since cellulose is not soluble in water. Carboxymethylcellulose is therefore usually used as a substrate. The level of cellulase activity in the soil is controlled by factors such as the temperature, the quantity and

quality of organic matter available and soil management practices (Doyle et al., 2006; Drewnik, 2006).

The enzyme **invertase** hydrolyses fructofuranosides, producing a β-D-fructofuranose (fructose) molecule and a moiety that recovers a hydroxyl group. The breakdown of sucrose, the most important fructofuranoside in the soil, results in a glucose molecule and a fructose molecule. Invertase can act either inside or outside the cell. Several authors have found a correlation between invertase activity and the carbon and nitrogen content of the soil (Sajjad et al., 2002; Gómez et al., 2006; Aswathy and Vimala, 2013).

The enzyme **β-glucosidase** is involved in the breakdown of cellulose to glucose, as we have previously described, by breaking the β 1-4 glycosidic bond. It has been determined that there is a close relationship between this activity and the availability of carbon in the soil (Stott et al., 2010; Pajares et al., 2011). Moreover, β-glucosidase activity is sensitive to the type of soil management practices (Madejón et al., 2001).

The enzyme **polyphenol oxidase** catalyses the oxidation of phenolic compounds to quinones using oxygen as an electron acceptor and also participates in the formation of humic substances. Furthermore, this enzyme is involved in the transformation of recalcitrant organic compounds such as lignin (Perucci et al., 2000). The behaviour of this enzyme is not clear, although it does not seem to correlate with either the hydrolase activity or the amount of soil organic matter (Sinsabaugh, 2010).

Lipase is a ubiquitous enzyme that is found in virtually all living things. It plays an essential role the digestion, transport and processing of lipids (triglycerides, waxes, fats and oils). Furthermore, it is part of a subclass of esterases involved in the catabolism of fats and oils, breaking and modifying the ester bonds of lipids and their derivatives. The main reaction performed by lipase is the hydrolysis of acylglycerides, i.e., the breakdown of triacylglycerol into diacylglycerol, monoacylglycerol, fatty acids and glycerol. Lipase also participates in the synthesis and transesterification of acylglycerides and phosphoglycerides. Lipase activity can be intracellular or extracellular and is responsible for cell reconstitution and lipoprotein metabolism. These functions are not only essential in the survival of microorganisms, but they also enable microorganisms to maintain their ecological role as decomposer organisms in natural biogeochemical cycles. Moreover, lipase activity is associated with increases in organic matter and hydrocarbons in the soil (Margesin, 2005). It has been reported that the levels of lipase are very low in plant waste, which is reflected after this waste is applied to the soil (Jurado et al., 2014).

Phosphatases make up a large group of enzymes that catalyse the hydrolysis of esters and phosphoric acid anhydrides, transforming them into various forms of inorganic phosphorus that can be assimilated by plants. Phosphatases play a very important role the phosphorus cycle in the soil, which is closely related to plant growth. The activity of this enzyme increases considerably with the addition of organic amendments to the soil (Tejada et al., 2006; Vinhal-Freitas et al., 2010).

The enzyme **urease** catalyses the hydrolysis of urea into ammonia and carbon dioxide and thus regulates the supply of nitrogen to plants. It can be an intracellular or extracellular enzyme. Urease activity also increases with the addition of organic matter to the soil (Tejada et al., 2006; Bastida et al., 2008a,d).

In the following table (Table 2) we can see some of the functions performed by the soil enzymes described above:

Table 2. The functions of some soil enzymes

Hydrolases	
Cellulase	Cellulase (crystalline) → cellulase (amorphous) → cellobiose
β-glucosidase	Cellobiase → glucose + ROH
Phosphatase	Porg → Pinorg
Urease	Urea + H_2O → CO_2 + NH_4^+
Invertase	Sacarose → glucose + fructose
Lipase	Triacylglycerol → fatty acids + glycerol
Oxidoreductases	
Dehydrogenase	$XH_2 + A \rightarrow X + AH_2$
Polyphenoloxidase	Monophenol + ½ O_2 → quinone + H_2O
Catalase	2 H_2O_2 → 2 H_2O + O_2

3.2. Biomass and structure of the soil microbial community

The microbial biomass represents between 1 and 3% of the total soil organic matter (Jenkinson and Ladd, 1981) and is the live component of this matter. Although this percentage is small, the microbial biomass nevertheless is one of the most active components of the soil organic matter, participating in the decomposition of organic matter entering the soil in the form of litter or animal or plant remains. By definition, the

biomass is part of the total organic carbon in the soil (labile fraction) and provides a relatively large amount of the total nutrients in the soil (Insam, 1990).

As described earlier, microorganisms play a key role in soil performance and fertility. Due to the high level of sensitivity of the soil microbial communities and their rapid response to changes in the soil, studying variations in these communities is a valuable tool for recognising early signs of soil disturbance (Anderson, 2003; Bastida et al., 2008, Ritz et al., 2009). Yet it is difficult to study soil microbial communities due to the fact that only 0.3% of the microorganisms can be cultivated (Ammann et al., 1995). Classical studies of microbial diversity based only on the isolation of microorganisms represent only a minority of the real diversity that can be found in the soil (Torsvik et al., 2003). To overcome this obstacle, a number of methods have been developed that do not rely on the cultivation of microorganisms such as phospholipid fatty acid (PLFA) analysis.

The following techniques were used in this Doctoral Thesis to evaluate both the microbial biomass and the structure of the microbial communities in the soil:

i) Phospholipid fatty acid (PLFA) analysis

This technique was first used on soil by Zelles et al. (1992) to study the microbial biomass and community structure in agricultural soils. PLFA analysis is based on the study of phospholipids, a class of lipids in the soil. Phospholipids are found in the membranes of living cells, and they break down rapidly when the cell dies, which means they do not survive for long enough to interact with the soil colloids (Zelles, 1999).

Changes in phospholipid fatty acid (PLFA) profiles are generally associated with variations in the abundance of microbial groups (Gram-positive, Gram-negative, Fungi or Actinobacteria), as some fatty acids are closely associated with these taxonomic groups of microorganisms. These profiles can thus provide information on the types of organisms present in an environmental sample (Frostergard and Bååth 1996; Rinnan and Baath, 2009; Frostegard et al., 2011). Furthermore, the results provide insight into the biomass of the microorganisms in the soil (Börjesson et al., 2012; Bowles et al., 2014). PLFAs are not only an extremely robust technique for studying the soil microbial biomass, but, with proper statistical treatment, they also provide reliable information on the structure of the microbial community. In fact, phospholipid fatty acid analysis is widely used to study the microbial biomass and changes in the soil microbial community after the addition of organic amendments or

any disturbance to the soil (Böhme et al., 2005; Elfstrand et al., 2007; Bastida et al., 2008), aspects that are closely linked to the focus of this Doctoral Thesis.

ii) Community Level Physiological Profiling (CLPP), Biolog

This is a broad-spectrum technique for evaluating the functional structure of the soil microbial community. Microbial community-level physiological profile (CLPP) analysis involves the direct inoculation of environmental samples in microtiter plates (Biolog plates) followed by incubation and spectrometric detection of heterotrophic microbial activity. The plate contains 31 different carbon sources in triplicate and a redox dye that makes it possible to evaluate the affinity of the microbial community for different carbon sources (Insam and Goberna, 2004).

This method was originally developed by Bochner (1989) to identify bacterial strains in medicine, and it was later adapted for use with microorganisms taken from environmental samples (Garland and Mills, 1991). The technique is characterised by its simplicity and rapidity. Nevertheless, it requires extreme care in data acquisition, analysis and interpretation. The carbon sources in the Biolog plates are either substrates obtained from plant root exudates or substrates that have a high discriminatory power among soil communities (Campbell et al., 1997; Hitzl et al., 1997).

To date, this technique has been used in many soil studies that have sought to detect functional changes in soil microbial communities after the addition of different sources of organic matter to the soil (Waldrop et al., 2000; Schutter and Dick, 2001; Gómez et al., 2006; Guenet et al., 2011) and to evaluate the impact of soil degradation processes (Chaer et al., 2009).

4. SOIL DEGRADATION IN ARID AND SEMIARID AREAS

In its natural state, soil is in dynamic equilibrium with its environment following a slow process called pedogenesis. In such a state of maximum evolution, the soil is more or less covered by vegetation that provides it with a gradual amount of organic matter and nutrients, helping it maintain and even improve its structure while also serving as protection against degradative processes such as erosion (Albaladejo et al., 2000; Bastida et al., 2006b). If such conditions are met, soils are able to maintain adequate quality and perform all of their functions properly. This state of equilibrium with the environment, however, may be altered as a result of natural or human disturbances, and such alterations, over time, lead to soil degradation.

Natural disturbances can be of minimal intensity, allowing the soil to adapt to the new conditions. Nevertheless, some natural disturbances can be extremely intense, producing drastic changes. Human activities tend to cause damage more quickly, disturbing the delicate balance between the soil and the environment and producing radical changes in soil properties and processes. A reduced amount of ground cover is one of the first signs of degradation that can be observed in the soil. This symptom is closely related to a shortage of soil organic matter, a loss of physical structure and decreased soil microbial activity (Cerdá et al., 1994; Caravaca et al., 2002; Tejada et al., 2006).

Agriculture produces alterations of different magnitudes in soils depending on the climate, relief and type of human activity. In most cases, human activity has a negative effect, leading to the rapid deterioration of the physical, chemical and biological properties found in the soil before its use for agricultural purposes. This deterioration can be seen in the fact that there are fewer nutrients and water available in the soil for plants. As a result, the crops yield less, and it is even possible for the soil to permanently lose its productive capacity. Removal of ground cover plants by humans greatly increases the risk of erosion by leaving the soil exposed to the wind and rain. Moreover, it is virtually impossible to replenish such eroded soils. We can thus define soil degradation as “an alteration in the equilibrium between the constituents of the soil due to changes in the soil’s physical, chemical, biological or biochemical properties, leading to the loss or diminution of soil fertility, thus decreasing the current or future capacity of the soil to produce goods or services, both in terms of quality and quantity” (González-Quiñones, 2006). A large part of the earth's surface is affected by processes of soil degradation (Barrow, 1994), which are especially pronounced in regions with an arid or semiarid climate. It is precisely these areas that are the focus of this Doctoral Thesis.

4.1. Extension and global importance of arid and semiarid lands

Drylands of the world occupy 6.31 10^9 ha or 47% of the earth’s land area (UNEP 1992) and are distributed among four climates: hyper-arid (1.0 10^9 ha); arid (1.62 10^9 ha); semi-arid (2.37 10^9 ha); and dry sub-humid (1.32 10^9 ha). Arid and semi-arid or sub-humid zones are characterised by low erratic rainfall, periodic droughts and different types of vegetative cover and soils. Interannual rainfall varies from 50-100% in the arid zones of the world with averages of up to 350 mm. In the semi-arid zones, interannual rainfall varies from 20-50% with averages of up to 700 mm.

In semiarid areas like south-east Spain, the degradation process is closely related to ground cover loss as a result of low rainfall in addition to the lithology in some areas. One of the most notable effects of this loss of vegetation is a loss of soil organic matter as a result of carbon mineralisation in the absence of organic matter input. Moreover, the organic matter content in soils is subject to strong and complex physical, chemical, biochemical and biological controls that are ultimately responsible for the stabilisation and mineralisation of carbon (Six et al., 2002; von Lutzow et al., 2006). Any alterations to the state of equilibrium in the soil resulting from land use (such as tillage) (Angers et al., 1993; Kandeler et al., 1999; Connant et al., 2004) and climate pressures may also alter the C stocks in the soil and potentially cause soil degradation. Such alterations thus affect the sustainability of the planet due to the fact that organic matter is a key component in determining the quality and sustainability of the soil. Without organic matter, the soil is thus condemned to a host of degradative processes that can even lead to a more intense and multifaceted state of degradation: desertification. Desertification is defined as "the degradation of land in arid, semiarid and dry sub-humid areas, caused primarily by human activities and climatic variations" (United Nations Convention to Combat Desertification [UNCCD], 1994).

Desertification involves the often irreparable loss of productive soil functions and the alteration of biological and hydrological cycles. Furthermore, this process of land degradation diminishes the level of environmental services provided by ecosystems. In addition to the loss of organic matter, microbiological soil properties are also negatively affected by drought since soil moisture plays a key role in the survival and activity of soil microorganisms (Bottner, 1985; Hueso et al., 2012). The different forms of land degradation resulting from desertification can include (UNCCD 2010): i) the loss of nutritive matter due to agricultural over-exploitation; ii) the loss of topsoil surface due to wind and water erosion, particularly due to the loss of vegetation; iii) landslides caused by the action of water and the effects of vegetation loss; iv) increased salinity and soil acidification due to inadequate irrigation, and; v) soil and water pollution due to excessive use of chemical fertilizers. Furthermore, declines in the agronomic productivity of soils in developing countries can be partly attributed to human-induced soil degradation and the attendant decline in soil quality (Lal, 2006).

The following map (Fig. 4) shows that desertification, the final stage of soil degradation, is not a local phenomenon but that it affects large areas of the planet. In fact, given the current situation of climate change, desertification may even expand in the coming years due to climate change. This is of particular concern in terms of soil organic

matter. Variations in temperature and precipitation may alter both biotic and abiotic factors that control carbon immobilisation in semiarid areas. The positive reaction of the soil microbial community in response to elevated CO_2 concentrations and soil warming can accelerate the microbial decomposition of soil organic matter and potentially lead to soil C losses (Nie et al., 2013). We should thus make a concerted effort to find environmentally sustainable alternatives in order to alleviate this process of extreme degradation.

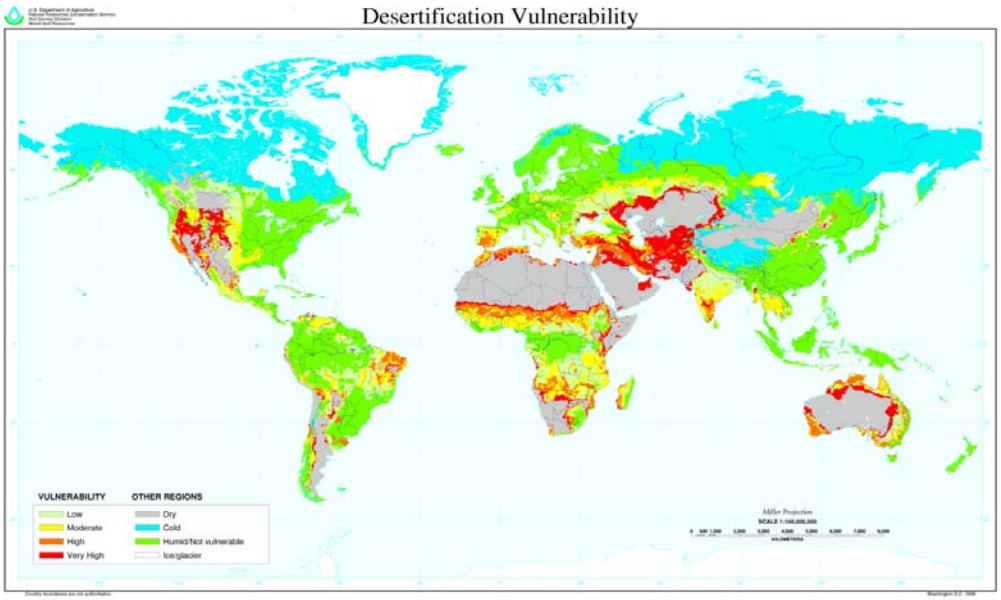


Fig. 4. Desertification vulnerability worldwide

4.2. Soil degradation and organic carbon in arid and semiarid lands

Soil is the largest pool of organic C in the biosphere, storing more C than plants and the atmosphere combined. Furthermore, soil also contains forms of inorganic C such as calcite and dolomite (Allison, 1965; Jobbagy and Jackson, 2000). Although arid and semiarid ecosystems have less vegetation and hence lower carbon accumulation than boreal or tropical areas, the dryland ecosystems cover around 30% of continental areas and are estimated to contain 20% of the global soil C pool (organic plus inorganic) (Rasmussen, 2006). Lal et al. (2004) concluded that the predicted amount of carbon in drylands is between 159 and 191 billion tons with a density of 35-42 (tons C ha^{-1}). If we compare these figures with the values estimated for boreal (247-344 tons C ha^{-1}), tropical (121-113 t tons C ha^{-1}) and tundra (121-127 tons C ha^{-1}) ecosystems, it remains clear that soils in arid and semiarid climates are depleted of carbon, both for “natural” or “anthropogenic reasons”.

Soil organic carbon sequestration is a potential tool in climate change mitigation, but it is finite in both time and duration (Lal, 2004). This soil function for carbon storage is different for each specific soil and depends on climate (Lal, 2009) and on land use (Conant et al., 2004). Arid and semiarid soils have been considered to be the lowest contributors to the global organic carbon stock (Janzen, 2004). The greatest contribution of these kinds of soil is through inorganic carbon in carbonate forms (Díaz-Hernández et al., 2003). The organic carbon concentration in arid and semiarid soils is already low, and degradation processes such as erosion are reducing this concentration even further (Cerda, 1997). However, the organic carbon sequestration potential for these ecosystems has been estimated to be high, through increases in organic matter (Lal, 2009). Increased organic matter in such arid and semiarid systems will provide a dual solution to land degradation and desertification by: i) improving soil quality and ii) enhancing soil organic carbon sequestration.

Spain suffers from a high rate of desertification in more than one third of its land area. In fact, according to the United Nations, Spain has more areas at risk of desertification than any other European country. This is because the entire southern half of Spain falls within the categories of arid, semiarid and dry sub-humid lands and is therefore highly susceptible to this phenomenon. The following Spanish Autonomous Communities are most affected by desertification: Murcia, Valencia and the Canary Islands. In these areas, desertification affects nearly 100% of the land area. One of the main causes of desertification in Spain is the climate: the areas mentioned above receive low annual rainfall (less than 300 mm a year in the case of Murcia), and when it does rain it tends to do so in torrents, falling so heavily that it can easily erode the land. As a result, the vegetation is sparse and thus provides little organic matter to the soil, further aggravating the situation. Once again, as noted earlier, the loss of organic carbon is a principal cause of soil degradation (Albaladejo and Diaz, 1990; García et al., 1992). Figure 5 shows the levels of organic C in the soil throughout Europe. We can see that large parts of Spain (particularly in the south and south-east), Italy, Greece and Cyprus have the lowest levels of organic C in the soil in Europe. Despite the irrefutable impact of the climate, there are also human factors causing soil degradation. Unsustainable agricultural practices are one example of a human action that diminishes the amount of organic matter in the soil, decreasing the soil quality and often leading to land abandonment.

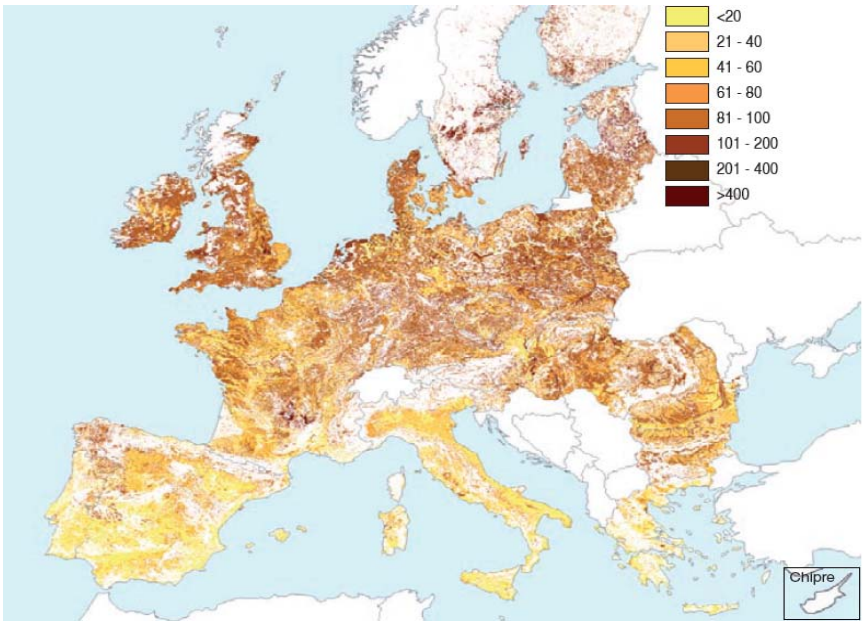


Fig. 5. Levels of organic C (tC ha^{-1}) in European soils.

4.3. Microbial biomass and enzyme activity in arid and semiarid areas

The microbial biomass of semiarid soils is usually restricted by the low amounts of vegetal input and limited water availability. The evaluation of microbial biomass by phospholipid fatty acid (PLFA) analysis is usually quite affordable in soil science and has revealed that total PLFAs range between 2.2-100 nmol fatty acids g^{-1} soil in arid and semiarid areas of the planet (Bastida et al., 2008d; Ben-David et al. 2011; Cotton et al., 2012; Drenovsky et al., 2010; Ford et al., 2007; Hueso et al., 2012; Hortal et al., 2013).

Generally, the level of microbial biomass in the soil correlates well with the amount of organic carbon and is also significantly related to the level of humidity in dryland soils. For instance, different authors have observed changes in microbial biomass linked to organic carbon levels after a change in land use (Entry et al., 2004; Jia et al., 2010). Similarly, the restoration of soil quality by the addition of organic waste byproducts has been observed to increase the microbial biomass by 1.6- to 3 times (Bastida et al., 2008d). Microbial biomass has also been reported to respond to vegetal growth and the parallel increase in soil organic matter (Ben-David et al., 2011; Hortal et al., 2013).

Interestingly, Drenovsky et al. (2010) pointed out that that dry soils had higher amounts of Gram-positive biomass, while pH was not an important feature controlling the biomass. Indeed, these authors showed that soils from dry ecosystems had a very different microbial community than soils from wetter ecosystems in more humid

regions. Deserts had the highest proportions of monounsaturated fatty acids (indicative of Gram-negative bacteria), and this proportion was 4.7-fold greater than that observed in wetlands. However, branched fatty acids displayed the opposite trend, and their amount was lowest in deserts. Interestingly, Hueso et al. (2012) concluded that water stress significantly influenced C and N transformation processes and microbial functionality. Furthermore, changes in community structure caused by drought were less pronounced when soils received organic amendments.

As stated above, microbial biomass is responsible for the production of enzymes that generate organic matter decomposition. These enzymes can be excreted in the extracellular micro-environment and can be protected by immobilisation in humic and clay colloids (Nannipieri, 2006). The presence of a stabilised pool of soil enzymes, particularly oxidative enzymes, can therefore be detected in the soil. The activity of these enzymes is less restricted by moisture than the activity of the decomposers themselves. In arid and semiarid soils, the potential activities of enzymes that decompose proteins (e.g. aminopeptidase) and recalcitrant C compounds such as lignin and humic substances (e.g. phenol oxidases) exceed the potential activities in mesic soils by more than an order of magnitude in both absolute terms and in relation to the activities of enzymes that breakdown cellulose, which generally dominate the EEA of mesic soils (Stursova et al., 2006; Zeglin et al., 2007; Stursova and Sinsabaugh, 2008). Because of carbonate accumulation, the pH of arid soils can reach 8 or above, which is optimal for phenol oxidase enzymes. The optimal pH for glycosidases (e.g. cellulase, chitinase) generally range from 4 to 6 while those of proteolytic and oxidative enzymes generally range from 7 to 9 (Sinsabaugh et al., 2002). Besides, soil texture and moisture determine the rates at which enzymes, substrates and reaction products diffuse.

5. REMEDIATION OF DEGRADED SOILS: THE EFFECTS OF APPLYING ORGANIC AMENDMENTS TO THE SOIL

Since the Kyoto Protocol of 1992, which identified soils as a possible sink of carbon, there has been much progress made in terms of the remediation of degraded soils. The recent Thematic Strategy for Soil Protection, which is a proposal for a new European directive (http://ec.europa.eu/environment/soil/pdf/ com_2006_0231 in pdf), concedes that soil may play a decisive ecological role. In developing this strategy, the European Commission identified the loss of organic matter due to anthropic actions and adverse climate as one of the principal causes of soil degradation, leading to decreased

vegetation and soil productivity (Albaladejo et al., 2000). A report on organic matter and biodiversity within the European Thematic Strategy (Van Camp, 2004) mentions that exogenous organic matter, i.e., organic materials added to a degraded soil in order to improve harvests or restore a degraded soil for subsequent use, constitutes an invaluable source of organic matter 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 elements, is considered to be one of the most effective methods in the fight against erosion and associated degradative processes.

The addition of organic amendments to the soil has many more beneficial effects. For example, it also improves soil fertility and favourably affects the functional capacity of the soil, enhancing soil quality and, as mentioned above, contributing to carbon sequestration in the soil. Applying organic materials to the soil also enhances the soil's nutrient status by serving as a source of macro-and micro-elements, and it also improves the physical properties of the soil by increasing soil porosity and water retention as result of the presence of humic-like substances, known as a polycondensate marcromolecular structure. One of the beneficial effects of such humic substances in the soil is that soil enzymes bound to humic fractions are protected in the long term against denaturation by proteolytic attack. Finally, organic matter addition encourages the development of microbial and biogeochemistry cycles in the soil (Carpenter-Boggs et al., 2000; Marschner et al., 2003; Lee et al., 2004; Bastida et al., 2008a,d) (Fig. 6).

Given these benefits, organic amendments have been widely used to improve soil quality and restore degraded lands (Garcia et al., 1992, Tejada et al., 2006, Bastida et al., 2007). In semiarid soils, the physical, chemical and biochemical properties have been studied in order to follow the evolution of the soil after adding organic amendments. Several studies have also been conducted to study the influence of amendments on aggregation in such semiarid soils (Roldán et al., 1996, Albiach et al., 2001). In such studies, it has been observed that the influence of organic matter on soil properties depends on the content, type and chemical composition of the organic material added. For instance, it is thought that fine soil particles have a critical role in C fixation, and indeed, some authors have observed an increase in carbon fixation in fine particles (clay or silt) after adding organic amendments (Caravaca et al., 2001, García et al., 2012; Nicolás et al., 2012). Moreover, recent studies based on carbon stable-

isotope probing have also suggested a protective role of clays (Hagelson et al., 2014). Organic amendments should be able to provide labile organic matter in quantities that are sufficient to activate the growth of existing soil microbial populations without producing adverse effects. The amendments added can thus reactivate all the processes carried out by the microorganisms in the soil. Materials that can be used as organic amendments to achieve this goal include manure, sludge, compost, agri-food waste, urban organic waste, plant litter, etc. The use of urban organic waste is potentially of great interest. Not only is this a way to take advantage of the high proportion of nutrient-rich organic matter in urban organic waste for improving soil quality, but it is also a rational means to dispose of such waste (Pascual et al., 1997; Crecchio et al., 2004; Bastida et al., 2008a,d). Nevertheless, urban waste contains toxic compounds, and recycling it in the soil can have a negative environmental impact if it is not used properly (Tejada et al., 2007). The negative effects include, most notably, a foul odour, increased heavy metal content, pathogenic microorganisms, salinity and either excess nutrients or nutrient deficiency or demand.

Besides the carbon input from the ground cover development, produced by adding organic amendments to the soil, organic amendments themselves can provide the soil with exogenous carbon that may persist. The stability and nature of the amendment can determine the residence time of the added organic carbon (Kiem and Kögel-Knabner, 2003; Abiven et al., 2009). In drylands ecosystems, due to the high potential for carbon sequestration, the stabilisation of soil organic matter is believed to be more controlled by the quantity of the organic amendment and its interaction with the soil matrix (i.e. texture) than the quality (Hassink et al., 1997; Gentile et al., 2011; Nicolás et al., 2012).

The main effects of organic amendments on soil can be summed up in the following points:

i) Effect on the physico-chemical properties of the soil

Amendments increase the buffer capacity of the soil, keeping the pH stable despite the addition of small amounts of strong acids or bases. Organic amendments have a buffering effect in the soil due to the presence of weakly acidic chemical functional groups in the organic molecules (Garcia-Gil et al., 2004). Furthermore, the cation exchange capacity increases in soils treated with organic materials (Weber et al., 2007). Any amendment that enhances the formation of humus will produce a significant increase in the cation exchange capacity, since this capacity is 3 to 6 times greater in humus than in soil clays (Harada and Inoko, 1975). This property is very

important in the soil as it controls the availability of nutrients for plants, is involved in the development of plant structure and aggregate stability, and allows for the retention of pollutants introduced into the soil (Weber et al., 2007). Some authors have observed that the ability of soil to retain cations significantly increases along with the amount of organic matter added (Díaz, 1992; Lopez-Pineiro et al., 2007).

ii) Effect on the physical properties of the soil

Generally, the addition of organic amendments to the soil has a positive influence on the soil's physical properties (Tejada et al., 2008; Annabi et al., 2011). Due to its low density and tendency to increase pore space in the soil, the organic matter in amendments reduces soil bulk density. Organic matter also has a positive influence on the formation and stability of aggregates in the soil (Albaladejo et al., 2000), both by increasing the cohesion between particles within the aggregates themselves and by increasing aggregate hydrophobicity. This reduces runoff, prevents the leaching of nutrients and improves plant growth (Albiach et al., 2001; García-Orenes et al., 2005; Annabi et al., 2006). Furthermore, the rich organic matter in the amendments, which is colloidal in nature, improves the water balance in the soil by increasing the soil's water retention capacity, thus making it possible for the soil to better withstand periods of drought (Mabuhay et al., 2006, Leon et al., 2006) (Fig. 6).

Ultimately improvements in the physical properties of the soil increase plant growth (which in turn supplies organic waste) and positively influence the microbiology and structure of the soil.

iii) Effect on the chemical properties of the soil

The addition of organic amendments to the soil provides the soil with macro- and micronutrients, such as N, P, K, etc. Yet the increase in these nutrients depends on the characteristics of the soil, the dose of organic material applied, and the method and frequency of application. Furthermore, adding organic amendments to the soil also favours the formation of complexes that retain these nutrients, preventing loss by leaching, thus making them more available to plants (Fig. 6). Adding amendments also influences the electric conductivity and pH of the soil depending on the material added. This soil management practice also increases the amount of total organic carbon and water soluble carbon in the soil, thereby increasing the amount of labile carbon in the soil and favouring the development of microorganisms (Bastida et al., 2008a).

iv) Effect on the microbiological and biochemical properties of the soil

The microbiological and biochemical properties are highly sensitive to changes that occur in the soil. As a result, these properties can be used to evaluate the effects of applying different types and amounts of organic matter on soil characteristics. Microorganisms play a major role in the decomposition of organic matter, nutrient cycling and other chemical transformations in the soil (Murphy et al., 2007). This is why, in general, both the quantity and quality of organic matter added to the soil are key factors in controlling the abundance and activity of the main microbial groups involved in nutrient cycling.

Organic amendments generally lead to an increase in the biomass and activity of the soil microbial community (Ros et al., 2003; Bastida et al., 2008a,d). This in turn results in an increase in the production of enzymes, which are responsible for most of the reactions involved in the processes of mineralisation and immobilisation of nutrients in the soil. The soil enzymes are thus related to the availability of nutrients in the soil for plants and therefore directly influence plant growth (Fig. 6). Many of the soil enzymes are protected from degradation and inactivation by being immobilised by the humic fraction of the added organic matter via the formation of complexes like enzyme-humus complexes (Nannipieri et al., 1990; Ceccanti and García, 1994; Benítez et al., 2005).

It is important to keep in mind that the addition of organic amendments to the soil must be controlled, avoiding the introduction of undesirable compounds like pollutants or heavy metals that would be extremely harmful and could even produce the opposite effect of soil remediation.

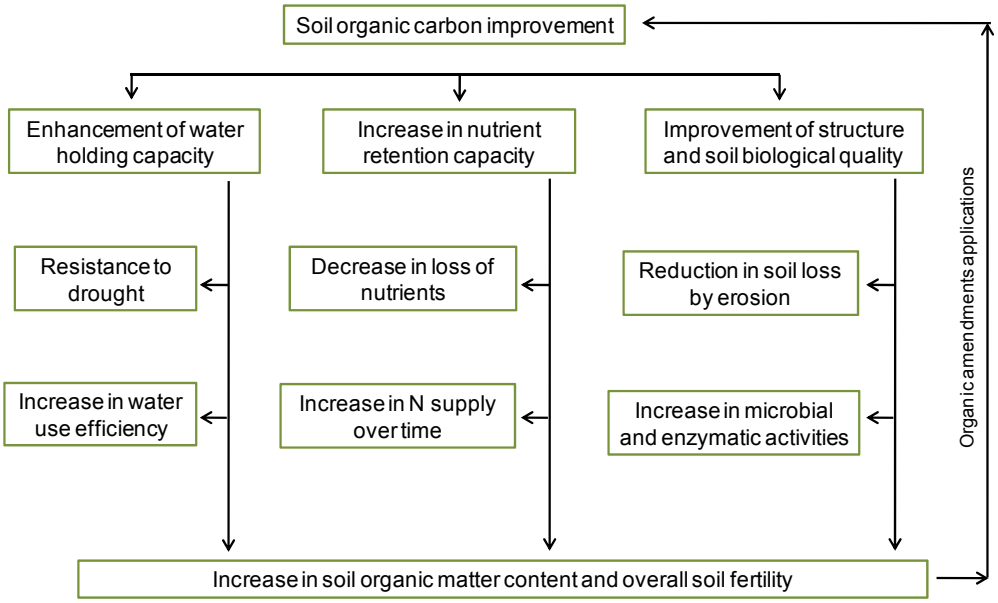


Fig.6. Effects of increasing soil organic matter content and overall soil fertility by soil organic carbon improvement (modification of Diacono 2010)

6. NEW METHODS FOR STUDYING THE SOIL MICROBIAL COMMUNITY AND THE CYCLING OF ORGANIC MATTER

Over time, the methods used for studying changes in the soil have progressed in order to obtain more extensive information concerning the microorganisms responsible for soil processes and, especially, regarding the biogeochemical carbon cycle, which is a fundamental part of this Doctoral Thesis. Some of these methods are briefly described below.

6.1. Isotopic methods

These methods are based on the use of stable isotopic labels such as ^{13}C. This makes it possible to track the carbon in the labelled compounds added to the soil. We can thus analyse the mineralisation of this carbon, its persistence in the soil and the microbial groups involved in breaking it down. Up until now, the application of isotopic methods in microbial ecology in semiarid soils had been virtually unexplored worldwide. In this Doctoral Thesis, isotopic methods, in part based on tandem mass spectrometry platforms, play a key role studying the microbial processes related to the C cycle.

i) PLFA-SIP (Phospholipid Fatty Acid Analysis-Stable Isotope Probing)

Boschker et al. (1998) published the first study using stable isotopic labels. This study identified the microbial groups responsible for the assimilation of carbon from the

greenhouse gas methane in freshwater sediments. When a microorganism grows and divides, the isotopic composition of the biomolecules in the new cells reflects the isotopic composition of the compounds on which the microorganism was grown. Therefore, the carbon atoms of a microorganism grown on ^{13}C-labelled substrates will be enriched in ^{13}C. This makes it possible to identify the microbial groups that have used a particular carbon source.

The PLFA-SIP technique has several advantages for use in evaluating the microbial biomass associated with the assimilation of a substrate: it is highly sensitive; it enables us to estimate the microbial population growth rate and which are the functional populations; and, using Isotope ratio mass spectrometry (IRMS), we can determine the amount of ^{13}C incorporated into each microbial group. In the past decade, the PLFA-SIP technique has been used to study the microbial groups involved in the breakdown of different organic substrates in the soil (Waldrop and Firestone, 2004; Brant et al., 2006; Rinnan and Bäth, 2009). Nevertheless, this technique has hardly been used in Spain and furthermore has rarely been applied in studies on semiarid soils.

ii) Isotopic composition of CO_2 and fractions of C

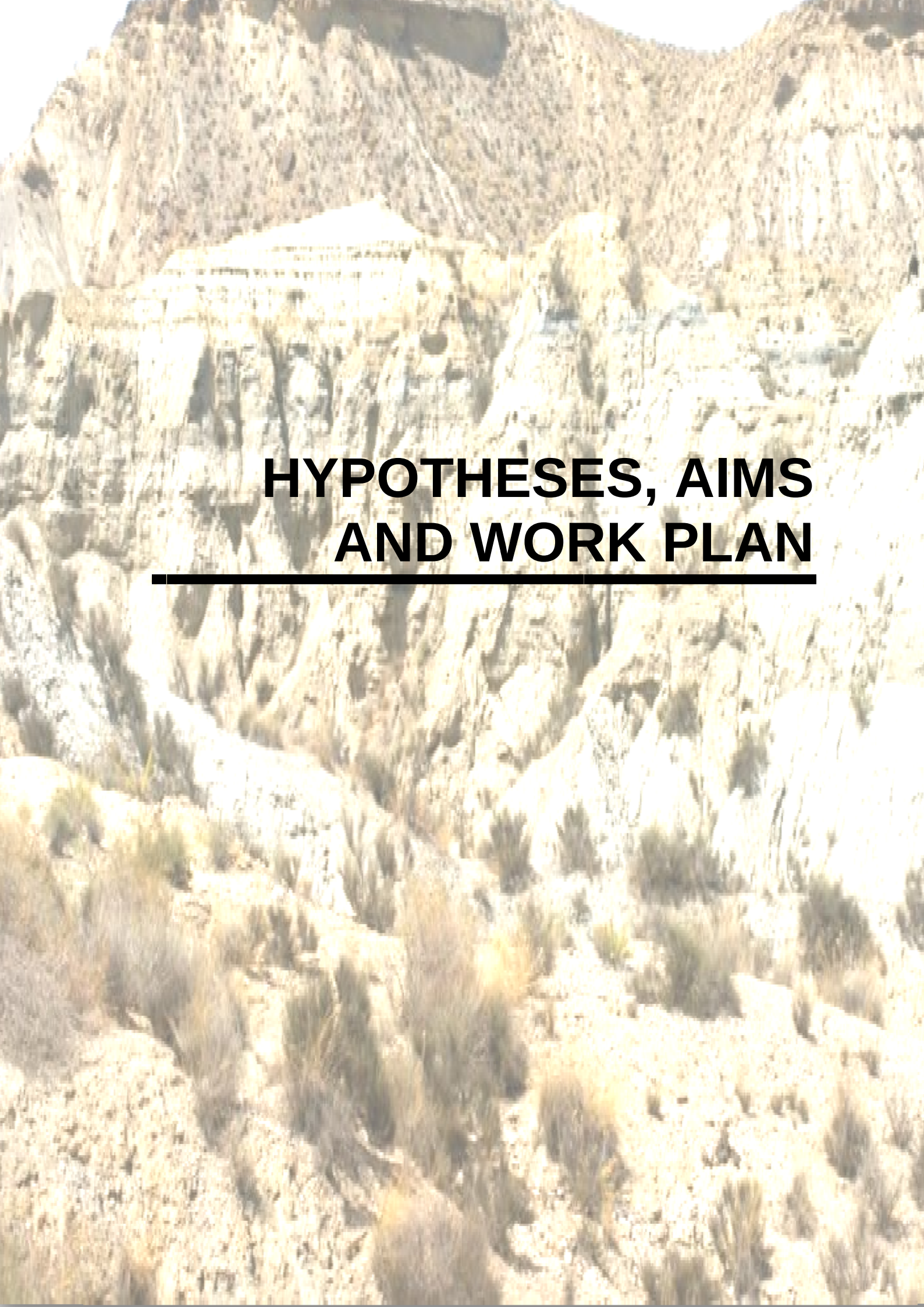
By analysing the carbon isotopic composition of the CO_2 emissions after the addition of ^{13}C-enriched compounds (commercially available), it is possible to determine how much of this carbon has been mineralised by microorganisms. Furthermore, it is also possible to determine the amount of CO_2 emitted from the soil organic matter itself. In this regard, it is worth noting that the addition of “fresh” organic matter sometimes stimulates microbial activity to a great extent, which not only results in the breakdown of a substantial portion of the newly added organic matter but also of the soil’s own organic matter. This process is known as the "priming effect" (Dalenberg and Jagger, 1989), and it is related to the ecological successions and dynamics of the microbial populations that occur in response to carbon molecules (Fontaine et al., 2003; Waldrop et al., 2004; Notthingham et al., 2009).

The analysis of the isotopic composition of the soil C and the humic and water-soluble fractions could provide critical information on the amount of added carbon that is not mineralised by the microbial community yet is preserved in the soil either in humic or soluble form.

6.2. Zymography

To date, the measurement of enzyme activities in the soil has made it feasibly possible estimate the potential of the microbial community with respect to various elements. Nevertheless, a wide variety enzymes of all classes have not yet been properly studied. This Doctoral Thesis, however, goes one step further in enzymatic determination in order to more deeply understand the influence of soil restoration processes on enzymatic diversity.

Zymography is a specific staining technique that makes it possible to observe the diversity of each enzyme. This technique involves extracting enzymes from the soil and separating them on a one-dimensional acrylamide gel. Once the different enzymes or isoforms are separated, the next step is staining. Specific colourimetric or fluorimetric methods are used for each enzyme. Although this technique is used in the basic characterisation of microbial cultures (Dutta et al., 2008; Nazir et al., 2010), it has rarely been applied in soil studies (Khalili et al., 2011; Cañizares et al., 2011).



HYPOTHESES, AIMS AND WORK PLAN

This Doctoral Thesis it is included in the framework of the Spanish Strategy for Science, Technology and Innovation (2013-2020), which emphasises R&D+i focused on the sustainability of soil and forest resources, inland water resources and adaptation to climate change. This Strategy explicitly refers to research on desertification and soil impoverishment. Furthermore, this Doctoral Thesis falls within the scope of European Union initiatives under the Horizon 2020 programme, which includes issues such as environmental protection, ecosystems and adaptation to climate change. The studies carried out and concepts addressed in this Thesis are thus of acute interest from a current political standpoint. Besides, this Doctoral Thesis not only addresses topics related to the possibility of restoring severely degraded arid or semiarid soils by amending these soils with more or less stabilised plant waste, but it also looks into the capacity of such soils to incorporate exogenous carbon derived from plant waste into their organic matter systems. The role played by soil microbial populations in the processes described above is an interesting aspect deeply considered in it.

1. HYPOTHESES

The research in this Doctoral Thesis is based on the following Hypotheses:

i) The level of soil degradation in semiarid environments is very high and mainly depends on the organic matter content in the soil, which is extremely low due to the absence of carbon inputs. Furthermore, this level of soil degradation is directly related to the size of the microbial biomass, and less degraded soils have more microbial biomass. Higher quality soils should have a more substrates available for microbial growth. The level of soil degradation could therefore have an impact on the assimilation and mineralisation of organic matter by the microbial community.

ii) In semiarid and arid soils, the carbon cycle should operate differently than in soils with a lower level of degradation (and of organic matter content). The organic waste that reaches such soils will be processed in different ways, which are highly dependent on the existing microbial populations. The contributions of the different microbial groups that make up the biodiversity of these types of soil in the processes of mineralisation, humification or fixation within colloids of different organic molecules depend on the “quality” or structure of these molecules.

iii) In the process of restoring semiarid soils by applying exogenous organic amendments, the quality and degree of stabilisation of these amendments and the dose applied may influence the effect of these amendments on the biological properties of the soil that are essential for restoration.

2. AIMS

The **Overall Goal** of this Doctoral Thesis is to contribute to knowledge about the ability of highly degraded semiarid soils to assimilate external sources of carbon derived from plant waste. Furthermore, we aim to better understand the role of the microbial community in this assimilation process in semiarid soils with different properties and levels of degradation, in addition to the relationship between the soil microbial community and the biogeochemical cycles of the major elements (C, N and P). The Thesis is particularly focused on carbon as a key element in the sustainability of the ecosystems studied. Indeed, comprehensive knowledge about the carbon cycle in semiarid soils is essential for developing organic management strategies to fight against soil degradation.

To achieve this overall goal, we have established the following **Specific Goals**:

i) To study the fate of the carbon contained in the different plant-derived organic molecules added to the soil. This will be done using isotopic methods that help determine how much plant-derived exogenous organic C is "fixed" in the soil and in its different chemical fractions in addition to what part of the organic C is mineralised and released into the atmosphere as CO_2.

ii) To study the changes produced in the biomass and structure of the microbial community, which are related to the assimilation and mineralisation of the added organic carbon.

iii) To provide comprehensive information on the enzymatic processes responsible for the carbon dynamics in semiarid soils. This goal will be achieved by studying the changes produced in the enzyme activity in the soil after the amendment or application of organic compounds of plant origin.

iv) To evaluate the effect of adding plant waste in different doses and with different levels of stabilisation, as a source of exogenous organic matter, to degraded semiarid soils at the field level. Studying the effect of these organic amendments on the biomass, structure and activity of the soil microbial community will be key in evaluating the level of restoration achieved in such degraded soils.

3. WORK PLAN

Murcia, where this thesis was carried out, is a Spanish Autonomous Community located in an area with a semiarid climate which is seriously affected by soil degradation processes. The average annual temperature in Murcia is around 18ºC,

and the average annual rainfall is below 300 mm. As a result of the climate, in addition to the soil lithology and past human actions such as misuse of the soil for agricultural purposes, there is a high proportion of soil in Murcia that is in an advanced state of degradation. Such soil contains very little organic matter and is practically devoid of vegetation.

The application of organic amendments has been widely studied in a general manner as a means to improve this situation in semiarid soils. In this Doctoral Thesis, however, we aim to improve our understanding of processes linked to carbon that occur in the soil, which we believe offers an acceptable level of scientific novelty. There is a significant lack of knowledge about the dynamics of the carbon cycle in such soils and the role of microorganisms in this cycle. To further knowledge about this subject, this Doctoral Thesis involves experiments both in the laboratory and in the field in order to obtain complete information at all levels on the dynamics of organic C and organic amendments with respect to the associated microbial populations.

We first conducted laboratory studies under controlled conditions using compounds enriched with ^{13}C (a stable isotope of carbon) (Fig. 7). These studies made it possible to thoroughly chart the C cycle, evaluating the persistence of plant-derived molecules that could be present in the rhizosphere of any soil in addition to the microbial populations associated with the dynamics of these molecules. In these two studies we applied, among other techniques, those based on the analysis of the isotopic composition of CO_2, water-soluble fractions of C and of humic substances and the isotopic composition of fatty acids (PLFA-SIP). In this Doctoral Thesis, these studies are described in detail in 2 chapters. The first of these chapters (Chapter 1) is dedicated to the dynamics of labile carbon (^{13}C-enriched glucose), a dominant type of carbon in the soil rhizosphere with a significant impact on the microbial community due to the fact that it is an easily assimilated energy source. Next, Chapter 2 evaluates the persistence and dynamics of more recalcitrant forms of plant-derived C by applying ^{13}C-enriched lignin and cellulose molecules. Furthermore, Chapter 3 contains a detailed analysis of the functional aspects of the dynamics of these organic molecules using enzymatic and metabolic indicators.

Once the C cycle was thoroughly understood via the experiments performed in Chapters 1, 2 and 3, “field” studies were conducted to understand the role of organic amendments in real conditions, both due to the plant origins of these amendments and to their indirect stimulation of vegetation growth, which has long-term effects (Fig. 7). Chapter 4 thus evaluates the effect of organic wastes with different levels of

stabilisation and in different doses on the soil microbial community (biomass, structure and function). Finally, Chapter 5 is focused on the effect of the organic amendments (urban biosolids) in the long term (24 years) on the enzymatic diversity and structure of the soil microbial community.

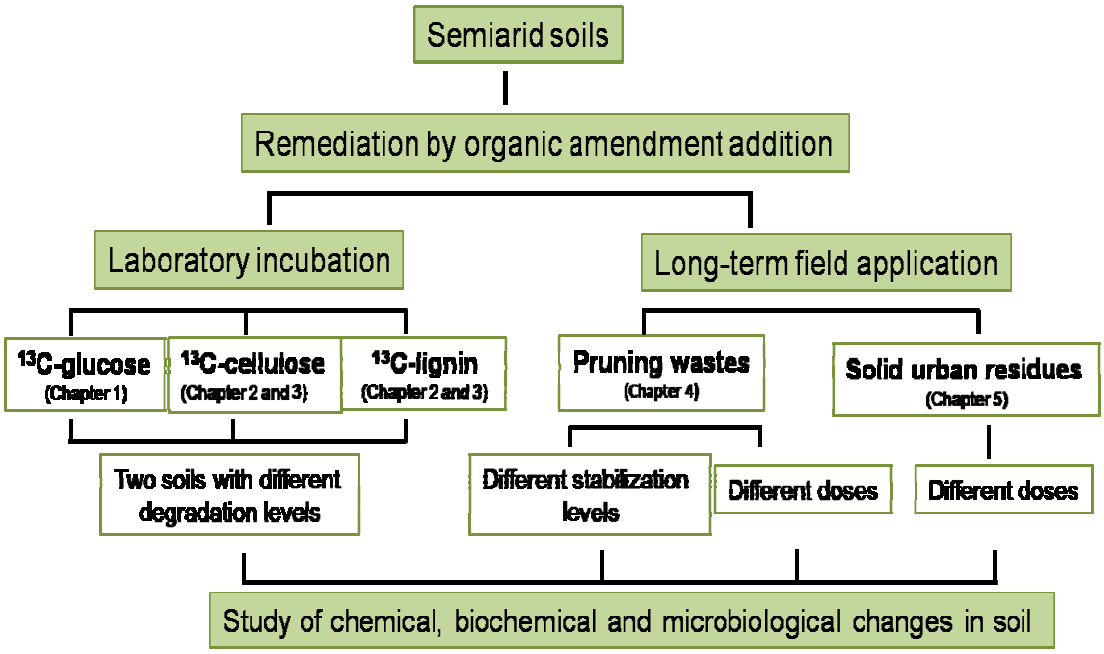


Fig. 7. Diagram of the proposed Doctoral Thesis research

CHAPTER 1

Can the labile carbon contribute to carbon immobilization in semiarid soils? Priming effects and microbial community dynamics.

Bastida, F., Torres, I.F., Hernández, T., Bombach, P., Richnow, H.H., García, C., 2013. Can the labile carbon contribute to carbon immobilization in semiarid soils? Priming effects and microbial community dynamics. Soil Biology and Biochemistry 57, 892-902.

ABSTRACT

Tracer experiments with isotopic-enriched carbon compounds can provide information regarding the carbon cycling in semiarid soils. We studied priming effects and microbial utilization of glucose as an example of bioavailable labile molecule in the carbon cycle of a semiarid soil. The soil, which has low content of total organic carbon (5.0 g kg^{-1}), was amended with $U^{13}C$-glucose (99 atom %) at concentration of 75 µg C g^{-1} soil (LD) or 300 µg C g^{-1} soil (HD). Glucose-derived carbon remained in soil after two months of incubation. The percentage of residual carbon stabilized was greater in LD with 40% of the initial ^{13}C added compared to 30% of the initial ^{13}C added in the HD. Comparison of ^{13}C content in water- and sodium-pyrophosphate extracts pointed to a significant humification of up to 2.4% of the initial ^{13}C-glucose. Glucose was subjected to an intense mineralization in the first 17-days of 22.8% and 40.94% for the LD and HD, respectively. The stable isotope probing (SIP) of phospholipid fatty acids (PLFAs) by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) showed that bacteria dominated glucose metabolism in comparison to fungi. Gram-negative populations were initially more involved in glucose assimilation than Gram-positive bacteria. In the fatty acids fraction, up to 95% of the ^{13}C was predominantly found in fatty acids typical for Gram-negative bacteria. However, after 4 and 17 days the ^{13}C-enrichment in Gram-positive biomarkers increased. The mineralization of soil organic matter triggered by glucose additions was more intense in HD (3.6% of soil TOC) than LD (1.0% of soil TOC) and reached the highest level after 4 days in HD. Priming was controlled by Gram-negative populations but fungi and, particularly actinobacteria played an important role in latter steps. Our data indicated that the intense metabolism of SOM due to priming phenomena compromises the potential carbon sequestration in this semiarid soil amended with glucose.

1. INTRODUCTION

Soils developed in arid and semiarid conditions are characterized by very low organic matter content (OM) from the scarce plant biomass (García et al., 1994) and it has been proposed that these soils have a big potential for carbon sequestration (Lal, 2004). However, such carbon sequestration would depend on the type of organic substrates, soil type and microbial community. Within organic carbon, labile compounds such glucose are quickly assimilated by microbial biomass (Perelo and Munch, 2005; Fischer et al., 2010). In boreal and temperate climates, isotopic evidences found in experiments with ^{13}C- or ^{14}C-labeled glucose indicate that carbon added to soils is not completely mineralized (Blagodatskaya et al., 2007; Schneckenberger et al., 2008; Fischer et al., 2010). However, the extension of glucose mineralization in semiarid soils has not been fully examined yet.

While a major part of the released CO_2 after organic amendment is supposed to derive from the mineralization of the exogenous organic matter added to soil, an additional CO_2 fraction may come from the mineralization of autochthonous soil organic matter as a consequence of increased microbial activity. This last mechanism, which is called “priming effect” (Kuzyakov, 2006), has been proved in temperate and boreal soils with high OM content and even in soils with low total carbon content (Hoyle et al., 2008), but not in soils from semiarid areas with a very low OM content in a pre-desertic state. In such environments a reduction of soil organic matter as a consequence of priming may have ecological significance.

Carbon fluxes and microbial community function can be tracked by stable isotope probing (SIP) methodologies which are mainly based on the incubation of an environmental sample with a ^{13}C-labeled substrate, followed by the analysis of ^{13}C-incorporation into biomarkers (Neufeld et al., 2007). In particular, the use of ^{13}C-labelled substrates in combination with phospholipid fatty acids analysis referred as PLFA-SIP (Boschker et al., 1998) can be useful to examine how soil microbial community transform carbon compounds (Waldrop and Firestone, 2004) and its relation to priming phenomena (Nottingham et al., 2009). Whether or not microbial populations in such semiarid, pre-desertic, environments are able to mineralize an additional part of SOM remains unknown, but it has been suggested that priming effects are related to the dynamics of microbial community (Fontaine et al., 2003). We hypothesized that the addition of glucose to a semiarid soil will stimulate priming effect and hence constraint the potential carbon sequestration at short term.

To trace the fate of labile-carbon in a pre-desertic area, semiarid-soil samples were incubated with ^{13}C-labeled glucose. The persistence of glucose derived carbon was analyzed in bulk soil, and in carbon extracted with water and sodium-pyrophosphate. Furthermore, the conversion of glucose-carbon into carbon dioxide and microbial biomass was assessed by analysis of the isotope enrichment of CO_2 and fatty acids, respectively. The aims of the study are: i) to evaluate the fate of glucose and the short-term accumulation of glucose derived carbon in a semiarid soil, ii) to elucidate the extent of short-term mineralization of glucose and priming effect in relation to the dose of glucose, and iii) to analyze the anabolic conversion of ^{13}C-glucose into microbial biomass.

2. MATERIAL AND METHODS

2.1. Study area and soil sampling

An area of 100 m^2 was selected for soil sampling in Abanilla, Province of Murcia (SE Spain, semiarid climate). This area was agriculturally abandoned since 1980. The soil in this area represents a typical marsh lithology. The mean annual rainfall is lower than 300 mm. Soil was selected due to its low organic carbon, microbial biomass content and microbial activity. Abanilla soil represents a highly degraded soil with no vegetation growing on it and it is therefore an adequate model to study processes of carbon transformation in pre-desertic conditions. The main physic-chemical characteristics of Abanilla soil are: pH (7.77), electrical conductivity (2.65 dS m^{-1}), clay-loam texture, total N (1.3 g kg^{-1}), total C (40 g kg^{-1}) and total organic C (5.0 g kg^{-1}). A detailed description of this soil is provided by Bastida et al. (2006a). Within this area, three plots were selected. In September-2010, 8 subsamples were taken from the top 15 cm of soil and pooled to obtain one composite sample per plot. Soil samples were sieved by < 2 mm and stored in lab conditions during 1 week until the beginning of the incubation experiments.

2.2. Experimental design and soil incubations

Three independent incubation experiments were performed. In all experiments, an aqueous solution of U^{13}C-glucose (99 atom %) (Cambridge Isotope Laboratories, Andover, MA) (^{13}C-glucose) was prepared. In order to compare the effect of glucose-dose, 75 µg C g^{-1} soil (low-dose treatment, LD) or 300 µg C g^{-1} soil (high-dose treatment, HD) were supplied. For a non-labeled control experiment, the same treatments but using glucose with natural isotopic abundance were prepared. The

doses correspond approximately to the same amount of microbial biomass C (around 80 µg C g^{-1} soil) in the case of LD or four-times the microbial biomass C content, in the case of HD. These amounts are similar to the doses of organic amendments applied in field restoration experiments in semiarid environments (Bastida et al., 2012). Furthermore, a control experiment soil without glucose was set-up using the same conditions than glucose-treatments. The water-holding capacity was gravimetrically controlled at 60 % during incubation for all treatments. All treatments were performed in triplicates.

The first phase aimed to evaluate the amount and form of the added carbon remaining in the soil after a two months incubation period. This experiment was carried out in plastic containers with 100 g of soil (Fig. 1.1). Each treatment was prepared in independent triplicates for each incubation time (1 day, 30 days and 60 days). Incubation was performed in chambers at 28 ºC in darkness. Total organic carbon, water-soluble carbon and sodium-pyrophosphate extracted carbon were analyzed for its total content and isotopic ratio.

Fig.1.1. Soil incubation in plastic containers.

The second incubation was devoted for the study of glucose mineralization and priming effects and was carried out in 12 ml-capped glass vials (Labco Limited, Lampeter, UK) containing 1 g of soil. The amount and the isotopic ratio of the CO_2 produced by cumulative mineralization were analyzed at each time as indicated below. Independent triplicates of each treatment were incubated for 1, 4, 7, 11, 14 and 17 days at 28 ºC in darkness. A higher time-resolution at the initial time points was designed in this experiment since it has been described that glucose mineralization is usually more intense at the initial stages (Schneckenberger et al., 2008).

The third incubation was dedicated to the study of microbial community dynamics related to glucose transformation. This assay was performed in Petri dishes with 25 g of soil. Assay was performed in chambers at 28 ºC in darkness. Each treatment was prepared in independent triplicates for each incubation time (1 day, 4 days and 17 days). Soil samples were used for analysis of phospholipid fatty acids and PLFA-SIP.

Preliminary analysis showed that the three experimental incubations (see below) were comparable (Tables A1.1 and A1.2, Annex).

2.3. Carbon analysis

The total organic carbon (TOC) of soil samples was determined after hydrolysis with 2N HCl in a Leco Truspec CN elemental analyzer (St. Joseph, MI). Analysis of carbon isotope ratios of bulk soil as well as water-soluble and sodium-pyrophosphate extracts were performed at the Stable Isotope Facility (University of California, Davis).

Bulk soil was analyzed for carbon isotope ratios using a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000 °C in a reactor packed with chromium oxide and silvered cobaltous/cobaltic oxide. N_2 and CO_2 were separated on a Carbosieve GC column (65 °C, 65 mL/min) before entering the IRMS. The final delta values used for ^{13}C-content calculations were expressed relative to international standards V-PDB (Vienna PeeDee Belemnite) (Coplen et al., 2006) according to:

$$\delta[(‰)] = \left(\frac{\text{Rsample}}{\text{Rreference}} - 1\right) \text{x } 1000 \qquad (1.1)$$

where R_{sample} and $R_{reference}$ are the ratios of the heavy isotope to the light isotope ($^{13}C/^{12}C$) in the sample and in the international standard, respectively. Delta units were converted to atom fraction of ^{13}C expressed as atom%:

$$\text{atom } \%\,^{13} = \frac{100}{\frac{1}{\left(\frac{\delta}{1000} + 1\right) R_{PDB}} + 1} \qquad (1.2)$$

where δ is the measured $\delta^{13}C$ (‰) of the sample using the delta notation and $R_{V\text{-}PDB}$ is the isotope ratio of V-PDB = 0.0112372 (Slater et al., 2001).

Water-soluble carbon was extracted with distilled water (1:5, solid:liquid) by shaking for 2 h at 50 ºC. The carbon was quantified with a Shimadzu TOC5050A Total Organic Carbon Analyzer after filtering. Sodium-pyrophosphate extractable carbon was extracted with 0.1 M sodium-pyrophosphate pH 9.8 (1:5 solid:liquid) by shaking for 4 h (Stevenson, 1982; Lucas-Borja et al., 2012). Subsequently, sodium-pyrophosphate

extracts were centrifuged at 13000 rpm for 10 min and the carbon content was determined using a Shimadzu TOC5050A Total Organic Carbon Analyzer. Hot-water and sodium-pyrophosphate extractions were performed in parallel samples, not sequentially. Water-soluble carbon represents the easy available carbon for microorganisms, while carbon contained in sodium-pyrophosphate extracts represents both labile and a more stable carbon presented in humic substances (Stevenson, 1982).

Of each extract, an aliquot of 8 ml was transferred to a 50 ml vial and was diluted with 22 ml of bi-distilled water. One ml of 30 mM sodium-azide was added to inhibit microbial growth. Samples were acidified and purged with helium off-line to remove all dissolved inorganic carbon (DIC). Depending on sample concentration, 1- to 4-mL aliquots were transferred to digestion vessel heated before and reacted with sodium persulfate to convert DOC into CO_2. The CO_2 was carried in a helium flow to the isotope ratio mass spectrometer where isotope ratios were measured. The analytical measurement was carried out by an Aurora 1030 TOC Analyzer (OI Analytical, College Station, TX) coupled to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) coupled to a GD-100 Gas Trap Interface (Graden Instruments).

2.4. Analysis of CO_2, glucose mineralization and calculations of priming effects

Four milliliters of headspace gas from each vial from the mineralization experiment were transferred to pre-evacuated glass vials (Labco Limited, UK) for analysis of the amount and isotopic composition of total CO_2. The isotope ratio of CO_2 was determined by gas chromatography–isotope ratio mass spectrometry as described elsewhere (Herrmann et al., 2010).

The isotopic ratio of CO_2 was used for the calculation of the percentage of CO_2-C derived from the added glucose or from SOM, as described by Waldrop and Firestone (2004). Priming effect was defined as the increase or decrease in the soil organic matter mineralization following substrate addition. Priming effect was calculated as the increase in total soil respiration following substrate addition minus the amount of carbon respired from the added substrate and from control soil without amendment (Blagodatskaya et al., 2007, Equation (1.3)). This was expressed as a percentage where 100% represents a doubling of the SOM-C respiration (Waldrop and Firestone, 2004; Brant et al., 2006).

$$\text{Priming effect} = \underbrace{(\text{total } CO_2 - \text{glucose derived } CO_2)}_{\text{Soil amended with glucose}} - \underbrace{\text{total } CO_2}_{\text{Unamended soil}} \qquad (1.3)$$

2.5. Phospholipid fatty acids analysis (PLFA)

Phospholipids were extracted from 8 g of soil using a chloroform-methanol extraction as described by Bligh and Dyer (1959), fractionated and quantified using the procedure described by Frostegard et al. (1993). Phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al., 1985) and designated as described by Frostegard et al. (1993). The complete dried FAME fraction was dissolved in *n*-hexane containing 20.06 mg ml^{-1} of 21:0 FAME as internal standard. Mass spectrometric analyses of FAMEs were carried out as described previously (Bastida et al., 2011). The absolute and relative amounts of FAMEs in the samples were determined according to the concentration of the internal standard added.

The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t are characteristic bacterial fatty acids and were chosen as bacterial biomarkers (Frostegard et al., 1993; Dungait et al., 2011). The 18:2ω6 was taken as indicator of fungal biomass (Rinnan and Baath, 2009). The ratio of bacterial to fungi PLFA was taken as an indicator of the dynamics between bacteria and fungi. The Gram-positive specific fatty acids i15:0, a15:0, i16:0, and i17:0 and the Gram-negative specific fatty acids cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t (Frostegard et al., 1993; Dungait et al., 2011) were taken as indicators for the ratio between Gram-positive and Gram-negative bacteria. 10Me-branched FAMES (10Me16:0, 10Me17:0 and 10Me18:0) were taken as specific actinobacterial biomarkers within Gram-positive bacteria (Dungait et al., 2011).

2.6. Isotopic analysis of fatty acids

Carbon isotope composition of FAMEs extracted from control and non-enriched glucose treatments were analyzed using a gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS) system as described previously (Bastida et al., 2011). The $\delta^{13}C$ values of the fatty acids reported were corrected for the carbon introduced during derivatization (Abraham et al., 1998) and converted to atom% following Equation (2). Samples were measured at least in triplicate with an analytical error smaller than ±0.5‰ standard deviation.

The incorporation of ^{13}C in FAMEs extracted from high dose treatments resulted in isotope ratios out of linearity limit of GC-C-IRMS. Because the isotope composition of the highly enriched ^{13}C-fatty acids could not be analyzed accurately using GC-combustion-isotope ratio mass MS, a 7890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used for the determination of isotope enrichment into fatty acids in the following way: the ^{13}C-incorporation into fatty acids caused the appearance of a series of isotopomers besides the natural molecular ion (M^+) (Annweiler et al., 2000; Fang et al., 2004). According to the distribution of these isotopomers, the percentage of labeled carbon (atom%) in the fatty acids could be calculated as described by Bombach et al. (2010).

Amount of ^{13}C incorporated into fatty acids was calculated as described by Boschker et al. (2004). The percentage of ^{13}C incorporated in specific Gram-positive (i15:0, i16:0, i17:0, 10Me16:0, 10Me17:0 and 10Me18:0), Gram-negative (cy17:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t), actinobacteria (10Me16:0, 10Me17:0 and 10Me18:0) and fungi (18:2ω6,9) relative to the total amount of ^{13}C in those fatty acids was used as an indication of the flow of ^{13}C in the different compartments of the microbial community.

2.7. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. The data were submitted to ANOVA of repeated measures. In the case of PLFA analysis, the intra-subject factor (time) was set up with 3 different levels and in the case of CO_2 analysis the intra-subject factor (time) was set up with 6 different levels. The inter-subject was defined as the treatment (dose). ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference at the 95% confidence interval in the lineal model). In order to determine significant differences among treatments at the same time, the data were subjected additionally to one-way ANOVA. Changes in the structure of the microbial community structure were evaluated by factor analysis using the relative abundances of all identified FAMES.

3. RESULTS

3.1. Carbon storage in soil

The fate of glucose was evaluated by analysis of total carbon and isotopic ratio in bulk soil as well as in water- and- sodium-pyrophosphate extracts. The TOC in the low-dose treatments (LD) and high-dose treatments (HD) was higher compared to the control receiving no glucose at the initial time (1 day) (Fig. 1.2A). After 60 days of incubation,

no significant differences were observed for the TOC content in soil with and without glucose.

At the beginning of the incubation, water-soluble carbon content was in mean 3.5-fold higher in the HD treatment in comparison to the control and LD treatment (Fig 1.2B). After 2 months of incubation, there were not significant differences in the content of extractable C with water and sodium-pyrophosphate (Figure 1.2B and 1.2C).

The persistence of ^{13}C derived from glucose was analyzed by means of ^{13}C enrichment in total organic carbon, water-soluble and sodium-pyrophosphate extractable fractions (Fig. 1.3A). The percentage of ^{13}C in bulk soil was significantly higher in the LD treatment than in the HD treatment ($P<0.05$) and tended to decrease during incubation. After 2 months, 29 µg ^{13}C g^{-1} which were equivalent to 40% of the initial ^{13}C amount added and 83 µg ^{13}C g^{-1} which were equivalent to 30% of the initial ^{13}C amount added still remained in soil for LD and HD, respectively (Fig. 1.3A).

The patterns of ^{13}C derived from glucose recovered with hot-water or sodium-pyrophosphate were similar. In both cases, a higher percentage of ^{13}C added to soil was found in the HD treatment compared to the LD treatment after 24 hours of incubation. These patterns were opposite to the percentage of ^{13}C in bulk soil. The amount of ^{13}C obtained by both extraction techniques (hot-water and sodium-pyrophosphate) was similar at the beginning of the incubation experiment. However, the ^{13}C content in the sodium-pyrophosphate extraction after 2 months was around 4-times higher (up to 2.4% of the initial ^{13}C-glucose) than the amount of ^{13}C extracted with water (Fig 1.3C). In particular, the total ^{13}C content of the sodium-pyrophosphate extract was higher in HD (3.95 ± 0.58 µg ^{13}C g^{-1}) than LD (1.83 ± 0.04 µg ^{13}C g^{-1}).

The ^{13}C-content of the non-extractable carbon fraction was calculated by subtracting the amount of ^{13}C found in the sodium-pyrophosphate extract from the amount of ^{13}C found in bulk soil. In absolute values, the non-extractable ^{13}C was always significantly higher ($P<0.05$) in HD than LD (78.89 ± 6.77 µg ^{13}C g^{-1} soil and 27.06 ± 0.70 µg ^{13}C g^{-1} soil, respectively). However, the percentage of immobilization of added ^{13}C-glucose was significantly higher in the LD than HD (Fig. 1.3D).

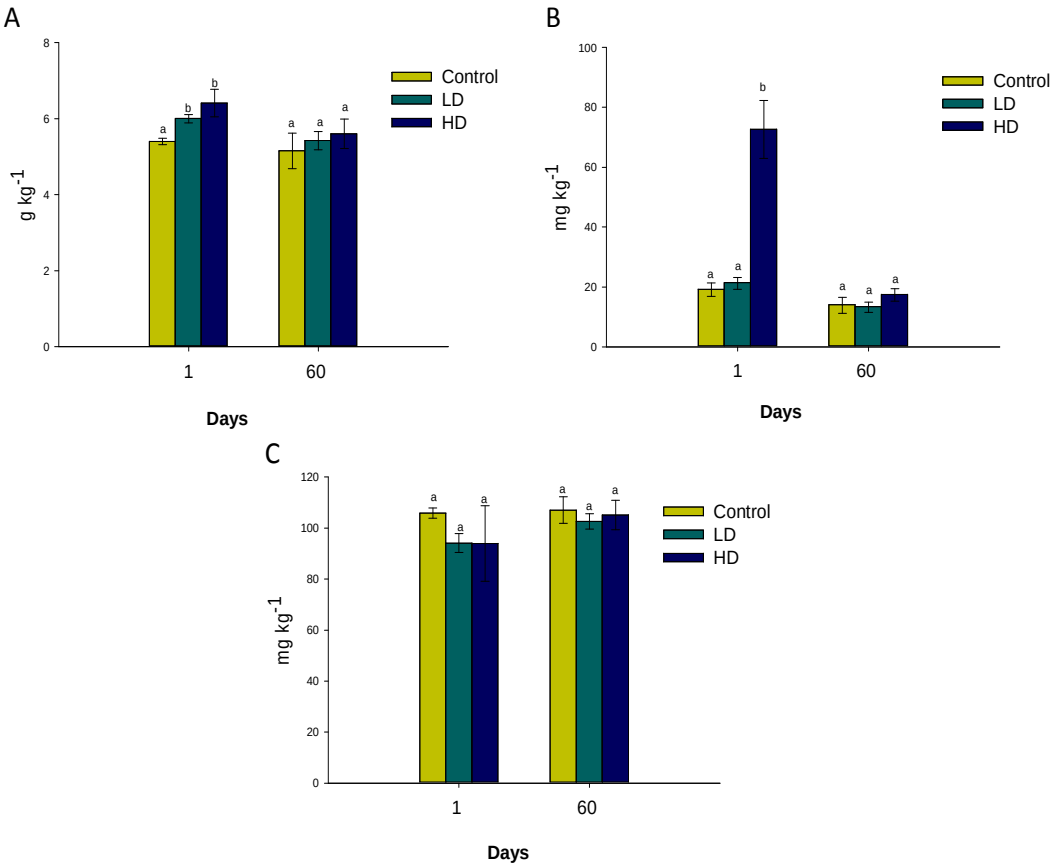


Fig.1.2. Total organic carbon (TOC) (A) and carbon content in water-soluble (B) and sodium pyrophosphate (C) in control and glucose-amended soils. LD (low-dose), HD (high-dose). For each incubation time, data followed by the same letter are not significantly different (P<0.05).

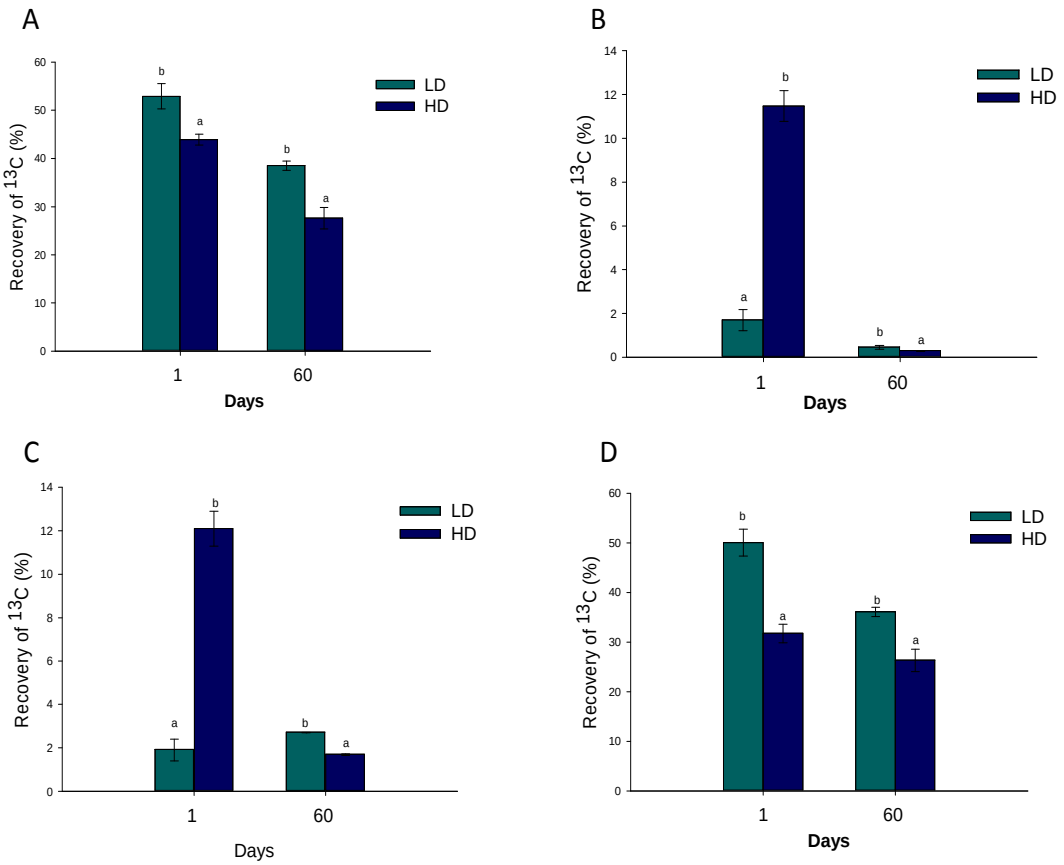


Fig.1.3. Recovery of ^{13}C in bulk soil (A), water soluble (B) and sodium pyrophosphate (C) extracts and calculated non-extractable ^{13}C (D) after addition of labeled glucose. LD (low-dose), HD (high-dose). For each incubation time, data followed by the same letter are not significantly different (P<0.05).

3.2. Glucose mineralization and priming effects

Soil respiration, glucose mineralization and priming effect were significantly influenced by treatment and time (P< 0.05) (Table 1.1). After 17 days of incubation, total respiration reached 113, 161 and 368 µg CO_2-C g^{-1} soil in control, LD and HD, respectively (Fig. 1.4A). Mineralization of the ^{13}C-glucose resulted in an enrichment of the ^{13}C within the CO_2 which was already detectable after 1 day of incubation. From the 4 days until the end of the incubation, glucose mineralization was always significantly higher in HD than LD treatments (P<0.05) (Fig 1.4B). In the course of the incubation, the evolution of $^{13}CO_2$ increased. After 17 days of incubation, 22.8% and 40.9% of the initially added glucose was mineralized in the LD and HD treatments.

With the exception of day 1, priming effect was significantly higher (P<0.05) in HD than LD treatment in the course of the incubation (Fig 1.4C). After 1 day, priming reached a maximum of 59.8% in the LD treatment, while in the HD treatment the highest priming occurred after 4 days of incubation (134.7%). As a consequence of such priming effect,

nearly 50 and 180 µg of autochthonous carbon per gram of soil were released in LD and HD treatments (Fig 1.4D). These amounts corresponded to a mineralization of 1.0 and 3.6% SOM, respectively.

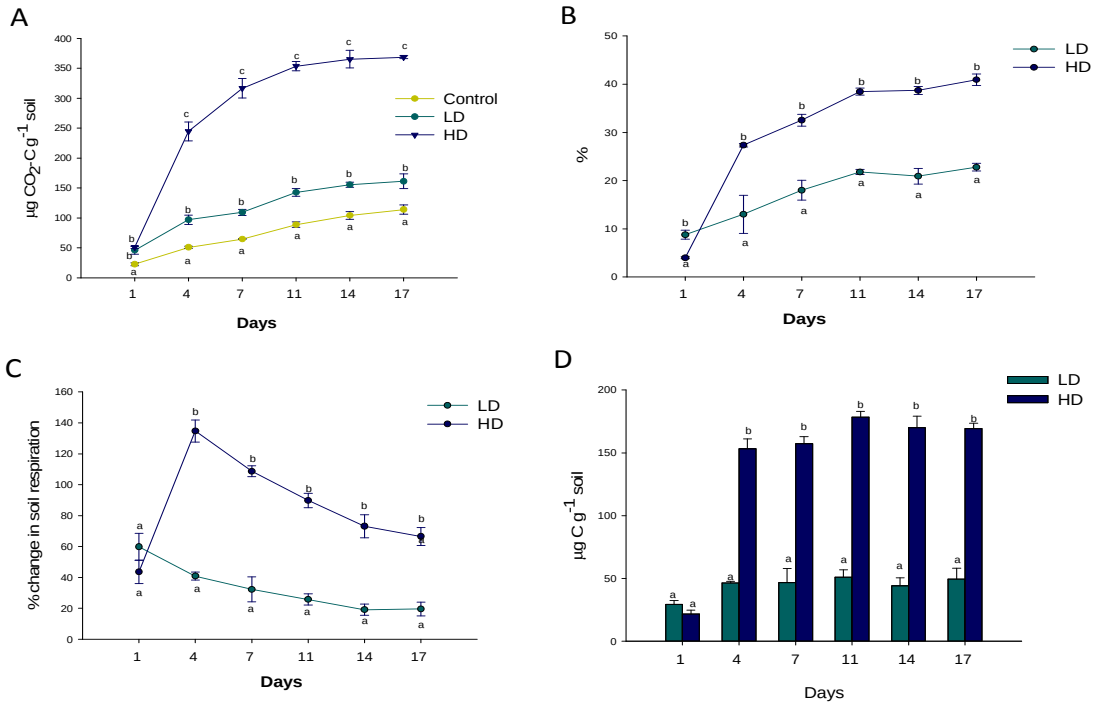


Fig.1.4. Soil respiration (A), glucose mineralization (B), cumulative priming (C), and carbon amount released by priming (D). LD (low-dose), HD (high-dose). For each incubation time, data followed by the same letter are not significantly different (P<0.05).

Table 1.1. Results of ANOVA of CO2 and PLFA analysis

Parameter [a]	SR		GM		PR		PR%		Bacteria		Fungi	
	F [b]	*P* [c]	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Factors												
Treatment (Tr)	2994.96	< 0.001	802.79	< 0.001	153.30	< 0.001	723.79	< 0.001	39.97	< 0.001	2.03	0.21
Time (Ti)	3445.32	< 0.001	888.48	< 0.001	24.29	0.008	2029.00	< 0.001	433.39	< 0.001	168.12	< 0.001
Tr x Ti	484.81	< 0.001	138.00	< 0.001	30.40	0.06	1238.17	< 0.001	1.67	0.26	0.45	0.65
	G+		**G-**		**Act**		**F/B**		**G+/G-**			
	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*		
Factors												
Treatment (Tr)	10.07	0.012	9.26	0.015	2.51	0.16	12.35	0.007	5.95	0.038		
Time (Ti)	309.01	< 0.001	239.25	< 0.001	3503.21	< 0.001	22.52	0.03	61.07	< 0.001		
Tr x Ti	2.04	0.21	0.98	0.43	13.22	0.06	11.66	0.009	4.84	0.056		

[a] SR (Soil respiration); GM (Glucose mineralization); PR (Priming effect, absolute values); PR% (Priming effect, %); G+ (Gram-positive bacteria); G- (Gram-negative bacteria); Act (Actinobacteria); F/B (Fungi to bacterial ratio); G+/G- (Gram-positive to Gram-negative ratio). [b] F-ratio. [c] *P* values.

3.3. PLFAs and community structure

Bacterial PLFA content, including Gram-positive and Gram-negative populations, were significantly influenced by both glucose-dose and incubation time ($P<0.05$) (Table 1.1). Glucose treatment lead an increase in bacterial PLFA from the beginning of the experiment to the day 4th and a further slightly increase in the course of incubation. HD showed significant higher values of Gram-positive PLFA content than LD, and LD showed higher values than control along the incubation ($P<0.05$) (Table 1.2). Gram-negative PLFA content was significantly higher in HD and LD compared to the control at 1 day (Table 1.2).

The ratio of Gram-positive to Gram-negative PLFA content was higher ($P<0.05$) in the soil with glucose than in the control. Fungi and actinobacteria were significantly ($P<0.05$) influenced by incubation time with a higher PLFA content after 4 and 17 days of incubation compared to the experiment at day 1. However, the glucose dose did not significantly influence the content of fungal and actinobacterial fatty acids (Table 1.1). The fungi to bacteria ratio was significantly lower in the HD treatment compared to the control and LD treatment at day 1 but no shifts in the ratios occurred during the course of incubation.

For estimating the effect of glucose treatment on whole microbial community structure, multifactorial analysis with the relative abundance of fatty acids was performed (Fig. 1.5). Factor 1 explained the 36.8 % of the variability of the results and Factor 2 explained 19%. At day 1, PLFA data of all three different treatments grouped closely together demonstrating a similar microbial community structure. Clear shifts in the community structure appeared at day 4. Regarding factor 1, the microbial community of HD after 4 days of incubation clustered in the same group as all samples after 17 days of incubation (Fig. 1.5).

Table 1.2. Changes in bacterial and fungal PLFAs (nmol g^{-1}), ratio of fungi to bacterial PLFAs (F/B) and ratio of Gram-positive to Gram-negative bacterial PLFAs (G+/G-) in control and glucose-treatments during the incubation period of 17 days.

	1 day						4 days						17 days					
	Control		LD		HD		Control		LD		HD		Control		LD		HD	
	mean	SD[1]	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Bacterial (B)	**2.38a**	0.27	**4.03b**	0.43	**9.61c**	2.14	**16.80a**	1.91	**19.57a**	3.56	**21.97**	3.48b	**17.10a**	1.77	**20.61b**	0.10	**22.99**	1.70b
Fungi (F)	**0.25a**	0.07	**0.29a**	0.06	**0.29a**	0.11	**0.72a**	0.09	**0.79a**	0.22	**1.04a**	0.30	**0.78a**	0.09	**0.92a**	0.10	**0.91a**	0.08
Gram-positive (G+)	**0.40a**	0.08	**0.92a**	0.31	**2.55b**	1.10	**5.30a**	0.76	**6.45a**	1.99	**7.70b**	2.18	**4.74a**	0.57	**6.05b**	0.03	**8.31c**	0.33
Gram-negative (G-)	**1.98a**	0.19	**3.11b**	0.19	**4.09c**	1.36	**9.37a**	0.96	**10.54a**	0.43	**12.42a**	1.79	**8.96a**	1.16	**11.65a**	0.16	**11.3a**	1.46
Actinobacteria	**n.d**		**n.d**		**0.57b**	0.11	**2.13a**	0.21	**2.59a**	1.60	**2.87a**	0.35	**3.41b**	0.15	**2.91a**	0.21	**3.37b**	0.13
F/B	**0.10b**	0.02	**0.07b**	0.01	**0.03a**	0.02	**0.04a**	0.00	**0.04a**	0.02	**0.04a**	0.01	**0.05a**	0.00	**0.04a**	0.00	**0.04a**	0.00
G+/G-	**0.20a**	0.02	**0.30a**	0.10	**0.63b**	0.16	**0.56a**	0.03	**0.61a**	0.19	**0.62a**	0.15	**0.53a**	0.01	**0.52a**	0.01	**0.74b**	0.07

For each incubation time, data followed by the same letter are not significantly different ($P<0.05$). [1]SD (standard deviation). LD (low-dose), HD (high-dose). n.d.: non-detected

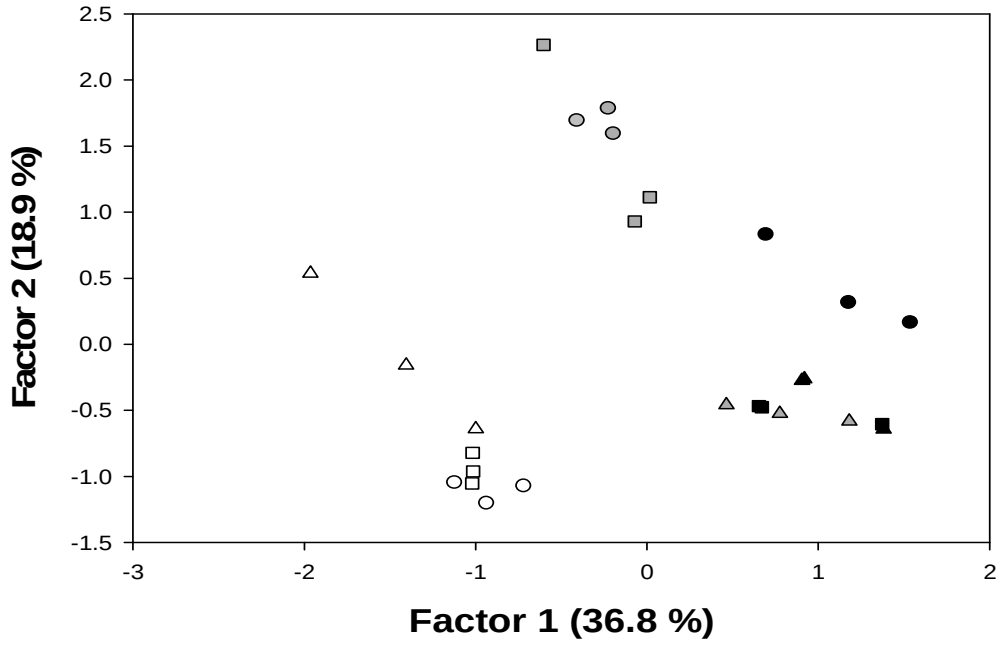


Fig.1.5. Community structure analysis by PLFAs of control and glucose amended samples at different incubation times. Legend: ○ (Control), □ (Low-dose, LD), Δ (High-dose, HD). Greyscale indicates incubation time: Open symbols (1 day), Grey symbols (4 days), Black symbols (17 days).

3.4. ^{13}C incorporation into PLFA (PLFA-SIP)

Fatty acids extracted from control and ^{12}C-glucose amended samples showed natural isotope of 1.08 ± 0.002 atom %. In soil amended with ^{13}C-glucose, a fast ^{13}C-labeling of PLFA occurred within 1 day of incubation. When comparing the degree of ^{13}C-labeling, two different statements can be made: (i) HD treatment showed always a significant higher ^{13}C-enrichment in each fatty acid compared to the LD treatment. (ii) fatty acid representatives of Gram-positive bacteria were more ^{13}C-enriched with values up to 50.96 atom% (i16:0) than fatty acids of Gram-negative fatty acids (43.53 atom%, 16,1ω7c) (Table A1.3, Annex).

Total amount of ^{13}C found in the different compartments of the microbial community followed a common trend for both LD and HD. Out of the total ^{13}C in fatty acids, 63% (LD) and 95% (HD) were presented in Gram-negative fatty acids at first day of incubation and the rest was in Gram-positive bacteria (Fig 1.6). No enrichment was found in actinobacteria and fungi at this time. However, after 4 days the ^{13}C content in Gram-positive increased compared to that level found in fatty acids representatives of Gram-negative at 1 day and reached higher values than typical Gram-negative bacterial fatty acids in the following period of the experiment (Fig 1.6). 2.05 and 3.02% of ^{13}C bound to the total ^{13}C Gram-positive fatty acid biomarkers were found in fatty

acid representatives of actinobacteria. Furthermore, a minimal portion of isotope labelling appeared in fungi fatty acids (4 and 2%, respectively for LD and HD). After 17 days, the amount of ^{13}C in Gram-positive decreased in comparison to 4th day of incubation whilst the amount in Gram-negative increased. The amount of ^{13}C in actinobacteria was 5.85 and 4.22% of the total ^{13}C found within the representative fatty acids.

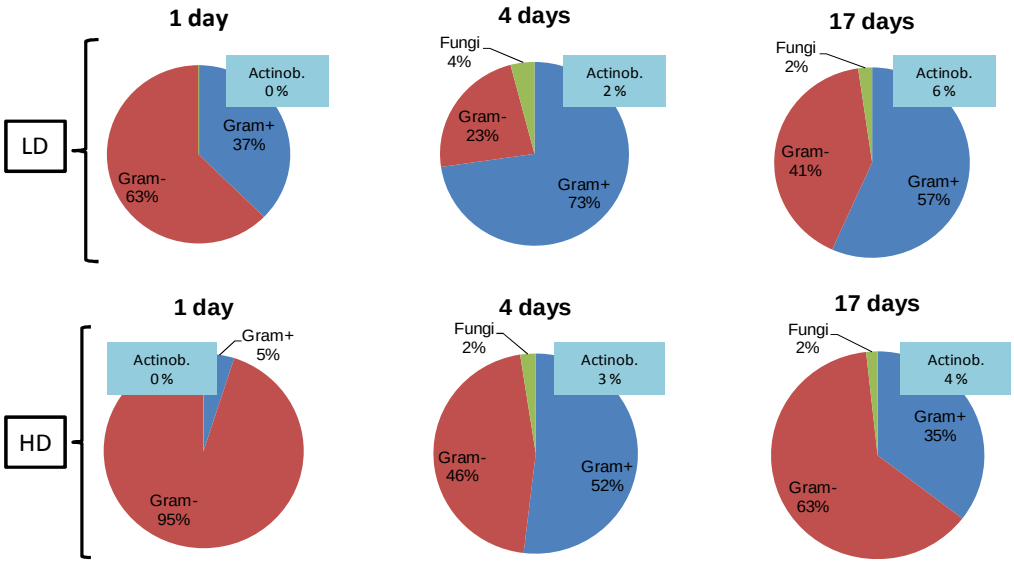


Fig.1.6. Percentage of ^{13}C incorporated into PLFA which is shared by specific groups of the community. LD (low-dose), HD (high-dose). The percentage of ^{13}C in actibobacteria out of the total Gram+ pool is showed in rectangles.

3.5. Soil-derived carbon incorporation into PLFA

Total amount of soil-derived carbon found in the different compartments of the microbial community followed a similar trend for both LD and HD. Overall, the absolute amount of soil-derived C in fatty acids increased with the incubation time. This amount was especially higher in some Gram-negative representatives (18:1ω9c and 18:1ω9t) than Gram-positive typical fatty acids (i15:0, i16:0, i17:0) (Fig. A1.1, Annex). Out of the total ^{13}C in fatty acids, the amount of soil-derived C was higher in Gram-negative than Gram-positive and fungi fatty acids for both LD and HD treatments during the experiment (Fig. 1.7). However, this amount decreased during incubation. Conversely, the amount of soil-derived C found in Gram-positive fatty acids increased during the incubation (Fig.1.7). Out of the total amount in Gram-positive bacteria, up to 18 and 22% of the soil-derived carbon was found within actinobacterial PLFAs after 17 days of incubation, respectively for LD and HD. The maximum amount of soil-derived C in fungi

(8% and 7%, respectively for LD and HD) was reached after 4 days of incubation (Fig. 1.7).

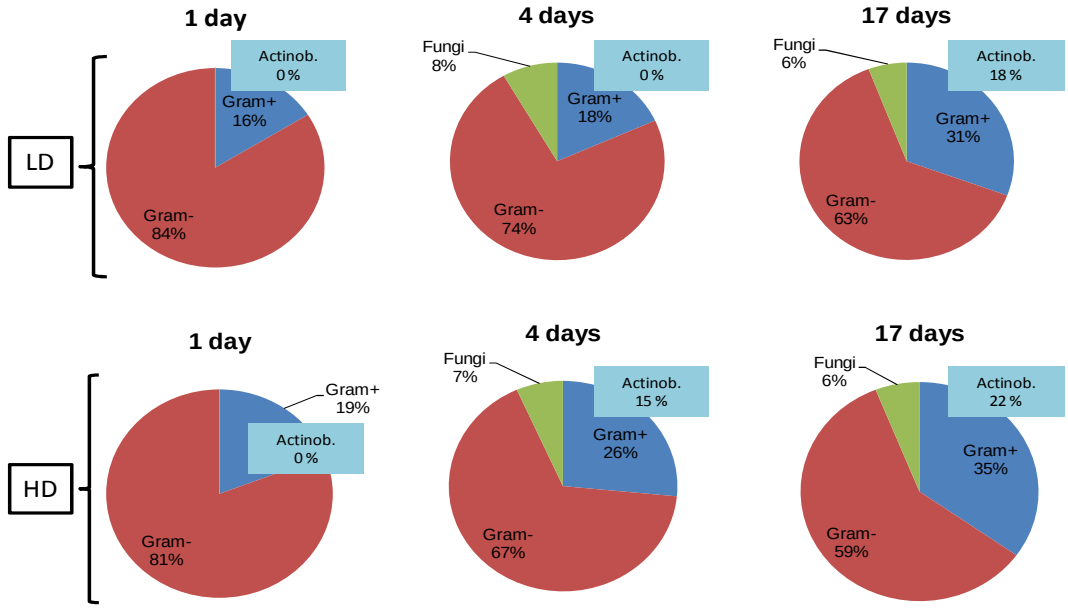


Fig.1.7. Percentage of soil-derived C incorporated into PLFA which is shared by specific groups of the community. LD (low-dose), HD (high-dose). The percentage of ^{13}C in actibobacteria out of the total Gram+ pool is showed in rectangles.

4. DISCUSSION

4.1. Carbon storage in soil

Glucose can be immobilized in microbial biomass (Perelo and Munch, 2005; Hoyle et al., 2008). Fischer et al. (2010) demonstrated that microbial immobilization of glucose out-competes physico-chemical sorption in soil. These authors observed that up to 59.1% of glucose-carbon can be stored in microbial biomass while only 6.7% was adsorbed in soil matrix. Glucose uptake by microbial cells can take 1-2 min (Hill et al., 2008; Fischer et al., 2010). However, in soils with low biomass, microbial immobilization of glucose can be limited or, at least, delayed. Hoyle et al. (2008) indicated that only 2.7% of added carbon was found within the microbial biomass after incubation for 2500 h in a soil with a microbial biomass C of 121 μg C g^{-1}. The remainder glucose-carbon was assumed to be retained within the soil organic matter pool. Accordingly, the low initial microbial biomass in Abanilla soil (80 μg C g^{-1}, Bastida et al., 2006a) might not be enough to quickly assimilate a big portion of added carbon that can be physically protected by soil particles (Jones and Edwards, 1998). In fact, a

great part of added glucose-carbon still remained in soil after two months of incubation. The higher percentage of ^{13}C recovered in liquid extracts in HD than LD at first day can be due to the need of a longer period for microbial assimilation and physical stabilization in soil particles when added carbon was abundant.

Carbon extracted with water includes the more labile fractions (Cook and Allan, 1992), while extractions with alkaline solutions, such sodium-pyrophosphate, additionally include also more stable carbon pools (Stevenson, 1982). After 2 months of incubation, the total amount of ^{13}C was higher in the sodium-pyrophosphate extracts than in the water-soluble extracts for both LD and HD. This data suggests a minor but significant humification of carbon derived from glucose whereas an important part might be physically protected in mineral colloids during the incubation (Derrien et al. 2006; Ekschmitt et al. 2008; Schmidt et al. 2011). Several authors have indicated out a chemical stabilization (i.e. humification) of carbohydrates (Gleixner et al., 1999; Piccolo et al., 1999). Contrarily, Schmidt et al. (2011) concluded that this neo-formation of stable organic matter is not quantitatively relevant for humus formation in soil.

4.2. Glucose mineralization and priming effects

Several authors have pointed out an increase in mineralization after glucose addition (Wu et al., 1993; Scheckenberger et al., 2008). Nevertheless, the increase in glucose mineralization was not proportional to the applied dose of glucose that was 4 times higher in HD than in LD. Accordingly, Bremer and Kuikman (1994) proposed the existence of an upper limit of glucose mineralization above which further addition do not lead to increasing mineralization rate. These results point to an inability of the microbial community for further metabolisation of high amounts of labile carbon in the HD treatment at short-time. However, glucose mineralization increased during incubation by the growing microbial community in the next days (Blagodatskaya et al., 2009).

Noteworthy, the released CO_2 is almost equally originated from glucose and primed SOM mineralization, as described above. The input of easily available organic substances in soil may change the turnover of native soil organic matter and cause priming effects (Kuzyakov et al., 2000; Blagodatskaya et al., 2007). An accelerated CO_2 lost as a consequence of intensified microbial metabolism could be of paramount ecological importance in pre-desertic areas with low OM content as SE-Spain. Two types of priming effects have been proposed: i) *apparent priming* which is due to the increased turnover of microbial biomass, and ii) *real priming* which is a consequence of

the SOM mineralization (Blagodatskaya and Kuzyakov, 2008). As in the case of García-Pausas and Paterson (2011) and Brant et al. (2006), the intensity of priming effect was highest at a short term (after 4d of incubation), especially in the high dose treatment. It has been suggested that apparent priming effect can be related to a non-increase in microbial biomass due to the accelerate turnover of microorganisms (Blagodatskaya et al., 2007). However, our study provides evidence that supports a real priming mechanism: (i) priming coincides with a noticeable microbial growth partially based on both ^{13}C-incorporation and soil-derived C into PLFAs (Nottingham et al., 2009), (ii) carbon released as a consequence of priming (up to 180 µg C g^{-1} soil) exceeds the original microbial biomass carbon of this soil of about 80 µg C g^{-1} (Bastida et al., 2006a).

4.3. Microbial dynamics in relation to glucose mineralization and priming effects

In some previous studies, no significant changes in community structure occurred after addition of glucose leading to the hypothesis that glucose is ubiquitously present in soil environments and do not preferentially support specific groups of the microbial community (Falchini et al., 2003; Nannipieri et al., 2003; Rinnan and Baath, 2009). Conversely, specific soil microbial populations which were unable to use the existing but hardly available soil organic carbon particularly in such a poor soil switched from their starving state and began to grow when an easily available substrate was provided (*r*-strategists) (Fontaine et al., 2003). It has been proposed that those changes might be associated with a change in the community structure (Schneckenberger et al., 2008). In our study, multivariate analysis of FAMEs revealed that the only variation in microbial community structure compared to control was observed in the HD treatment after 4 days of incubation. Possibly (i) an excess of glucose induces the growth of specific components of the microbial populations (*r*-strategists) that change the community structure; and/or (ii) *K*-strategists populations begin to grow when energy-rich compounds are already exhausted (Fontaine et al., 2003) or become unavailable for microbes (Schmidt et al., 2011).

In relation to microbial biomass, several authors did not find changes in the concentration of PLFAs after glucose addition (Rinnan and Baath, 2009). In other cases, the responses were different and depended on soil type and biomass (Dungait et al., 2011). In our study, incubation conditions (i.e. moisture) influenced PLFA content as stated by the evolution of control treatment during the incubation. In any case, the addition of glucose to an extremely poor soil had an important impact in the microbial

biomass in comparison to control treatment at each specific incubation time. It is noteworthy to mention that this semiarid soil has almost no vegetal cover and hence soil is not adapted to carbon inputs.

The low ^{13}C-enrichment into fungi and actinobacteria representative fatty acids may suggest that those groups are marginally competitors for glucose or low-intense cross-feeding phenomena over bacteria. It is noteworthy to mention that this conclusion is valid for the studied desert soil with low microbial biomass and presumably low proportion of fungal biomass. Gram-negative bacteria played a more important role than Gram-positive bacteria in the initial biodegradation of glucose, as previously highlighted (Brant et al., 2006; García-Pausas and Paterson, 2011). Contrarily, these results disagree with the findings of Dungait et al. (2011). After 4 days of incubation the higher ^{13}C bound in Gram-positive than Gram-negative bacterial fatty acids despite its lower PLFA content indicates a high but delayed efficiency of Gram-positive bacteria in the use ^{13}C-derived carbon (Brant et al., 2006; Fontaine et al., 2003).

Fontaine and Barot (2005) proposed that real priming effect is caused by the increase in microbial biomass of *K*-strategists when energy-rich compounds are unavailable. In our study, the significant increase of microbial biomass during the experiment that courses with an increase of soil-derived C within certain PLFA biomarkers supports a real priming effect (Nottingham et al., 2009). Overall, Gram-negative populations were particularly important in metabolizing soil organic carbon in comparison to Gram-positive bacteria. Similar results were found by other authors (Waldrop and Firestone, 2004; Nottingham et al., 2009). However, the importance of Gram-positive bacteria in relation to priming effect increased during incubation time. As in the case of glucose-mineralization, Gram-positive bacteria showed a delayed response in the mineralization of SOM. Moreover, within Gram-positive, actinobacteria played an important role in the mineralization of SOM. In the case of HD treatment, the peak of priming coincided with the highest increase of soil-derived C in actinobacterial and fungal PLFAs. These results suggest that these populations act as *K*-strategists in the degradation of SOM. Nevertheless, from the high content of ^{13}C found in Gram-positive bacterial PLFAs, it is obvious that the so-called *K*-strategists populations are also using ^{13}C-derived carbon from glucose itself, glucose metabolites or glucose-derived biomass.

The use of isotope-based methodologies allowed a deep understanding of carbon-cycling in a semiarid soil subjected to a labile carbon amendment in form of glucose. According to the proposed objectives, we conclude:

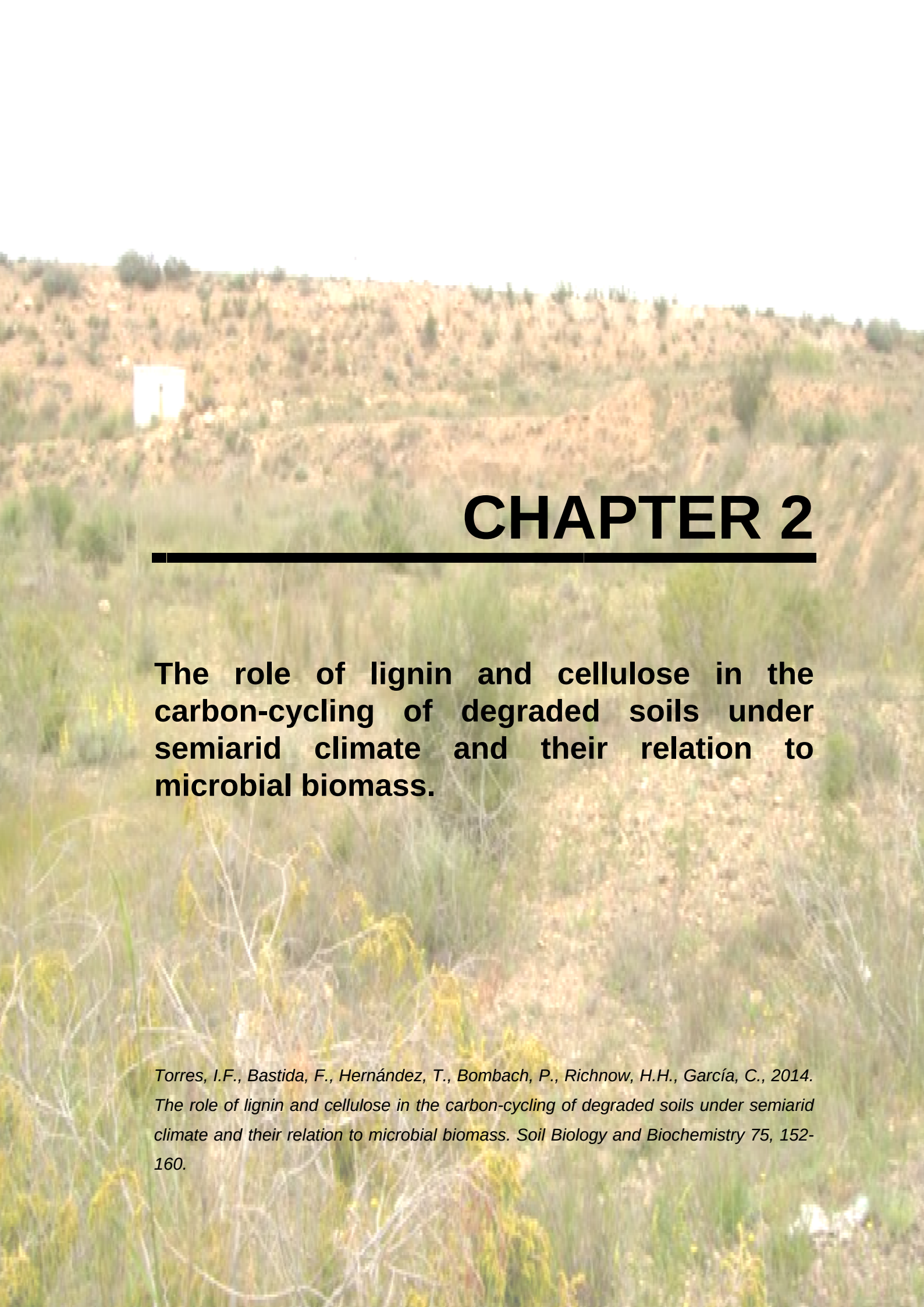
i) After 2 months, up to 40 % of glucose-derived carbon remained in soil.

ii) Gram-negative bacteria initially drove glucose mineralization and they were actively followed by Gram-positive bacteria, while fungi populations play only if at all a minor role in glucose transformation in the tested soil.

iii) Priming effects induced by glucose seem to be controlled by Gram-negative populations but fungi and, particularly actinobacteria played an important role in latter steps.

iv) Soil organic matter represents a major substrate for priming effect in semiarid areas when microbial community is stimulated with high doses of glucose. Indeed, the importance of SOM in the total budget of mineralization was quantitatively comparable to the amount of mineralized glucose. Up to 180 µg C of soil organic matter was mineralized as a consequence of priming, while up to 120 µg of CO_2-C evolved from glucose.

At short-term, the potential for carbon sequestration derived from glucose is strongly affected by the metabolism of autochthonous SOM in a semiarid soil. However, further long-term studies are necessary to assess the impact of labile compounds in the carbon stocks of soil and the possibility of the formation of stable humic substances. From the practical point of view, a possible SOM degradation should be considered when fresh organic amendments are used for soil restoration in semiarid areas.



CHAPTER 2

The role of lignin and cellulose in the carbon-cycling of degraded soils under semiarid climate and their relation to microbial biomass.

Torres, I.F., Bastida, F., Hernández, T., Bombach, P., Richnow, H.H., García, C., 2014. The role of lignin and cellulose in the carbon-cycling of degraded soils under semiarid climate and their relation to microbial biomass. Soil Biology and Biochemistry 75, 152-160.

ABSTRACT

A high level of biological degradation is usually observed in soils under semiarid climate where the low inputs of vegetal debris constraint the development of microbiota. Among vegetal inputs, cellulose and lignin are dominant substrates but their assimilation by the microbial community of semiarid soils is yet not understood. In the present study, ^{13}C-labeled cellulose and ^{13}C-labeled lignin (75 mg ^{13}C g^{-1} soil) were added to two semiarid soils with different properties and degradation level. Abanilla soil is a bare, highly degraded soil without plant cover growing on it and a total organic C content of 5.0 g kg^{-1}; Santomera soil is covered by plants (20% coverage) based on xerophytic shrubs and has a total organic C content of 12.0 g kg^{-1}. The fate of added carbon was evaluated by analysis of the carbon isotope signature of bulk soil-derived carbon and extractable carbon fractions (water and sodium-pyrophosphate extracts). At long-term (120 days), we observed that the stability of cellulose- and lignin-derived carbon was dependent on their chemical nature. The contribution of lignin-derived carbon to the pool of humic substances was higher than that of cellulose. However, at short-term (30 days), the mineralization of the added substrates was more related to the degradation level of soils (i.e. microbial biomass). Stable isotope probing (SIP) of phospholipid fatty acids (PLFA-SIP) analysis revealed that just a minor part of the microbial community assimilated the carbon derived from cellulose and lignin. Moreover, the relative contribution of each microbial group to the assimilation of lignin-derived carbon was different in each soil.

1. INTRODUCTION

Poor vegetal cover, inadequate texture and the high salt content in arid and semiarid environments constrain the development of soil microbial communities (Albadalejo and Díaz, 1990; García et al., 1994) and cause severe biological degradation of soils, which affects the carbon cycling of these environments (Bastida et al., 2006a). In this scenario, the scarce and dormant microbial biomass must efficiently deal with low inputs from the poor above-ground plant communities in order to obtain energy for its maintenance. Among such vegetal inputs, cellulose and lignin are dominant components. Lignin is a cross-linked polyphenol macromolecule with molecular masses exceeding 10.000 amu. It is relatively hydrophobic and aromatic in nature. Cellulose is a polysaccharide consisting of a linear chain of several hundred to over ten thousand β (1→4) linked D-glucose units (Updegraf, 1969). Recent evidence suggests that the stability and low degradability of lignin in soils seems to be overestimated and that their contribution to humus is exaggerated (Stevenson, 1982; Thevenot et al., 2010). Indeed, several authors have concluded that lignin is not stabilized in mineral soil horizons (Rumpel et al., 2004; Vancampenhout et al., 2012) and observed a weak contribution of lignin to the stable carbon pool (Thevenot et al., 2010). In contrast, Hoffman et al. (2009) found a relatively high persistence of lignin in soil. In any case, both soil characteristics (i.e., texture, clay content, etc.) and biotic environment can influence the stabilization of carbon in soil (Rumpel et al., 2004).

Despite the dynamics of these compounds are fundamental for terrestrial carbon cycling (Rodriguez et al., 1997; Eichorst and Kuske, 2012), the fate of lignin and cellulose are not fully understood particularly in carbon-limited soil ecosystems, i.e. semiarid climates. Previous studies have highlighted a key role for fungi in the biodegradation of cellulose (Fontaine et al., 2011). However, other authors have suggested an ecological succession driven firstly by bacteria and secondly by fungi (Hu & van Bruggen, 1997; Schutter and Dick, 2001). Conversely, Snajdr et al. (2010) and Schutter and Dick (2001) affirmed that fungi played a less important role than expected in lignin degradation.

In this study, the fate of cellulose and lignin was traced in degraded soils under semiarid climate conditions using stable isotope probing (SIP) methodologies. For these purposes, ^{13}C-labeled-cellulase and ^{13}C-labeled-lignin were added to two soils with different level of degradation and distinct characteristics such as texture, electrical conductivity and level of microbial biomass. The combination of SIP experiments with phospholipid fatty acids analysis (PLFA-SIP) allows gaining quantitative information on

the microbial assimilation of lignin- and cellulose derived carbon and to trace the microbial groups involved in the degradation (Boschker et al., 1998; Waldrop and Firestone, 2004; Rinnan and Baath, 2009).

We aim to shed light on the fate of cellulose and lignin in degraded soils under semiarid climate conditions and the role of the soil microbial community on these processes. The specific objectives of this study were: i) to evaluate the stability and humification of cellulose and lignin in semiarid soils with different levels of degradation at long-term; ii) to evaluate the mineralization of these substrates at short-term, and iii) to identify the microbial groups responsible for the transformation of both compounds.

We hypothesize that the fate of cellulose- and lignin-derived carbon is related to the level of degradation of both soils. Furthermore, we expect that mineralization and microbial assimilation of carbon derived from both substrates will be higher in the soil with a lower degradation level due to its initially greater microbial biomass. Moreover, the relative contribution of each particular microbial group to the assimilation of substrates is expected to be similar in both soils and dependent on the substrate type.

2. MATERIAL AND METHODS

2.1. Study area and soil sampling

Two different soils were chosen in the Province of Murcia located in the South-East of Spain. Both soils are subjected to a semiarid climate with a mean annual rainfall of less than 300 mm and a mean annual temperature of 18 ºC. Both soils were agriculturally used in the past and were subjected to natural degradation processes due to the adverse climate conditions of South-East Spain. In the basis of total organic C, nitrogen content, vegetal cover and microbial biomass and its activity, the two soils have different levels of degradation (Bastida et al., 2006a), which may influence the dynamics of cellulose and lignin.

The first soil was taken in an area of 100 m^2 located in Abanilla in the Province of Murcia, SE Spain. This soil is classified as Calcaric regosol (Soil Survey Staff, 1998) and represents a highly degraded soil with no vegetation growing on it (Bastida et al., 2006a). It was selected as an adequate model for studying processes of carbon transformation under pre-desertic conditions. The soil particle distribution was 34.6% clay; 7.7% silt, and 57.7% sand. Abanilla soil has a pH of 7.8, an electrical conductivity of 2.6 dS m^{-1}, a total N content of 1.3 g kg^{-1}, a total C content of 40 g kg^{-1} and total organic C was 5.0 g kg^{-1} (Fig. 2.1).

The second soil was taken in an area of 100 m^2 located in Santomera in the Province of Murcia, SE Spain. The soil was classified as Haplic calcisol (Soil Survey Staff, 1998) and represents a low degraded soil (Bastida et al., 2006a). Plant cover was around 20% and was dominated by xerophytic shrubs. The soil particle distribution was 18.8% clay, 9.5% silt, and 71.7% sand. Santomera soil has a pH of 7.7, an electrical conductivity of 0.3 dS m^{-1}, a total N content of 1.0 g kg^{-1}, a total C content of 71.2 g kg^{-1} and total organic C was 12.0 g kg^{-1}. A detailed description of these soils is provided by Bastida et al. (2006a) (Fig. 2.1).

Within these areas, three plots (n = 3) of 20 m^2 each were selected. Six subsamples were taken from the upper 15 cm of each plot and pooled to obtain one composite sample per plot. Soil samples were sieved by < 2 mm and stored at 4 ºC until the beginning of the incubation experiments.

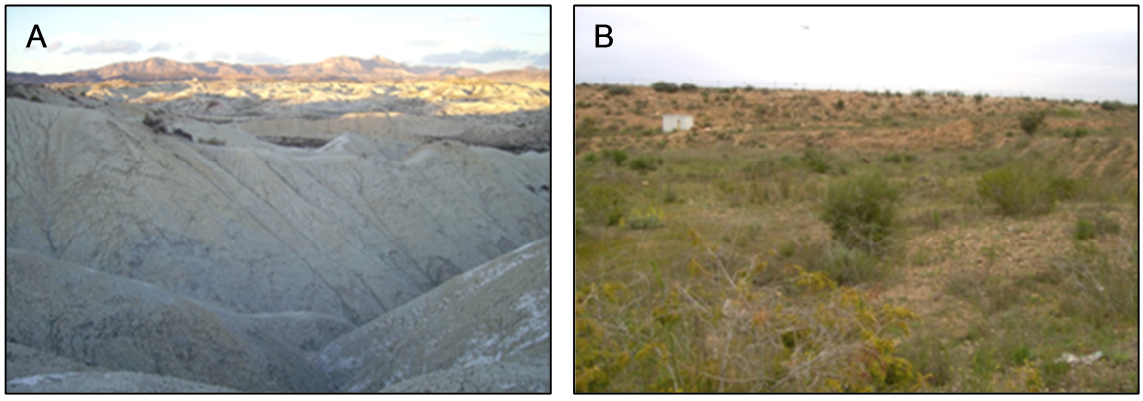


Fig.2.1. Abanilla (A) and Santomera (B) soil.

2.2. Experimental design and soil incubations

Two independent incubation experiments with each of the two soils were performed. The first incubation was carried out in containers with 100 g of soil. Each treatment was prepared in identical triplicates for each incubation time (1, 4, 20, 60, and 120 days). Incubation was performed in chambers at 28 ºC in darkness and controlled moisture. Soil samples were used for the measurement of the content and the isotopic composition of carbon in bulk soil, water-soluble and sodium-pyrophosphate extracts at long-term, as well as for PLFA and PLFA-SIP analysis.

In the second incubation experiment, the mineralization of cellulose and lignin was studied at short-term. This experiment was carried out in 12 ml-capped glass vials (Labco Limited, Lampeter, UK) containing 1 g of soil. Each treatment was incubated in

triplicates at 28 ºC in darkness. The concentration and carbon isotope ratio of CO_2 were analyzed after 1, 4, 10, 20 and 30 days of incubation.

In both experiments, two solutions were prepared with uniformly ^{13}C-labeled substrates (>97 atom%) from maize, one of C^{13}-cellulose and the other of C^{13}-lignin (Isolife, Wageningen, The Netherlands). 75 µg C g^{-1} soil of cellulose or lignin was supplied. For non-labeled control experiments, the same concentrations of these substrates were prepared, but using cellulose and lignin with natural isotopic abundance. The amount of substrates is in the same range as carbon applied in field restoration experiments and in previous isotope labeling experiments (Chapter 1). Furthermore, a control experiment consisting of soil without cellulose or lignin was set up, using the same conditions as the other two treatments. The water-holding capacity of the soil was gravimetrically adjusted to 60% during incubation for all treatments. Before the substrates were added, soils were pre-incubated with distilled water during two weeks in order to avoid drastic effects of moisture on the measured parameters when adding the substrates. All treatments were performed in replicates (n = 3).

2.3. Analysis of carbon fractions

The total organic carbon (TOC) of soil samples was determined after acidification with 2N HCl to pH 2 in a Leco Truspec CN elemental analyzer (St. Joseph, MI) (Chapter 1). Hot water-soluble carbon was extracted with distilled water (1:5, w:v) by shaking for 2 h at 50 ºC. Sodium-pyrophosphate extractable carbon was extracted with 0.1 M sodium-pyrophosphate pH 9.8 (1:5, w:v) by shaking for 4 h (Stevenson, 1982; Lucas-Borja et al., 2012). Subsequently, carbon content in both extracts was determined using a Shimadzu TOC5050A Total Organic Carbon Analyzer. Hot water and sodium pyrophosphate extractions were performed in parallel samples, not sequentially. Hot water-soluble carbon represents the easily available carbon for microorganisms, while carbon in sodium pyrophosphate extracts represents both a labile and more stable carbon presented for example in humic substances (Stevenson, 1982).

2.4. Carbon isotope analysis of carbon fractions and CO_2

Analysis of carbon isotope ratios of water-soluble and sodium-pyrophosphate fractions were performed as described in chapter 1. Carbon isotope composition of bulk soil and carbon fractions were reported in the δ notation in per mil relative to the Vienna PeeDee Belemnite standard (V-PDB) (Coplen, 2011):

$$\delta^{13}C\ [‰] = \frac{R_S}{R_{Std}} - 1 \qquad (2.1)$$

where R_S and R_{Std} are the ratios of the heavy isotope to the light isotope ($^{13}C/^{12}C$) in the sample and in the international standard, respectively. Delta values were converted to ^{13}C fraction expressed as % (Coplen, 2011) (Equation (2.2)):

$$^{13}C\ [\%] = \frac{100}{\frac{1}{\left(\frac{\delta^{13}C}{1000}+1\right)Rstd} + 1} \qquad (2.2)$$

where $\delta^{13}C$ is the measured isotope value of the sample and R_{Std} is the carbon isotope ratio of the V-PDB standard with 0.0112372 (Slater et al., 2001).

Four milliliters of headspace gas from each vial of the mineralization experiment was transferred to pre-evacuated glass vials (Labco Limited, UK) for analysis of the amount and isotopic composition of CO_2. Carbon isotope analysis of CO_2 was performed using a Thermo Scientific GasBench-PreCon trace gas system interfaced to a Delta V Plus IRMS (ThermoScientific, Bremen, DE). CO_2 was sampled by a six-port rotary valve (Valco, Houston TX) with a 100 µL loop programmed to switch at the maximum CO_2 concentration in the helium carrier gas. The CO_2 was then separated from N_2O and other residual gases isothermically at 45 ºC using a Poraplot Q column (25 m x 0.32 mm ID) and helium as carrier gas at a flow rate of 2.5 mL min^{-1}). Two laboratory standards were analyzed with every 10 samples. The laboratory standards were calibrated directly against NIST 8545.

The $\delta^{13}C$-values of CO_2 were used for calculating the percentage of CO_2-C derived from the added cellulose or lignin, as described previously (Waldrop and Firestone, 2004).

2.5. Phospholipid fatty acids analysis (PLFA)

Phospholipids were extracted from 6 g of soil using chloroform-methanol-water extraction as described by Bligh and Dyer (1959). After lipid fractionation on silicic acid columns (Sep-Pak Silica, Waters), the phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al., 1985) and designated as described by Frostegård et al. (1993). The complete dried FAME fraction was dissolved in n-hexane containing 0.23 mg ml^{-1} of 21:0 FAME as internal standard. Gas chromatographic-mass spectrometric analysis of FAMEs was carried out as described previously (Bastida et al., 2013). The absolute and relative amounts of FAMEs in the

samples were determined according to the concentration of the internal standard added.

The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t are characteristic bacterial fatty acids and were chosen as bacterial biomarkers (Frostegård et al., 1993; Dungait et al., 2011). The fatty acids i15:0, a15:0, i16:0 were taken as representatives for Gram-positive bacteria; and i17:0 cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t were taken as representatives for Gram-negative bacteria (Frostegård et al., 1993; Dungait et al., 2011). The 18:2ω6 was taken as indicator of fungal biomass (Brant et al., 2006; Rinnan and Baath, 2009).

2.6. Carbon isotope analysis of fatty acids

The carbon isotope composition of FAMEs extracted from control, ^{12}C-substrate and ^{13}C-substrate incubations was analyzed using gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS) system as described previously (Bastida et al., 2011). The $\delta^{13}C$ values of the fatty acids reported were corrected for the carbon introduced during derivatization (Abraham et al., 1998) and converted to ^{13}C-fraction (%) following Equation (2.2). Samples were measured at least in triplicate with an analytical error of less than ± 0.5‰ standard deviation.

The amount of ^{13}C incorporated into fatty acids was calculated as described by Boschker (2004). The percentage of ^{13}C incorporated into fatty acids taken as representatives for Gram-positive bacteria (i15:0, i16:0, i17:0, 10Me16:0, 10Me17:0 and 10Me18:0), Gram-negative bacteria (cy17:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t), and fungi (18:2ω6,9) relative to the total amount of ^{13}C in those fatty acids was used as an indication of the flow of ^{13}C in the different compartments of the microbial community.

2.7. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. The data were submitted to ANOVA of repeated measures (Table A2.1). In the case of PLFA analysis, the intra-subject factor (time) was set up with 5 different levels, and in the case of CO_2 analysis, the intra-subject factor (time) was set up with 6 different levels. The inter-subjects were defined as the treatment (substrate added) and soil (Table A2.1). ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference, at the 95% confidence interval in the lineal

model). In order to determine significant differences among treatments and soils at the same time, the data were also subjected to one-way ANOVA.

3. RESULTS

3.1. Carbon in bulk soil, water extracts and sodium-pyrophosphate extracts

In general, the amount of carbon extracted by hot water was greater in the Santomera soil than in the Abanilla soil and decreased in all microcosms during the incubation (Fig. A2.1, annex). The Santomera soil also exhibited about 10 times higher carbon content of sodium-pyrophosphate extracts than the Abanilla soil. Incubation with ^{13}C-lignin resulted in a higher ^{13}C-enrichment within the bulk soil carbon than incubation with ^{13}C-cellulose (Fig. 2.2). Up to 78% and 25% of the added ^{13}C was found in bulk soil after 4 months of incubation with labeled lignin and cellulose, respectively. In both soils, the ^{13}C-fraction in bulk soil carbon decreased during incubation (Fig. 2.2).

The ^{13}C enrichment was analyzed in water-soluble and sodium-pyrophosphate extractable fractions. Overall, the percentage of ^{13}C recovered with sodium-pyrophosphate was much higher than the amount recovered with water in both soils (Fig. 2.2).

The percentage of ^{13}C recovered with hot water was higher in Santomera soil (up to 1.6%) than in Abanilla soil (up to 1.1%). Noticeably, the recovery of ^{13}C in hot water was higher in soils amended with lignin than in those amended with cellulose in both soils (P<0.05). The percentage of ^{13}C recovered with sodium-pyrophosphate was also higher in Santomera than in Abanilla soil. The percentage of lignin-derived ^{13}C in sodium-pyrophosphate extracts reached up to 11% and 13% in Abanilla and Santomera soils, respectively (Fig. 2.2). However, the maximum recovery of cellulose-derived ^{13}C in sodium-pyrophosphate extracts was 3% in Santomera soil.

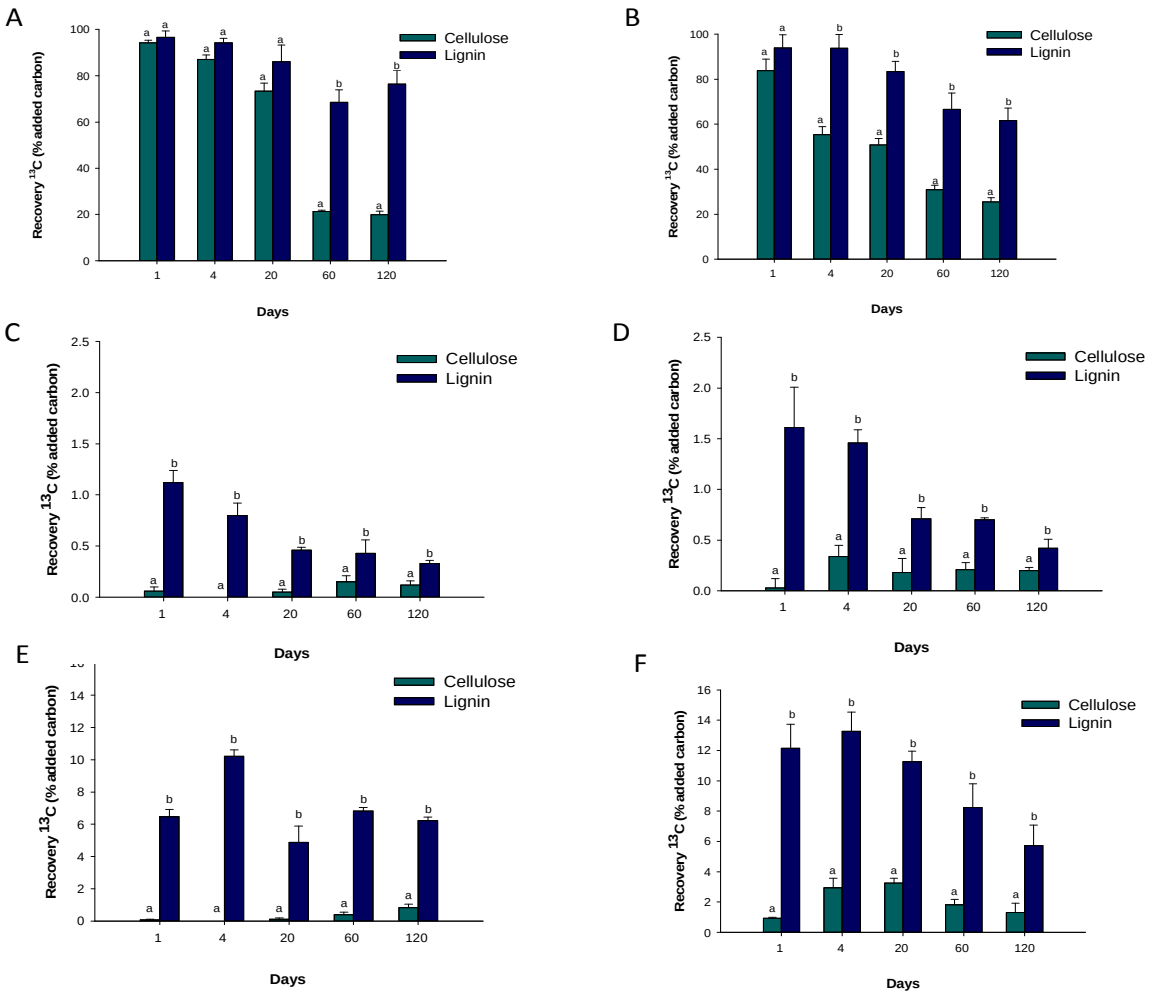


Fig.2.2. Recovery of ^{13}C in bulk soil in Abanilla and Santomera, respectively (A and B), water soluble extract (C and D) and sodium pyrophosphate extract (E and F) after addition of ^{13}C-cellulose and lignin. For each incubation time, data followed by the same letter are not significantly different (P<0.05).

3.2. Substrate mineralization

Mineralization of ^{13}C-lignin or ^{13}C-cellulose was already detectable after 0.5 days of incubation but the highest percentage of substrate mineralization occurred after 4 days of incubation. After 30 days of incubation in Santomera and Abanilla soils, 39.7% and 9.3% of the added ^{13}C-cellulose and 34.9% and 8.1% of the ^{13}C-lignin were mineralized, respectively (Fig. 2.3).

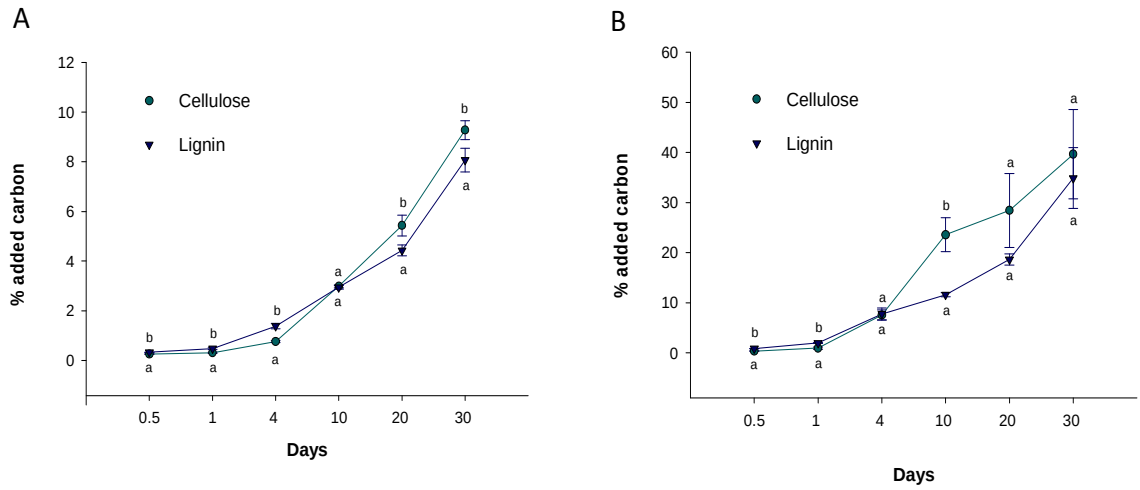


Fig.2.3. Cellulose and lignin mineralization in Abanilla (A) and Santomera (B) soil. For each incubation time, data followed by the same letter are not significantly different (P<0.05).

3.3. PLFA concentration and ^{13}C incorporation into PLFA (PLFA-SIP)

Microbial biomass, as estimated by PLFA concentration, was higher in the Santomera soil than in Abanilla soil (Fig. A2.2 and A2.3, annex). In both soils, the PLFA concentration of all microbial groups tended to decrease during incubation. The PLFA content of both soils did not respond immediately to substrate addition. Indeed, an increase in the PLFA content of soils with substrates was only observed after 20 days of incubation (Fig. A2.2 and A2.3, annex).

Fatty acids extracted from controls as well as from ^{12}C-cellulose and ^{12}C-lignin amended soil exhibited a ^{13}C-fraction of 1.08% ± 0.002. Incubation of soils with ^{13}C-cellulose or ^{13}C-lignin resulted in a fast ^{13}C-labeling of PLFAs already after 1 day of incubation (Fig. 2.4). For instance, the enrichment in the fungal 18:2ω6,9 fatty acid ranged from ^{13}C-fraction of 10%-6% during the incubation of Abanilla soil with lignin, and this enrichment was significantly higher (P<0.05) than in Gram-positive and Gram-negative bacteria representative fatty acids. However, the enrichment of fungal fatty acid did not significantly differ from those of bacterial fatty acids in Santomera soil (Fig. 2.4). In soils amended with cellulose, the enrichment in the fatty acid 18:2ω6,9 reached up to a ^{13}C-fraction of 4% both in cellulose and lignin treatments after 20 days of incubation.

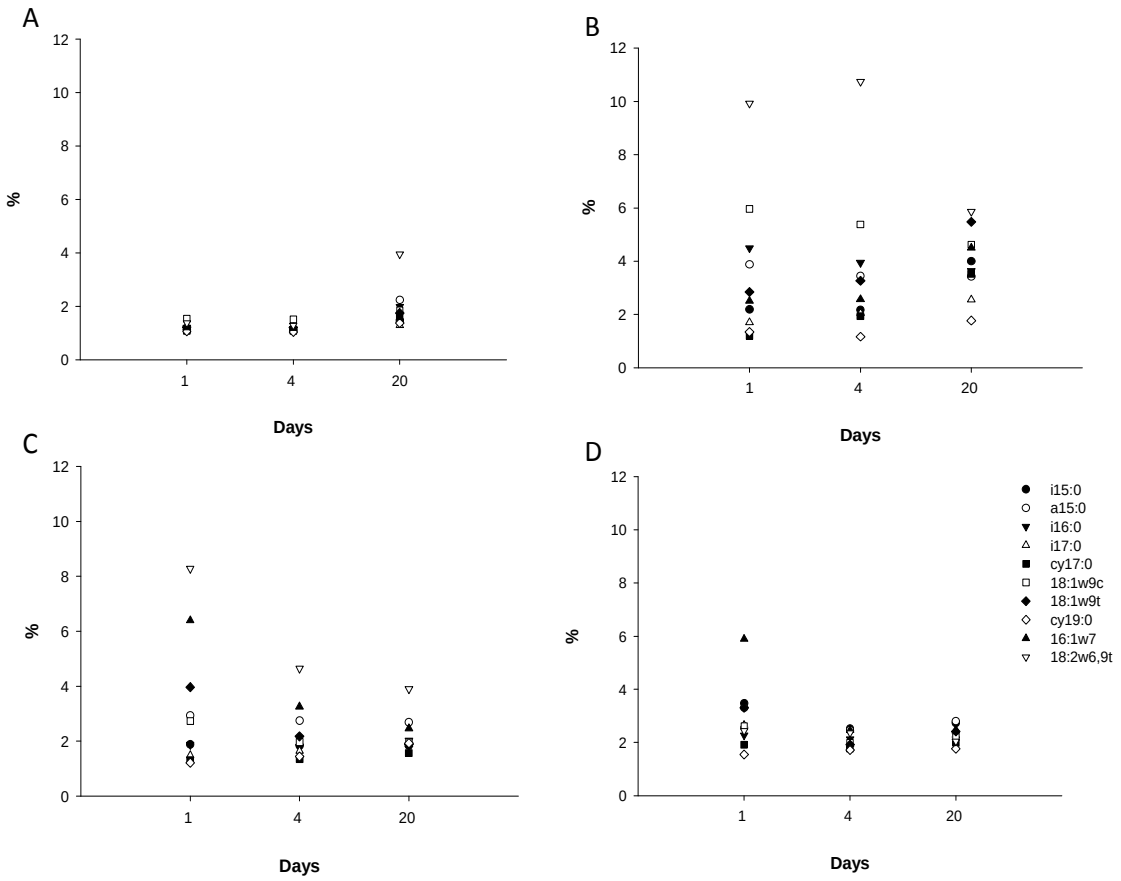


Fig.2.4. ^{13}C-fractions in fatty acids given in %. (A) Abanilla soil amended with ^{13}C-cellulose, (B) Abanilla soil amended with ^{13}C-lignin, (C) Santomera soil amended with ^{13}C-cellulose and (D) Santomera soil amended with ^{13}C-lignin. Standard deviation was always below 10% of the mean. i15:0, a15:0, i16:0, i17:0 were taken as indicator for Gram-positive bacteria, cy17:0, 18:1w9c, 18:1w9t, cy19:0, 16:1w7 as measure of Gram-negative bacteria and 18:2w6,9 corresponded to Fungi.

The total amount of ^{13}C found in fatty acids was much lower than the amount of ^{12}C (please, note the differences units in (Tables 2.1 and 2.2). Overall, the absolute amount of ^{13}C incorporated into PLFAs was higher in Santomera soil than in Abanilla soil. In Abanilla soil, the amount of lignin-derived ^{13}C in the microbial community was significantly higher (with values up to 448 ng ^{13}C allocated in bacterial PLFAs) than the amount of cellulose-derived ^{13}C (maximally up to 210 ng ^{13}C in bacterial PLFAs) (P<0.05). Both bacteria and fungi assimilated a higher amount of lignin-derived ^{13}C than cellulose-derived ^{13}C in Abanilla soil (Table 2.1). In contrast, fungi assimilated more ^{13}C from cellulose than lignin in Santomera soil during incubation. Regarding bacterial groups, both Gram-positive and Gram-negative bacteria hosted a higher amount of lignin-derived ^{13}C than cellulose-derived ^{13}C in Abanilla soil. However, Gram-negative bacteria in Santomera soil did not differentially assimilate ^{13}C derived from cellulose or lignin during incubation (Table 2.1).

Table 2.1. Nanograms of ^{13}C content in microbial biomass in Abanilla and Santomera soil during incubation.

		1 day		4 days		20 days	
		Cellulose	**Lignin**	**Cellulose**	**Lignin**	**Cellulose**	**Lignin**
Abanilla soil	**Bacteria**	121.9a	326.0b	82.4a	356.8b	211.1a	448.8b
	Gram-negative	83.6a	261.7a	59.9a	258.6b	141.7a	330.2b
	Gram-positive	38.3a	64.3b	22.4a	98.2b	69.3a	118.6b
	Fungi	9.4a	85.9b	7.1a	70.2a	30.5a	45.8b
Santomera soil	**Bacteria**	563.6a	669.8a	519.4a	528.2a	445.8a	700.5a
	Gram-negative	364.9a	339.3a	346.2a	321.3a	277.6a	283.7a
	Gram-positive	198.7a	330.5b	173.2a	206.9a	168.2a	416.8b
	Fungi	111.8a	42.6a	103.5b	65.6a	131.7a	49.4a

For each incubation time and microbial group, data followed by the same letter are not significantly different ($P<0.05$).

Out of the total ^{13}C in PLFAs, the percentage of ^{13}C was higher in Gram-negative than in Gram-positive biomarkers, both in cellulose- (up to 67%) and lignin- (up to 68%) amended Abanilla soil (Fig. 2.5). Out of the total ^{13}C in fatty acids, the percentage of ^{13}C in Gram-positive biomarkers reached its highest value at the first day (25-29%) in Abanilla soil treated with cellulose. In the case of lignin, the percentage of ^{13}C in Gram-positive bacteria increased over time and reached up to 24% at 20 days (Fig. 2.5).

In Santomera soil, the percentage of ^{13}C was higher in Gram-negative than in Gram-positive bacteria for the cellulose treatment. However, in lignin-treated soils, the percentage of ^{13}C in Gram-positive fatty acids was equal to or higher than the percentage in Gram-negative fatty acids (Fig. 2.5).

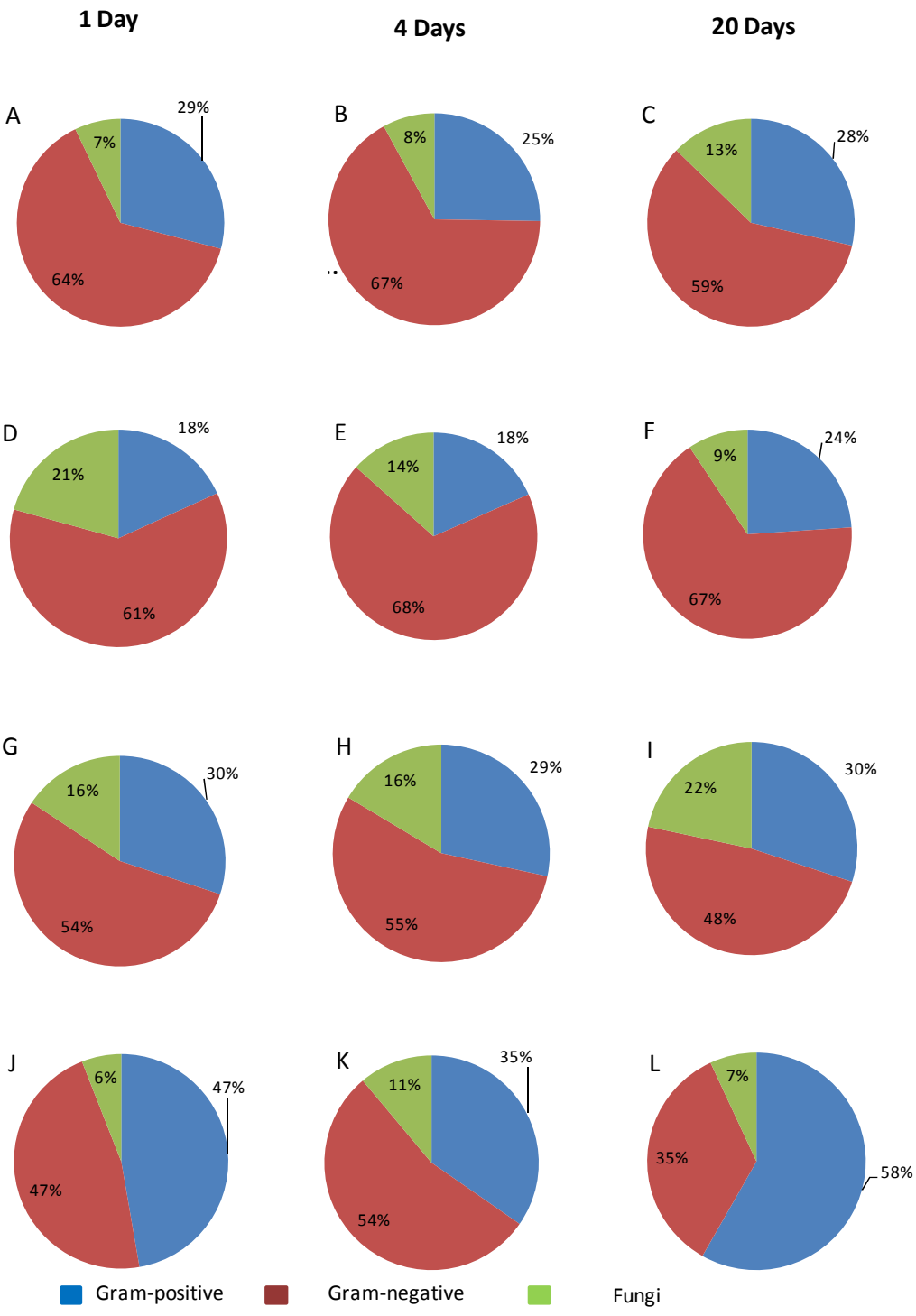


Fig.2.5. Percentage distribution of ^{13}C incorporated into PLFA which is shared by specific groups of the community after 1, 4 and 20 days of incubation. (A, B and C) Abanilla soil with added cellulose, (D, E and F) Abanilla soil with added lignin, (G, H and I) Santomera soil with added cellulose, (J, K and L) Santomera soil with added lignin.

Table 2.2. Micrograms of ^{12}C content in microbial biomass in Abanilla and Santomera soil during incubation.

		1 day			4 days			20 days		
		Control	**Cellulose**	**Lignin**	**Control**	**Cellulose**	**Lignin**	**Control**	**Cellulose**	**Lignin**
Abanilla soil	**Bacteria**	13.9b	8.9a	9.6a	18.2b	5.9a	10.1a	10.8a	11.2a	9.8a
	Gram-positive	4.6b	3.2a	2.7a	6.1b	1.9a	2.8a	3.3a	3.8a	3.4a
	Gram-negative	9.3c	5.7a	6.9b	12.1b	4.1a	7.3a	7.5a	7.4a	6.4a
	Fungi	1.3a	0.6a	0.7a	1.2b	0.5a	0.7a	0.6a	0.8a	0.7a
Santomera soil	**Bacteria**	38.9c	19.3a	24.2b	37.0b	23.1a	23.4a	23.9a	21.1a	30.8b
	Gram-positive	11.8c	8.6a	10.1b	13.9b	8.2a	7.9a	9.0a	7.6a	15.7b
	Gram-negative	27.1c	10.7a	14.2b	23.1b	14.9a	15.6a	14.9a	13.5a	15.0a
	Fungi	2.6c	1.3a	1.9b	2.0a	2.2a	2.5a	1.2a	2.1b	2.2b

For each incubation time and microbial group, data followed by the same letter are not significantly different ($P<0.05$).

4. DISCUSSION

4.1. Long-term fate of added carbon in semiarid soils

Considering the results of this study together with previous research in the same soil types but amended with ^{13}C-glucose (Chapter 1), we noticed that lignin-carbon lasted longer than cellulose or glucose carbon in semiarid soils. Hence, our result points to a major importance of the molecular structure of fresh organic matter in its stability in soil, at least in short-term. In contrast, other studies have highlighted that molecular structure alone does not control the stability of soil organic matter at long term (Schmidt et al., 2011).

The recovery of ^{13}C in sodium-pyrophosphate extracts was always higher than in water extracts (Fig 2.1.). Water-soluble extracts include the most labile and available fractions (Cook and Allan, 1992), while sodium-pyrophosphate extracts include additionally more stable carbon pools (Stevenson, 1982; Chapter 1). Our results indicate that a minor portion of cellulose and lignin was quickly subjected to humification. Nevertheless, the contribution of lignin-derived C to the pool of humic substances was higher than that of cellulose in both soils.

The process of humification may involve the degradation of macromolecular compounds into small compounds that are stabilized in soil (Eusterhues et al., 2003). Additionally, compounds derived from the microbial biomass that has been enriched in ^{13}C may be incorporated into the humic substance fraction after cell death.

The recovery of ^{13}C-derived in water-extracts from lignin or cellulose was very low in both cases and followed a different time response. The amount of carbon extracted was higher at the beginning and then progressively declines particularly in the case of lignin. This phenomenon could be attributed to several factors: physical entrapment (Thevenot et al., 2010), chemical transformation and partial mineralization during incubation. However, cellulose behaved differently and the amount of ^{13}C-derived from this substrate extracted with water increased with time. Despite the initially higher level of un-extractability of cellulose-derived carbon, the generation of secondary metabolites by microbial metabolism could explain the higher recovery of cellulose-derived carbon at long-term (Jenkinson et al., 1987).

4.2. Short-term mineralization of lignin and cellulose

A low ^{13}C-enrichment of CO_2 was observed in soils with ^{13}C-cellulose and ^{13}C-lignin at the initial incubation times. These results indicated that, even if the substrate

mineralization was low at the beginning, a minor part of the microbial community kept the metabolic capacity for processing these vegetal-like compounds in soils that are highly degraded. In any case, the initial mineralization of these substrates was slow, particularly if we compared with the fast mineralization of cellulose found by other authors (Fontaine et al., 2004). The slow mineralization of substrates at the beginning may be due to the low amount of microbial biomass present in degraded soils (which ranged between 16 and 49 mg C kg^{-1} soil in Abanilla and 200-450 mg C kg^{-1} soil in Santomera) (Bastida et al., 2006a). Moreover, a correlation between biomass and substrate respiration has been observed by other authors (Waldrop and Firestone, 2004; Brant et al., 2006; Guenet et al., 2011). Furthermore, the initial un-adaptation of the major part of the microbial community for the mineralization of plant-derived substrates would explain the low mineralization capacity at the initial incubation times. In contrast, the slope of substrate mineralization increased from the 4th day of incubation until 30 days, which could suggest an adaptation of microbial community for the mineralization of added substrates.

It is worth of mentioning that the amount of ^{13}C-derived from substrates in microbial biomass at the first day was remarkable in comparison to the scarce amount of substrates that was respired. Products of cellulose and lignin degradation might be stored (and not mineralized) in cell and serve as precursors for fatty acid biosynthesis (Hershberger et al., 1956). Indeed, some authors have noticed that carbon substrates are preferentially stored rather than used for growth (Nguyen and Guckert, 2001) or that respiration is delayed in comparison to storage within microbial cell (Hill et al., 2008).

4.3. Microbial dynamics in relation to cellulose and lignin mineralization

The microbial biomass decreased during incubation in control and amended soils. This decrease could be due, among other factors, to low availability of substrates, as illustrated by the decrease in water-soluble carbon in both soils, and particularly in Abanilla. Water-soluble carbon contains easily available carbon that can be used as an energy source by microorganisms (Cook and Allan, 1992).

It is important to note that the addition of substrates did not generally support the development of microbial biomass, which goes against logic, since microbial growth in semiarid degraded soils is usually hampered by the scarcity of organic matter (Bastida et al., 2006a). Indeed, an increase in the PLFA content has been observed after the addition of a similar dose of carbon in the form of glucose in previous studies (Bastida

et al., 2013). Noteworthy, carbon quality is particularly important because it restricts the supply of energy for microbial growth in soil (Fontaine et al., 2003). Some authors have even observed a decrease in microbial biomass after the addition of cellulose and lignin (Schutter and Dick, 2001). For instance, DeAngelis et al. (2011) affirmed that lignin can create a non-favorable chemical environment for the development of microbial biomass, with the exception of certain populations that are able to use lignin. In our study, the only positive response to cellulose or lignin amendment was found at 20 days and was dependent on the soil properties, substrate and microbial group. Besides the fact that an important fraction of added carbon could be immobilized in soil mineral particles (Derrien et al., 2006; Ekschmitt et al., 2008), we also found that only a small fraction of the microbial community assimilated carbon from the added substrates. This is logical, since the enzymatic degradation of macromolecular organic carbon compounds occurs in a limited number of microbial populations (Hammel, 1997; Hu and van Bruggen, 1997; Waldrop et al., 2000). It makes even more sense in our desert-like ecosystems, where the microbial community is not adapted to abundant vegetal inputs. However, it is important to note that the ecological value of such microbial biomass able to assimilate vegetal-like compounds is fundamental, since they are the starters of carbon cycling.

Despite the lower fungal biomass in comparison to bacteria, Fontaine et al. (2011) noted that cellulose decomposition was mainly processed by soil fungi due to stronger competitive advantages over bacteria. We agree with the important role of fungi in cellulose degradation in Santomera soil from the first day of incubation with respect to the high ^{13}C-enrichment of the 18:2ω6,9 biomarker in comparison to Gram-negative and Gram-positive bacteria (Fig. 2.3 and 2.4). However, in Abanilla soil, fungi were the main drivers of lignin assimilation. In both cases, the assimilation of substrate-derived C into fungal biomass occurred rapidly and decayed during incubation, but always maintained a higher level of enrichment than the bacterial fatty acids. In the case of Abanilla soil, the final decay of fungi enrichment in lignin-amended soil occurred in parallel to a peak of ^{13}C-assimilation in Gram-positive bacteria. This pattern also been found after amendment with ^{13}C-phenol (Brant et al., 2006), and can be explained by an ecological succession related to the degradation of lignin or by a carbon flux from fungi to such bacterial groups as a consequence of grazing phenomena. Rinnan and Baath (2009) explored the assimilation of vanillin in fatty acids of tundra soil. The carbon derived from vanillin, a common product of lignin depolimerization, was quickly assimilated by fungi in tundra soil, but there was a lower level of enrichment in the fungal biomarker (18:2ω6,9) than that found in some Gram-negative biomarkers.

However, phenol and vanillin are simple structural units constitutive or derived parts of lignin. Nonetheless, the absence of studies based on the stable isotope probing of labeled lignin limits the extension of our discussion.

If we consider the total amount of ^{13}C found in the different microbial groups (Fig 2.4), Gram-negative bacteria followed by Gram-positive bacteria and fungi are important for the assimilation of lignin and cellulose. These results are in agreement with those found by Haichar et al. (2007) using a DNA-SIP approach with labeled-cellulose. These authors found a higher number of gene sequences related to Gram-negative bacteria in the heavy fractions (i.e. Sphingomonadaceae, Mesorhizobium sp., Flavobacterium sp, Myxobacterium sp., etc.) than to Gram-positive bacteria (Streptomyces sp.).

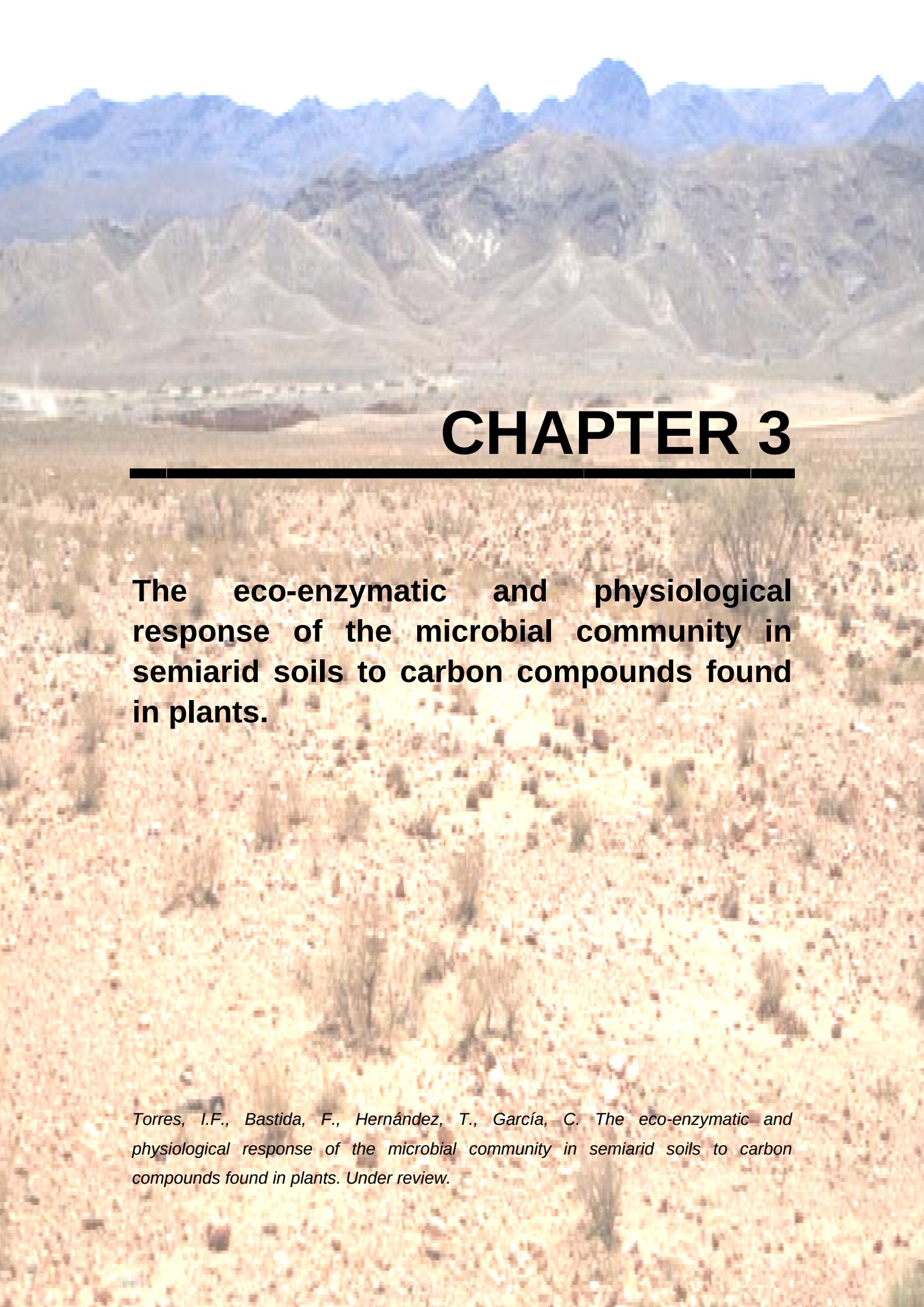
5. CONCLUSIONS

A major proportion of cellulose- and lignin-derived carbon remained in soil, and only a small fraction of the microbial community was able to assimilate the carbon derived from those macromolecular substrates. However, this community was fundamental for starting the biogeochemical cycling of carbon in desert-like ecosystems. According to the proposed objectives and hypothesis we conclude that:

i) At long-term, the stability of carbon was highly dependent on the chemical nature of the substrate, lignin being more stable than cellulose in both types of soils at long-term. Lignin contributed more than cellulose to the pool of humic substances.

ii) At short-term, substrate mineralization was more related to the soil properties (i.e. microbial biomass) and soil degradation level than on the chemical structure of the substrate.

iii) Microbial assimilation of cellulose- and lignin-derived carbon was higher in the soil with the highest level of biomass (Santomera). The relative contribution of each microbial group to the assimilation of cellulose-derived C was common in both soils. Cellulose assimilation was highly mediated by fungi in both soils, while Gram-negative rather than Gram-positive bacteria exerted an important role on the assimilation of this substrate. However, contrary to the suggested hypothesis, the relative contribution of each microbial group to the assimilation of lignin-derived carbon was different in each soil.



CHAPTER 3

The eco-enzymatic and physiological response of the microbial community in semiarid soils to carbon compounds found in plants.

Torres, I.F., Bastida, F., Hernández, T., García, C. The eco-enzymatic and physiological response of the microbial community in semiarid soils to carbon compounds found in plants. Under review.

ABSTRACT

Cellulose and lignin are the main energy and nutrient sources available for soil microorganisms. In semiarid soils, however, the transformation of organic matter is limited by climatic conditions and minimal amounts of microbial biomass. In the present study, cellulose and lignin were added to two semiarid soils showing different levels of degradation. One of these soils, Abanilla soil, has a total organic C content of 5.0 kg^{-1} while the other soil, Santomera soil, has a total organic C content of 12.0 kg^{-1}. Soil enzyme activities, respiration and community-level physiological profiles were analysed in both soils after the addition of cellulose and lignin. Abanilla soil, the most degraded soil, showed a fast response to the addition of cellulose and lignin molecules at both the enzymatic and catabolic levels. On the other hand, while there were notable differences in the functional structure of the microbial community in Santomera soil at 120 days, changes were slower than in Abanilla soil. Furthermore, catabolic diversity was higher in Abanilla soil, suggesting that degraded semiarid soils have a high potential to degrade vegetal input even if they have not received plant residue for a long time.

1. INTRODUCTION

Cellulose is one of the major components of plant material (between 30-60%), together with hemicelluloses and lignin. Cellulose $(C_6H_{10}O_5)_n$ consists of long polymers of β-1,4-linked glucose units, which in turn form higher order fibular structures (Nazir et al., 2010). Lignin $(C_{31}H_{34}O_{11})_n$ is a complex organo-aromatic polymer of phenylpropane residues linked by a variety of chemical bonds (Kirk and Farrell, 1987). These two compounds are introduced into soil by plant residue and are the main energy and nutrient sources available for soil microorganisms. The decomposition of these organic materials plays an important role in the biogeochemical cycling of carbon and in the structure and activity of the soil microbial communities (Swift et al., 1979; Paul and Clark, 1996; Bardgett et al., 2005). However, under arid and semiarid conditions, litter deposition is limited by scarce vegetal growth as a consequence of climatic constraints. Under these circumstances, microbial biomass is strongly limited by the lack of input of energy sources into the soil system. Furthermore, the responsiveness of the microbial communities in these ecosystems to compounds derived from plants is not widely known.

The oxidation of several substrates in the community-level physiological profile (CLPP) plates by microorganisms can produce utilisation patterns that may differ according to the microbial community and/or habitat. CLPP analysis has been used by a number of researchers to study functional changes in the soil microbial community after the addition of organic matter (Waldrop et al., 2000; Schutter and Dick, 2001; Gómez et al., 2006; Guenet et al., 2011). Several studies have indicated that changes in the functional structure of the soil microbial community as a consequence of vegetal input may determine variations in the rate of eco-enzyme and metabolic processes involved in the biogeochemistry of the soil. For instance, Degens (1998) showed that the addition of simple organic substrates to soil changed the metabolic profiles and metabolic diversity of soil microbial communities. Overall, our aim was to elucidate whether the microbial communities of degraded soils with minimal or non-existent vegetal cover and scarce organic matter are potentially able to manage plant-derived carbon polymers. The specific aims of this study are: i) to compare the impact of cellulose and lignin addition on the enzyme activities related to the C, N and P cycles of two soils showing different levels of degradation; and ii) to explore the connections between enzyme activities and the physiological capabilities of the microbial community in response to cellulose and lignin. For these purposes, we have combined the use of different enzymes linked to the C, N and P cycles with an analysis of the

metabolic capabilities of the microbial community using CLPPs. Due to the low carbon content in the soils studied, we hypothesised that the C-enzymes would have low sensitivity to the addition of trace amounts of polymeric carbon compounds and that the enzymes involved in the N and P cycles would not be affected.

2. MATERIAL AND METHODS

2.1. Study area and soil sampling

The two different soils studied (Abanilla and Santomera) were selected in the region of Murcia located in the south-east of Spain. Both soils are subjected to a semiarid climate with a mean annual rainfall of less than 300 mm and a mean annual temperature of 18ºC. Furthermore, both soils were used for agricultural purposes in the past, and both have undergone natural degradation processes due to the adverse climatic conditions found in south-east Spain. The two soils show different levels of degradation in terms of total organic C and nitrogen content, vegetal cover and microbial biomass and activity (Bastida et al., 2006a). Moreover, these differences may influence the dynamics of cellulose and lignin in the two soils.

The first soil, Abanilla, was located in the municipality of Abanilla in the region of Murcia (SE Spain). This soil is classified as Calcaric regosol (Soil Survey Staff, 1998) and is highly degraded with no vegetation growing on it (Bastida et al., 2006a). This soil was thus selected as an adequate model for studying the processes of carbon transformation in a pre-desert environment. The soil particle distribution was as follows: 34.6% clay, 7.7% silt and 57.7% sand. Abanilla soil has a pH of 7.8, an electrical conductivity of 2.6 dS m^{-1}, a total N content of 1.3 g kg^{-1}, a total C content of 40 g kg^{-1} and a total organic C content of 5.0 g kg^{-1}.

The second soil, Santomera, was located in the municipality of Santomera, also located in the region of Murcia (SE Spain). The soil is classified as Haplic calcisol (Soil Survey Staff, 1998) and represents a less degraded soil (Bastida et al., 2006a). The plant cover on Santomera soil was around 20% and was dominated by xerophytic shrubs such as Helianthemum almeriense, *Moricandia arvensis*, *Anthyllis cytisoide*s and *Asparagus horridus*. The soil particle distribution was 18.8% clay, 9.5% silt and 71.7% sand. Santomera soil has a pH of 7.7, an electrical conductivity of 0.3 dS m^{-1}, a total N content of 1.0 g kg^{-1}, a total C content of 71.2 g kg^{-1} and a total organic C content of 12.0 g kg^{-1}.

Within these two areas described above, three plots (n=3) of 20 m^2 each were selected. Six subsamples were taken from the upper 15 cm of each plot and pooled to obtain one composite sample per plot. These soil samples were sieved through a < 2 mm mesh and stored at 4ºC until the beginning of the incubation experiments.

2.2. Experimental design and soil incubation

An incubation experiment was carried out in containers with 100 g of soil. Each treatment was prepared in independent replicates (n=3) for each incubation time (1, 60, and 120 days). Incubation was performed in chambers at 28ºC with controlled moisture. Either cellulose or lignin (75 µg C g^{-1} soil) was added to each sample. The amount of substrates used was in the same range as the carbon applied in field restoration experiments and in previous isotope labelling experiments (Chapter 1 and 2). This amount is considered as a trace dose and represents less than 1.5% of the autochthonous organic carbon of the soils. The water-holding capacity of the soil was gravimetrically adjusted to 60% during incubation for all treatments. Furthermore, a control experiment was also conducted on soil without added cellulose or lignin which was subjected to the same conditions as the other two treatments. Before the substrates were added, soils were pre-incubated with distilled water for two weeks in order to avoid drastic effects of moisture on the measured parameters when adding the substrates.

2.3. Enzyme activities

The urease activity in the soil was determined by the buffered method of Kandeler and Gerber (1988). In this procedure, 0.5 mL of a solution of urea (0.48%) and 4 mL of borate buffer (pH 10) were added to 1 g of soil and then incubated for 2 h at 37 ºC. The ammonium concentration of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without urea to determine the amount of ammonium produced in the absence of this substrate (Fig. 3.1).

Fig.3.1. Urease activity

Phosphomonoesterase activity was determined following the method described by Tabatabai and Bremner (1969), by adding 2 mL of MUB (modified universal buffer) pH 11 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl phosphate to 0.5 g of soil. The level of β-glucosidase activity, on the other hand, was determined according to a modification of Tabatabai's method (1982), by adding 2 mL of MUB pH 6 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl *β*-D-glucopiranoside to 0.5 g of soil. The mixtures were then incubated at 37 ºC for 1 h, after which time the enzymatic reactions were stopped by cooling on ice for 15 min. Then, 0.5 mL of 0.5 mol L^{-1} $CaCl_2$ and 2 mL of 0.5 mol L^{-1} NaOH were added for the phosphatase activity assay and 2 mL of 0.1 mol L^{-1} tris(hydroxymethyl)aminomethane-sodium hydroxide (THAM-NaOH) of pH 12 were added for the for *β*-glucosidase assay. For the control test, the respective substrates were added before the addition of $CaCl_2$ and NaOH.

Polyphenol oxidase activity was determined by Allison's method (2006), using 50 mM pyrogallol/50 mM EDTA as the substrate. The absorbance of the supernatant from the reaction was determined directly at 460 nm after 1 h.

Lipase was measured according to the method of Margesin et al. (2002) by reaction with p-nitrophenyl butyrate 100 mM. Cellulase activity, on the other hand, was determined by a modification of the method of Deng and Tabatabai (1994), using carboxymethylcellulose as substrate. Finally, catalase activity was determined by the method of Johnson and Temple (1964), using H_2O_2 as substrate, which was shaken for 20 min. The filtrate was titrated with 0.1 M $KMnO_4$.

2.4. Microbial respiration

Microbial respiration (CO_2 emission) was measured in 10 ml capped tubes containing 1 g of soil. Soil samples were humified with distilled water at 60% of their water-holding capacity. Vials were hermetically sealed and then incubated in the dark at 28 ºC for 19 days. The concentration of CO_2 was periodically analysed with a gas chromatograph [Trace Ultra Thermo Scientific, Milan (Italy)] using a packed column [Trace PLOT TG-BOND Q GC, Trace Ultra Thermo Scientific, Milan (Italy)] (Fig.3.2).

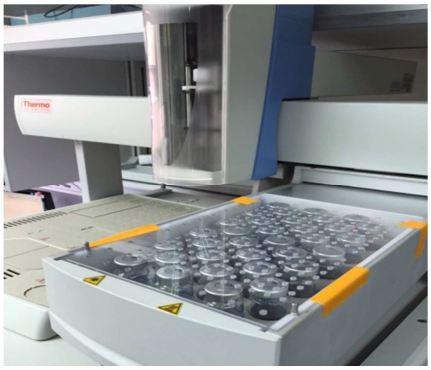


Fig.3.2. Microbial respiration mesure

2.5. Community-level physiological profiles (CLPPs) of the microbial community

Biolog EcoPlates (Biolog, Inc., Hayward, CA, USA) containing 31 different C sources and water were used to perform microbial community-level physiological profiling based on carbon source utilisation (Hitzl et al., 1997). The carbon sources belonged to the following groups: carbohydrates, polymers, carboxylic acids, aromatic compounds, phosphorylated chemicals, amino acids, esters and amines. Two grams of wet samples were shaken in 20 ml of sterile water at 150 rpm for 15 min at 4 ºC. After incubation, a liquid extract was obtained by centrifuging at 2000 rpm for 10 min; 100 µl were inoculated on each Biolog plate well, and the plates were incubated at 28 ºC for 7 days. The microbial biomass of cell extracts was equalised by considering the PLFA content of each sample (Chapter 2). Such standardisation prevents the microbial biomass level from influencing the oxidation of substrates and thus makes it possible to adequately compare the functional community structures of each treatment. The rate of utilisation of the different substrates was indicated by the reduction of tetrazolium, a redox indicator dye which changes from colourless to purple. Data were recorded for one week at 590 nm in an automated plate reader (Multiskan Ascen) until a plateau was reached. Microbial activity was expressed as the average well colour development (AWCD), as described by

Garland (1996). The catabolic diversity (Hbio) for each sample was estimated using the Shannon-Weaver index, calculated from the normalised AWCD data after 70 h of incubation in Biolog EcoPlates (Insam and Goberna, 2004).

2.6. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. In order to determine significant differences between the means of the treatments, data was subjected to one-way ANOVA. Moreover, two-way ANOVA was performed to ascertain the influence of soil type and molecule type. ANOVA was followed by Tukey's significant difference post-hoc test (HSD, honestly significant difference, at the 95% confidence interval in the lineal model). A factor analysis was carried out on the CLPP data to explore possible differences in the functional structure of the microbial communities.

3. Results

3.1. Chemical variables

The total organic C and N content did not vary through the incubation period, and no differences between treatments were observed ($P<0.05$). The applied trace-dose was not sufficient to vary the content of C and N (the amounts were likely below the sensitivity level of the analytical methods used).

3.2. Enzyme activities

Nearly all enzyme activities were higher in Santomera soil than in Abanilla soil, except for polyphenol oxidase activity. Furthermore, the enzyme activity trends were different in both soils and depended on the type of molecule added ($P<0.05$) (Table 3.1). In the case of both soils studied, the enzyme activities did not differ at 120 days ($P<0.05$).

Table 3.1. ANOVA of repeated measures with material stabilization and dose as main factors.

	β-glucosidase		Cellulase		Lipase		Polyphenoloxidase		Phosphatase	
	F	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Molecule	0.00	0.96	132.40	<0.001	0.06	0.81	0.64	0.45	26.57	0.001
Soil	18536.54	<0.001	3229.67	<0.001	2877.79	<0.001	3519.67	<0.001	12862.16	<0.001
molec x soil	0.20	0.67	29.94	0.001	0.86	0.38	0.11	0.75	6.84	0.03
	Urease		**Catalase**		**H**		**Basal respiration**			
	F	***P***	**F**	***P***	**F**	***P***	**F**	***P***		
Molecule	62.11	<0.001	31.08	0.001	38.89	<0.001	12.24	0.01		
Soil	50074.88	<0.001	2206.68	<0.001	270.17	<0.001	4181.68	<0.001		
molec x soil	83.27	<0.001	15.31	0.004	10.46	<0.001	64.51	<0.001		

H: Shannon-Weaver index

i) Enzymes related to carbon cycle

Cellulase activity begins cellulose degradation process. Thus, in Abanilla soil, cellulose addition showed in a significantly increase of cellulose activity over control soil and soil amended with lignin at the first day of incubation (P<0.05). Moreover, at 60 days of incubation, both the cellulose and lignin treatments showed significantly higher cellulase activity levels than the control soil (Fig. 3.3A). In Santomera soil, molecule addition resulted in a significantly increase of cellulase activity regarding control soil after 60 days of incubation (P<0.05) and this activity was higher in soil amended with cellulose than in soil amended with lignin. However, at 120 days, only cellulase activity in soil amended with cellulose remained significantly higher than control soil (P<0.05) (Fig. 3.3B).

β-glucosidase activity catalyses the last stage of cellulose degradation that consists in the hydrolysis of cellobiose to glucose. In Abanilla soil, cellulose addition increased β-glucosidase activity significantly compared to control soil and soil amended with lignin at 60 days of incubation (P<0.05). However, lignin addition did not vary β-glucosidase activity respecting control soil during incubation time (Fig. 3.3C). In Santomera soil, β-glucosidase activity showed significant increases in both the cellulose and lignin treatments with respect to the control soil at the beginning of the incubation period (P<0.05). Moreover, this activity remained higher in soil amended with cellulose regard to control soil until 60 days of incubation but not in soil amended with lignin (Fig. 3.3D).

Polyphenol oxidase activity seems to be involved on the transformation of recalcitrant compounds and humic substances formation. However, in Abanilla soil, the addition of cellulose or lignin only affected polyphenol oxidase activity on the first day of incubation. At this time, soil polyphenol oxidase activity was higher in both treatments than in the control soil (Fig. 3.3E). In Santomera soil, on the other hand, polyphenol oxidase activity levels decreased significantly in the treated soils regarding control soil on the first day. Nevertheless, the activity level of this enzyme was significantly higher in the Santomera soil amended with lignin than in the soil amended with cellulose at 120 days of incubation (Fig. 3.3F).

In Abanilla soil, lipase activity was higher in the soil amended with cellulose than in the other treatments at 1 and 60 days of incubation, although all treatments were equal at 120 days (Fig. 3.3G). In Santomera soil, on the other hand, soil amended with lignin showed the highest levels of lipase activity on the first day of incubation. At 60 days of incubation, however, the lipase activity in the Santomera soil amended

with cellulose was greater than that in the control soil and soil amended with lignin (Fig. 3.3H).

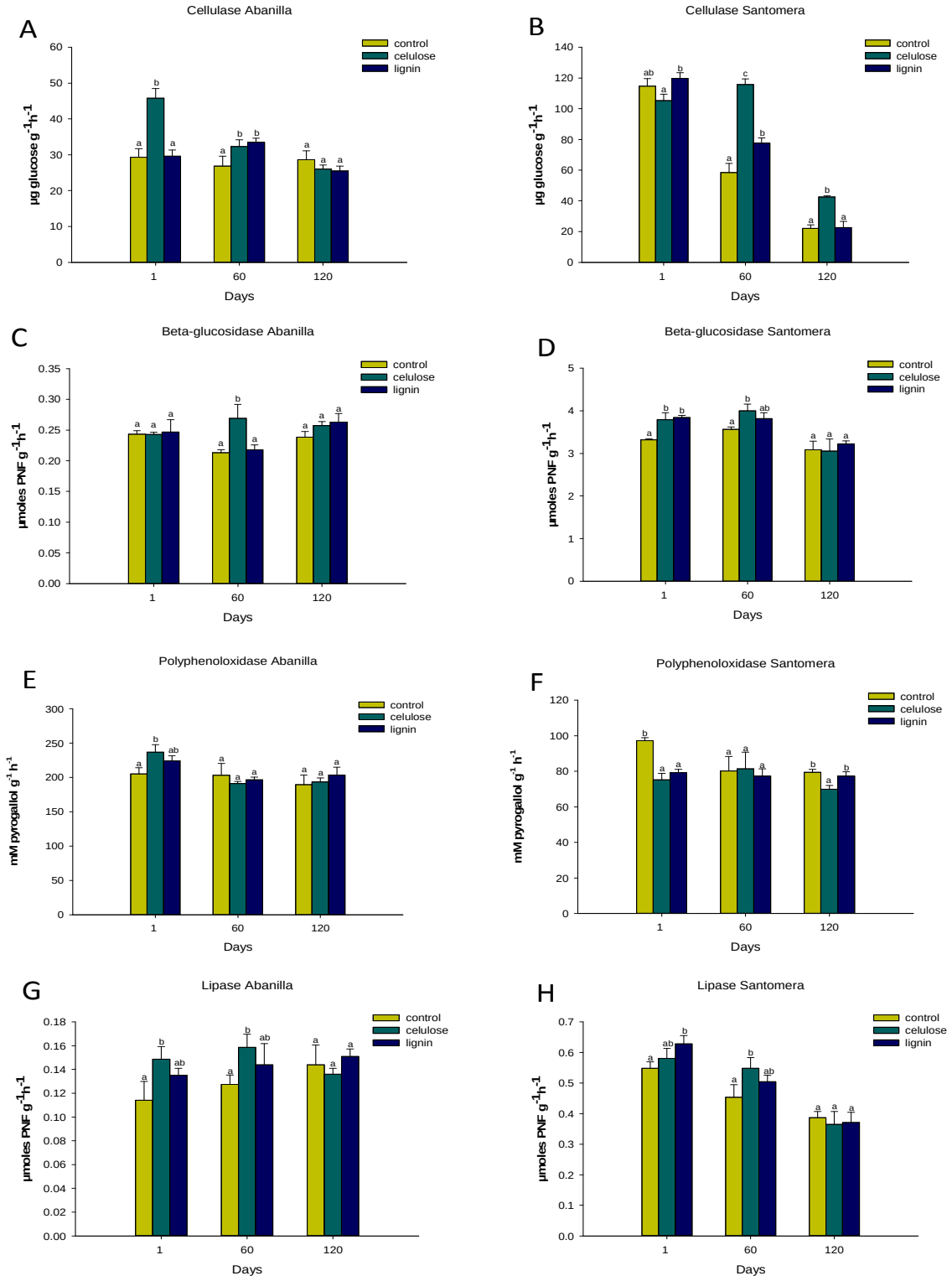


Fig.3.3. Enzymatic activities related to carbon cycle after cellulose and lignin addition in Abanilla and Santomera soil.

ii) Catalase activity

Catalase activity is considered an overall indicator of microbial activity. In the case of Abanilla soil, catalase activity was lower in Abanilla soil with added cellulose and lignin than in the control soil on first day of incubation (P<0.05). Even so, soil amended with lignin showed significantly higher catalase activity levels than the soil amended with cellulose (P<0.05). Nevertheless, soil amended with cellulose showed a greater level of catalase activity than the other treatments at 60 days (Fig. 3.4A). Similarly, in Santomera soil, cellulose addition induced a decrease in catalase activity in comparison to the control soil at the beginning of incubation (P<0.05). Nevertheless, at 60 days of incubation, the catalase activity levels in soil with lignin treatment were equal to levels in the control soil and significantly higher than levels in the soil amended with cellulose (P<0.05) (Fig. 3.4B).

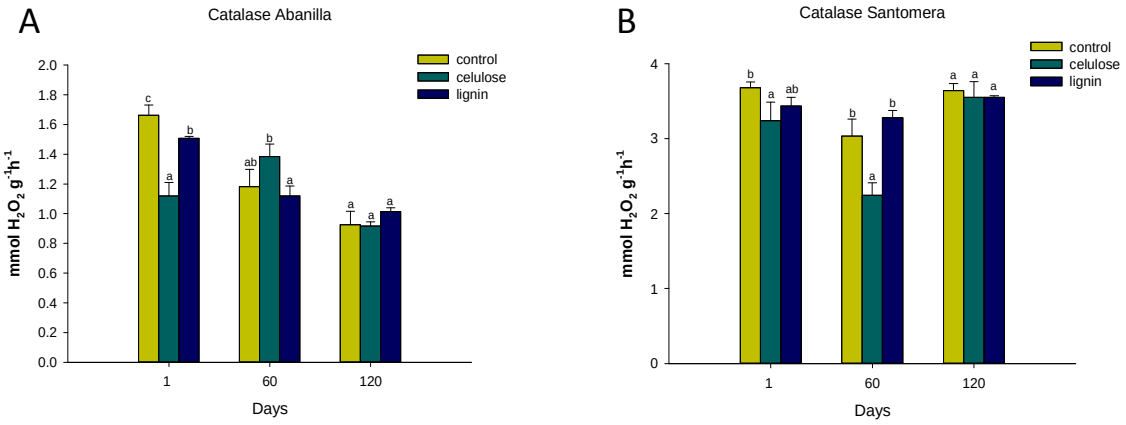


Fig. 3.4. Catalase activity after cellulose and lignin addition in Abanilla and Santomera soil.

iii) Enzymes related to phosphorus and nitrogen cycles

Phosphatase enzyme is responsible for transforming different forms of organic phosphorus to simple forms easily assimilated by plants. In Abanilla soil, phosphatase activity increased significantly with cellulose and lignin addition on the first day of incubation (P<0.05). Nonetheless, by 60 days the activity of this enzyme decreased significantly in the soil treated with organic molecules with respect to the control soil (P<0.05). At this time, phosphatase activity was significantly higher in the soil amended with cellulose than in the soil amended with lignin (Fig. 3.5A). In Santomera soil, soil amended with cellulose showed significantly higher levels of phosphatase activity than the control soil and soil amended with lignin at 60 days of

incubation. No significant differences between treatments appeared at other times (Fig. 3.5B).

Urease enzyme catalyses the hydrolysis of urea to ammonium, In Abanilla soil, urease activity was significantly higher in the control soil and the soil amended with lignin than in the soil amended with cellulose at 1 day of incubation (P<0.05). However, at 60 days, the soil amended with cellulose showed significantly more urease activity than the other treatments (Fig. 3.5C). However, in Santomera soil, on the first day, the levels of urease activity in the soil amended with cellulose and the control soil were significantly higher than in the soil amended with lignin (P<0.05). At 60 days of incubation, there was more urease activity in the soil amended with cellulose than in the other treatments (Fig. 3.5D).

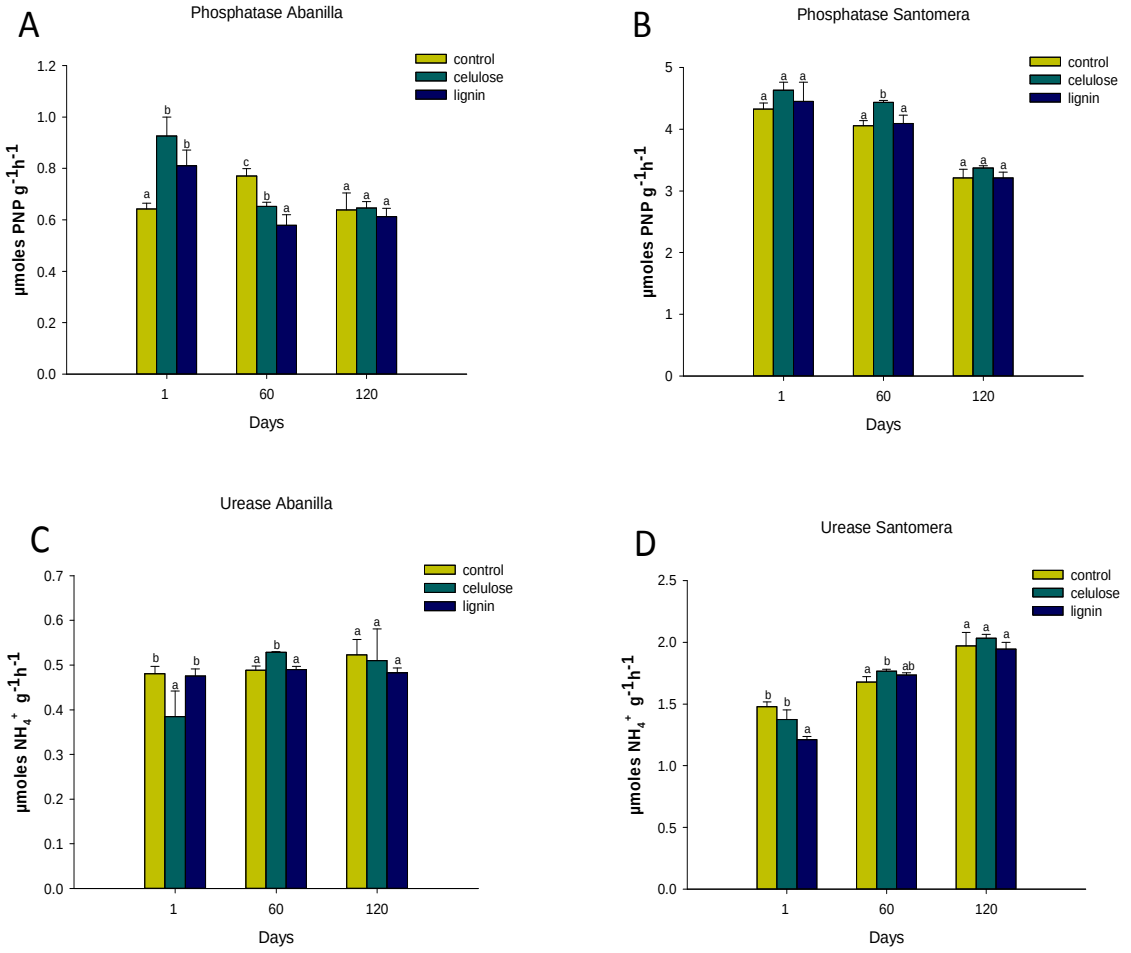


Fig. 3.5. Phosphatase and urease activities after cellulose and lignin addition in Abanilla and Santomera soil.

3.3. Basal respiration of the soil

Basal respiration showed different trends depending on the soil type. Nevertheless, it can be said that the basal soil respiration rate decreased in both Abanilla and Santomera soils over time. Furthermore, in Abanilla soil, CO_2 emissions were significantly higher in the soil amended with cellulose at 1 day of incubation than in the rest of the treatments (P<0.05) (Fig. 3.6). In Santomera soil, the soil amended with lignin showed the highest basal respiration rate at 1 day of incubation (P<0.05). As referred to above, the basal respiration rate was lower in samples from 120 days in both Abanilla and Santomera soils. This trend, however, was particularly marked in Santomera samples.

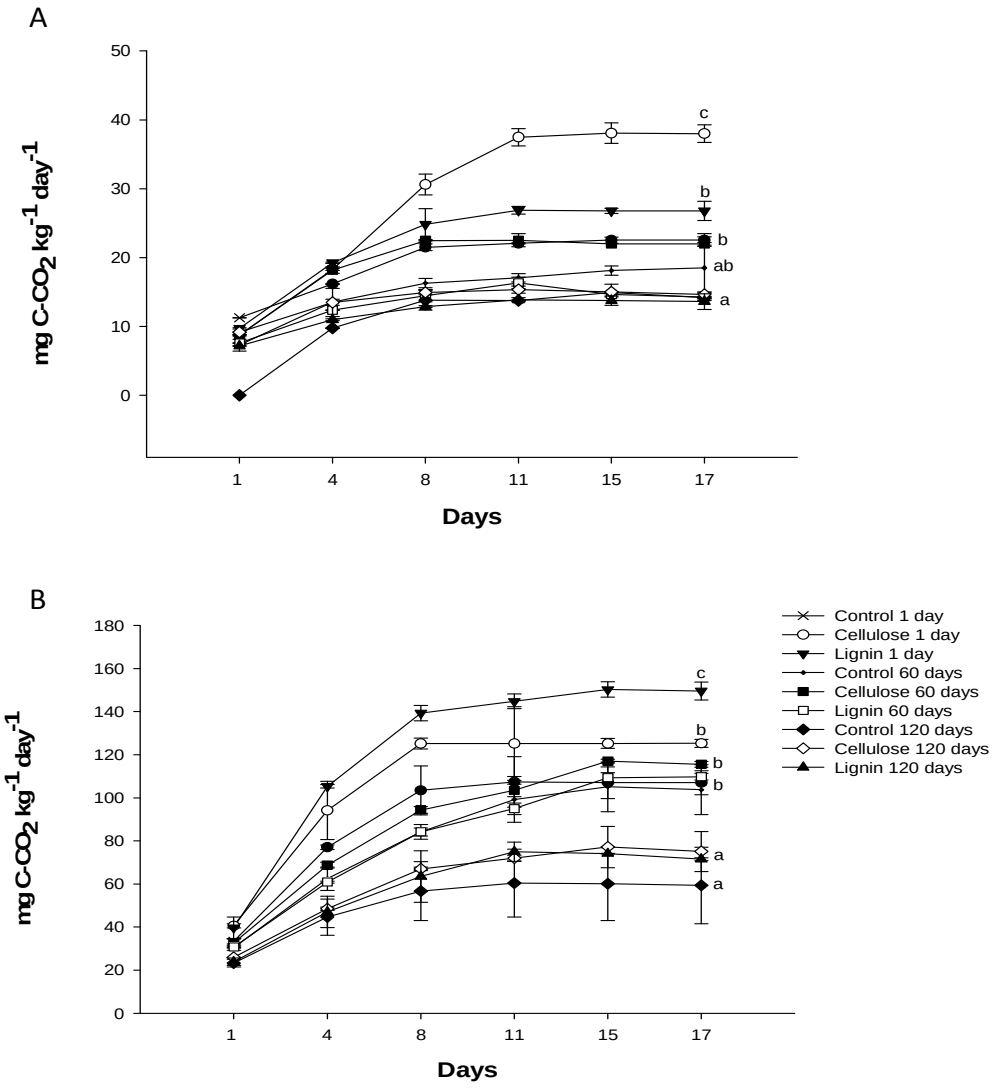


Fig. 3.6. Basal respiration after cellulose and lignin addition in Abanilla and Santomera soil.

3.4. Community-level physiological profiles (CLPPs) of the microbial community

The functional diversity and functional structure of each microbial community were analysed using Biolog EcoPlates. Based on carbon utilisation patterns, the functional catabolic diversity was calculated using the Shannon-Weaver index. The catabolic diversity (H) depended on the type of molecule and soil (Table 3.1) and was higher in Abanilla soil than in Santomera soil. The trend was different in both soils over time, but overall, catabolic diversity increased with the addition of cellulose and lignin (Table 3.2).

Table 3.2. Catabolic diversity (H) of microbial communities after cellulose and lignin addition in Abanilla and Santomera soil.

		1 day	60 days	120 days
Abanilla	**Control**	3.24 a (0.08)	3.04 a (0.04)	3.02 a (0.02)
	Cellulose	3.37 b (0.01)	3.86 c (0.05)	3.69 b (0.38)
	Lignin	3.36 b (0.03)	3.52 b (0.15)	3.07 a (0.01)
Santomera	**Control**	2.66 a (0.03)	2.68 a (0.09)	2.65 a (0.09)
	Cellulose	2.89 b (0.10)	2.85 b (0.05)	2.83 b (0.02)
	Lignin	2.85 b (0.03)	2.86 b (0.04)	2.86 b (0.06)

In Abanilla soil, catabolic diversity was significantly higher with the addition of cellulose and lignin than in the control soil at 1 and 60 days of incubation. Nevertheless, Abanilla soil amended with cellulose showed significantly higher catabolic diversity than soil amended with lignin at 60 days (P<0.05). At 120 days, catabolic diversity levels in Abanilla soil were similar in the control soil and the soil amended with lignin. In Santomera soil, catabolic diversity did not vary over time and was significantly higher in amended soil than in control soil (P<0.05).

The functional structure of the microbial communities was evaluated using a factor analysis of the normalised AWCD values for each single substrate. In Abanilla soil, Factor 1 accounted for 59.84% of the total system variance and Factor 2 accounted for 14.94% (Fig. 3.7A). With respect to Factor 1, Abanilla soil amended with cellulose at 60 and 120 days and soil amended with lignin at 60 days were separated from the rest of the samples. Furthermore, polymers, carbohydrates and aromatic compounds received

a high loading score in Factor 1. On the other hand, amines and esters received a high loading score in Factor 2.

In Santomera soil, Factor 1 accounted for 39.03% of the total system variance and Factor 2 accounted for 21.91% (Fig. 3.7B). Factor 1 separated the Santomera soil treatments into three groups, one formed by soil amended with lignin at 120 days, another formed by control soil at 120 days, and a last group made up of the rest of the samples. Esters and aromatic compounds received a high loading score in Factor 1 while phosphorylated chemicals, amino acids and carbohydrates received a high loading score in Factor 2.

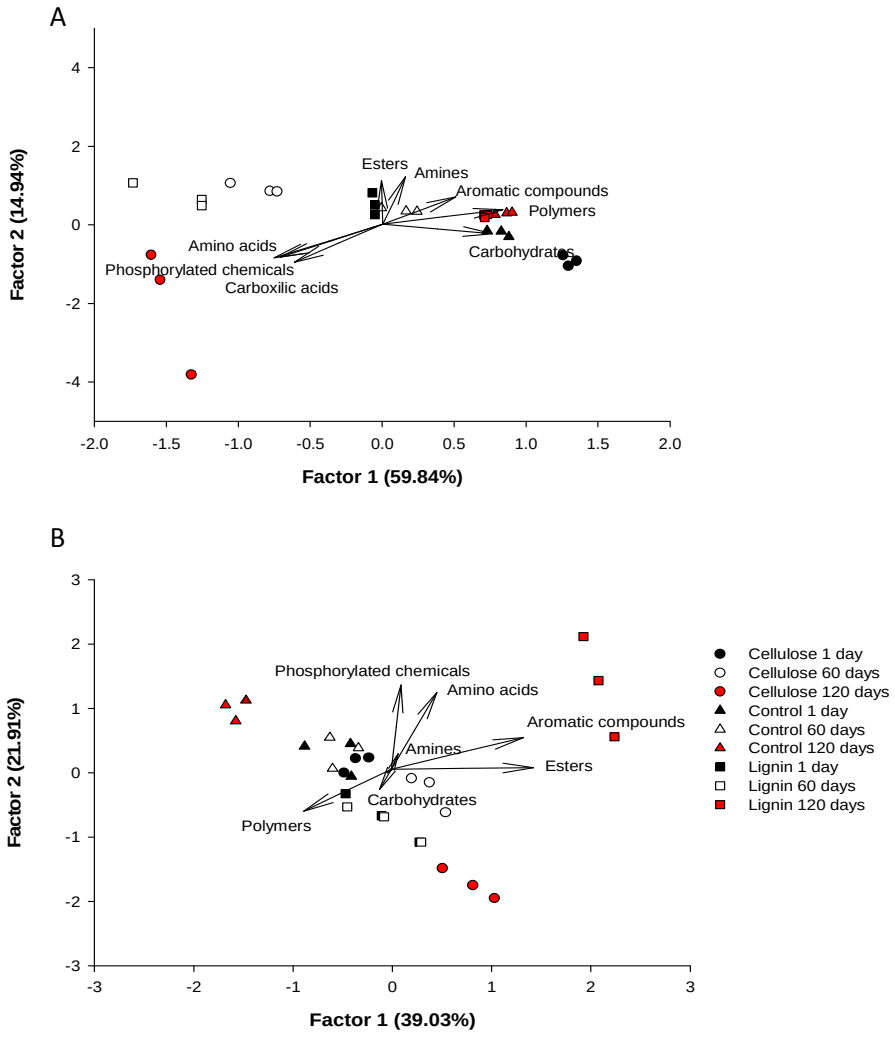


Fig.3.7. Principal Component Analysis of CLPP after cellulose and lignin addition in Abanilla and Santomera soil.

4. DISCUSSION

4.1. Enzyme activities

Overall, there was a higher level of enzyme activity observed in Santomera soil, which is related to the fact that this soil has a higher organic C content and greater microbial biomass than Abanilla soil (Bastida et al., 2006a; Chapter 2). Nevertheless, the addition of cellulose led to a proportionately larger increase in cellulase activity in Abanilla soil on the first day compared to Santomera soil. This fact could indicate that the more degraded soil (Abanilla) is particularly predisposed and sensitive to the addition of cellulose, although this soil is not naturally adapted to plant input, which agrees with results obtained by other authors (Hirsch et al., 2009; Guenet et al., 2011). It is also possible that the large amount of clay in Abanilla soil, with its significant active surface area (Dilustro et al., 2005; Wei et al., 2014), contributes to the rapid response of cellulase in reaction to cellulose in this soil. The immobilisation of cellulase in clay might be an important process for maintaining the carbon cycle in Abanilla soil. Cellulase showed a much longer response time in Santomera soil, although this response was sustained over a longer period of time. In fact, cellulase was one of the few enzymes showing higher activity levels at 120 days in Santomera soil. These results may be linked to the presence of a microbial community and certain enzymatic systems that are better adapted to the plant remains used in this soil. In fact, Santomera soil is less degraded than Abanilla and has a certain level of plant cover.

β-glucosidase is an enzyme that acts upon glucose dimers present in the soil *per se* or as a result of the biomolecules added. In Abanilla soil, the β-glucosidase activity was only observed to be affected by the addition of cellulose at the 60 day mark. This enzyme catalyses the degradation of compounds of low complexity, which could be by-products of cellulose decomposition in the soil (Jimenez et al., 2004). These compounds may no longer be available at 120 days, either because they have already been consumed or have been retained in the soil. The fact that the peak in β-glucosidase activity in Abanilla soil (in the soil with added cellulose) occurred at 60 days could be the result of increased cellulase activity on the first day of incubation.

Within the C cycle, it is important to emphasise co-metabolic phenomena involving relationships between various enzymes. For example, it is possible that the addition of lignin produced increased levels of cellulase activity in Santomera soil (which is better adapted to plant waste than Abanilla soil) due to increased metabolic activity in the soil microbial community. Similarly, studies demonstrated that the addition of both cellulose and lignin induced microbial turnover and storage of these substrates by

microorganisms but did not produce an increase in biomass (Nguyen and Guckert, 2001; Chapter 2). This increased activity in the microbial community could be responsible for the recycling of phospholipid fatty acids in the microbial membranes. It is therefore logical that the addition of cellulose and lignin has been linked to increased levels of lipase activity in some cases.

Polyphenol oxidase is another enzyme involved in the C cycle. Nevertheless, it is not entirely clear how this enzyme operates as it often does not correlate with hydrolase activity (Sinsabaugh, 2010). This enzyme has many functions, including making it possible for microorganisms to degrade lignin and humic substances by providing access to C, N and P (Moorhead and Sinsabaugh, 2006). For this reason, it was unusual to observe in Santomera soil that polyphenol oxidase activity levels decreased more in soils amended with cellulose and lignin than in the control soil. These results agree with the findings of several authors who have also observed that polyphenol oxidase activity can decrease or be inhibited after the addition of exogenous organic matter (Sinsabaugh et al., 2005; Stursova and Sinsabaugh, 2008). Moreover, this decrease could be due to the decrease in fungal biomass that has been observed in this soil after the addition of cellulose and lignin (Chapter 2), given that polyphenol oxidase activity has been widely linked to fungi (Allison and Jastrow, 2006; Sinsabaugh, 2010; Weand et al., 2010). In Abanilla soil, the increase in polyphenol oxidase activity could be due to the large amount of clay in the soil composition (Allison and Jastrow, 2006).

With regard to catalase, our results are consistent with those described by several other authors who have shown that the activity of this enzyme is necessary for lignin to be degraded by fungi and bacteria, but not for cellulose (Bourdais et al., 2012; DeAngelis et al., 2013). In fact, the inhibition of catalase we observed after cellulose was added to both Abanilla and Santomera soils coincides with the results of Matasyoh et al. (2011). These authors found that the inactivation of certain catalase genes actually increases the capacity of fungi to degrade nutrients from wood.

The alteration of C-cycling enzymes resulting from low doses of C amendments may have an impact on the biogeochemistry of other cycles. For instance, urease is an enzyme linked to N mineralisation (Simpson and Freney, 1988; Vahed et al., 2011). The activity of this enzyme was only observed to increase in soils with added cellulose at 60 days. In contrast, the response of phosphatase was much faster (1 day) and was consistent in both Abanilla and Santomera soils.

Both cellulose and lignin induced higher phosphatase activity in Abanilla soil, and cellulose (but not lignin) increased the activity of this enzyme in Santomera soil. Other authors have observed variations in phosphatase activity as a result of C or N inputs (Burke et al., 2012; Marklein and Houlton, 2012; Kivlin and Treseder, 2014; Godin et al., 2015). The same relationships were also observed by Asmar et al. (1994), who demonstrated that adding carbon to the soil can stimulate both enzyme activities and N mineralisation. One explanation for this phenomenon may be that the addition of carbon to the soil leads to a decrease in other nutrients such as N or P, thus inducing the microbial community to produce enzymes to help mitigate this decrease (Allison and Vitousek, 2005; Sinsabaugh and Shah, 2011).

4.2. Relationships between enzyme patterns and physiological profiles

As has been noted in several studies (Manici et al., 2004; Nourbakhsh; 2006. Elfstrand et al., 2007), the addition of compounds such as hemicellulose, cellulose and lignin, can increase the enzyme activity and alter the structure of the microbial community in the soil. Satti et al. (2003), on the other hand, observed that recalcitrant natural compounds like lignin can actually inhibit microbial activity, thereby decreasing the rate of decomposition and mineralisation of the organic matter present in the soil. Notwithstanding, far less is known about the response of semiarid soils devoid of vegetation to the addition of such compounds.

In our case, the addition of cellulose and lignin generally triggered changes in the enzyme activity of the soil starting from the first day of incubation, although the direction of these changes depended on the soil type (and its degradation level), the enzyme involved and the type of molecule added. Nevertheless, the impact of these biomolecules at the enzyme level was not long-term, as no consistent variations were observed (relative to the control) after 120 days of incubation. This may be due to the inability of recalcitrant substrates such as cellulose and lignin to induce long-term changes in the enzyme activity of semiarid soils because such soils adapt poorly to plant remains, despite the fact that the substrates have not been completely mineralised by this time, as has been observed in previous studies. In fact, after 30 days of incubation in Santomera soil, 39.7% of the added cellulose and 34.9% of the added lignin were mineralised; in Abanilla soil, 9.3% of the added cellulose and 8.1% of the added lignin were by this time (Chapter 2). The lack of changes in the level of enzyme activity in the long term can be explained by two facts. Firstly, lignin and cellulose do not produce an increase in microbial biomass (Chapter 2). Secondly, these

substrates could have been trapped in the soil either physically or via the formation of humic substances (Schmidt et al., 2011). In both cases, the substrates would not have been broken down due to their complexity and to the difficulty microorganisms would have had in reaching them. In fact, the level of mineralisation at 120 days was generally lower than at other times. Furthermore, differences with the control soils at 120 days were minimal, especially in Abanilla soil, which contains clays that can trap the added biomolecules and their metabolites. It is interesting to note the fact that in Santomera soil there were remarkable differences between the control soil and the soils with added lignin or cellulose at the functional and structural level (CLPP) at 120 days, even though there were minimal enzymatic differences at this same point in time.

By contrast, in Abanilla soil, which is more degraded and has high clay content, a rapid enzymatic response correlated with changes in the physiological profile of the soil starting from the first day. One possible explanation for these differences between the two soils could be the fact that Santomera soil has lower clay content and contributes to a lesser extent to fixing the added substrates. As a result, the metabolites derived from the added substrates are more bioavailable in the long term and the CLPPs are affected to a greater extent. Araújo et al. (2013) observed that less degraded soils have more stable microbial properties, so it thus makes sense that in Santomera soil the reaction to the addition of cellulose and lignin would be slower yet more long-lasting.

4.3. Catabolic diversity

In addition to studying the enzyme activity in Abanilla and Santomera soils after the addition of cellulose and lignin, we also studied variations in the catabolic diversity (H) of the microbial communities of the two soils after these amendments were added. Consistent with results obtained by other researchers studying the application of organic amendments (Gómez et al., 2006; Ros et al., 2006; Hu et al., 2011), we observed an increase in catabolic diversity after the addition of cellulose and lignin. Furthermore, this increase varied depending on the soil. In Abanilla soil, catabolic diversity was greater in the soil amended with cellulose than in the other treatments (after two months). In Santomera soil, on the other hand, the addition of substrates produced a greater increase in catabolic diversity than in the control soil regardless of the type of molecule applied.

It is worth noting that there was a greater level of catabolic diversity in Abanilla soil than in Santomera soil at all of the times studied. This shows that Abanilla soil, despite the fact that it is devoid of vegetation and generally does not receive carbon input, has

great potential in terms of its capacity to respond to new organic input. Our results agree with the data obtained by Guenet et al. (2011) and Hirsch et al. (2009), who introduced plant waste and cellulose into soils that had not received plant input for 80 and 50 years, respectively.

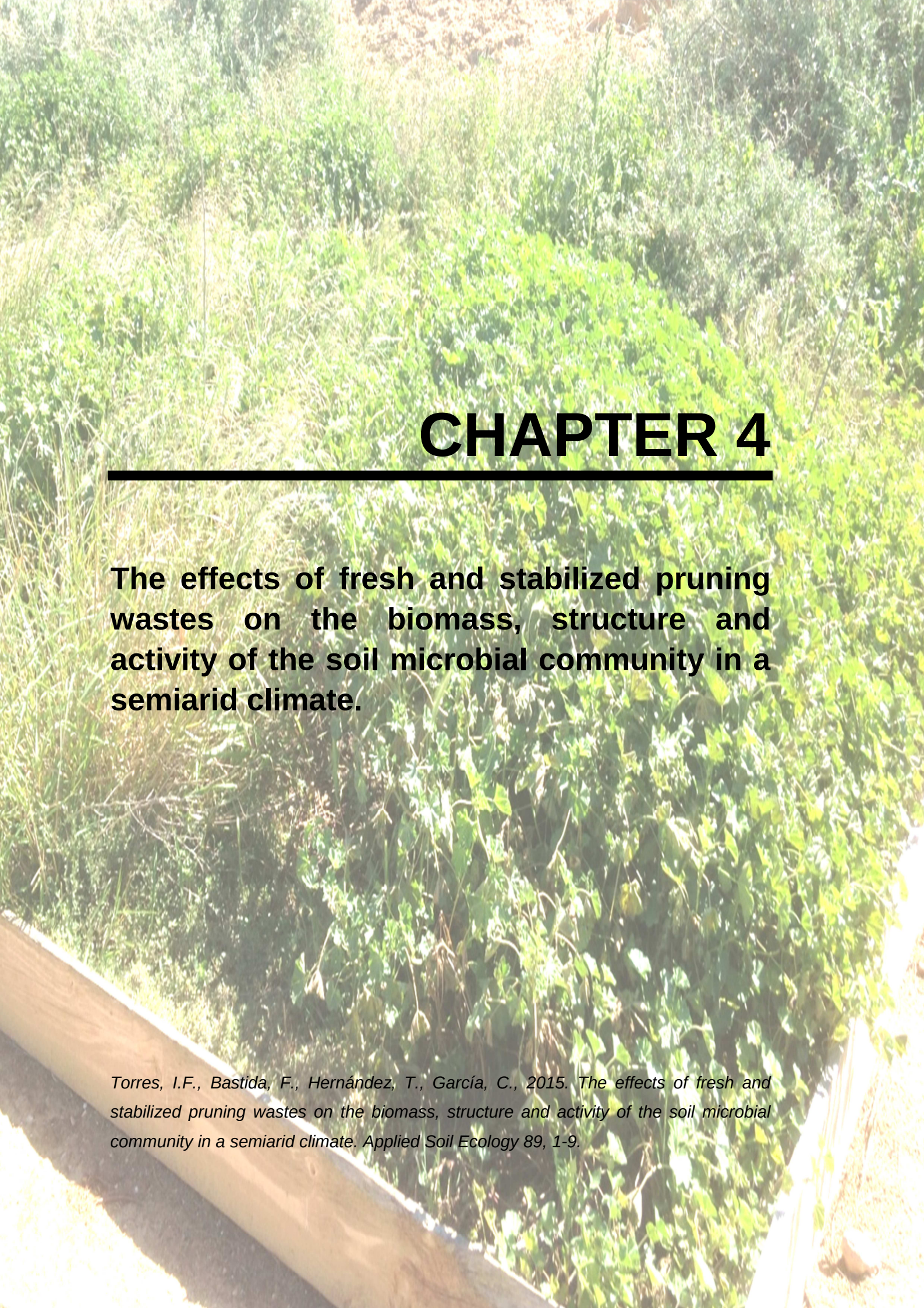
5. Conclusions

Adding cellulose and lignin to semiarid soils revealed several fundamental aspects of the functionality of these substrates at the microbial level in terms of biogeochemistry. Our findings are summarised below:

i) The impact of the addition of cellulose and lignin at the enzyme level depended on the type of molecule and soil. Neither substrate, however, had a long-term effect on the enzyme activity of the soils studied. On the other hand, the addition of these two substrates did have a long-term effect on the functional structure of the soil microbial community.

ii) The level of soil degradation influenced the response time of the microbial community to the addition of cellulose and lignin. The most degraded soil (Abanilla) was more predisposed and sensitive to the addition of the substrates. These results demonstrate that soils do not lose their potential to respond to organic input despite not having received any such input for a long time.

iii) The addition of small amounts of carbon to the soil was found to have an impact on the biochemistry of the cycles of other elements such as P or N.



CHAPTER 4

The effects of fresh and stabilized pruning wastes on the biomass, structure and activity of the soil microbial community in a semiarid climate.

Torres, I.F., Bastida, F., Hernández, T., García, C., 2015. The effects of fresh and stabilized pruning wastes on the biomass, structure and activity of the soil microbial community in a semiarid climate. Applied Soil Ecology 89, 1-9.

ABSTRACT

The incorporation of organic amendments from pruning waste into soil may help to mitigate soil degradation and to improve soil fertility in semiarid ecosystems. However, the effects of pruning wastes on the biomass, structure and activity of the soil microbial community are not fully known. In this study, we evaluate the response of the microbial community of a semiarid soil to fresh and composted vegetal wastes that were added as organic amendments at different doses (150 and 300 t ha^{-1}) five years ago. The effects on the soil microbial community were evaluated through a suite of different chemical, microbiological and biochemical indicators, including enzyme activities, community-level physiological profiles (CLPPs) and phospholipid fatty acid analysis (PLFA). Our results evidenced a long-term legacy of the added materials in terms of soil microbial biomass and enzyme activity. For instance, cellulase activity reached 633 µg and 283 µg glucose g^{-1} h^{-1} in the soils amended with fresh and composted waste, respectively. Similarly, bacterial biomass reached 116 nmol g^{-1} in the soil treated with a high dose of fresh waste, while it reached just 66 nmol g^{-1} in the soil amended with a high dose of composted waste. Organic amendments produced a long-term increase in microbiological activity and a change in the structure of the microbial community, which was largely dependent on the stabilization level of the pruning waste but not on the applied dose. Ultimately, the addition of fresh pruning waste was more effective than the application of composted waste for improving the microbiological soil quality in semiarid soils.

1. INTRODUCTION

Soil degradation is one of the main threats to arid and semiarid ecosystems and is characterized by loss of organic matter (OM) as a consequence of scarce vegetal growth (García et al., 1996). Yet organic matter plays a central role in maintaining key soil functions and global biochemical cycles. Furthermore, it is an essential determinant of soil fertility and resistance to erosion (Ros et al., 2006; Fonte et al., 2009). In scenarios of soil degradation, the application of organic wastes to the soil constitutes an environmental and agricultural means to maintain soil organic matter, reclaim degraded soils and supply plant nutrients. The fresh organic matter present in organic wastes stimulates the development and activity of the soil microbial community (Yang et al., 2003; Bonilla et al., 2012) and can incorporate also exogenous microbes to the soil environment. Moreover, organic matter improves the physical structure of the soil and contributes to carbon sequestration (Foley and Cooperband, 2002; Ros et al., 2006). Nevertheless, the biostimulant capacity of organic amendments depends on their chemical composition (Ajwa and Tabatabai, 1994).

Agricultural and gardening activities produce large quantities of vegetal residues and by-products. These residues cause serious environmental and visual pollution such as formation of pests that can move to new crops, gas emissions, toxic particle accumulation, and so on (Blázquez et al., 2011); particularly in agricultural areas like southeast Spain. A sustainable valorization of this vegetal waste is therefore need. It has been observed that the incorporation of green wastes derived from pruning into soil may improve long term soil fertility and quality (Doran et al., 1988). Moreover, composting these vegetal wastes can also help to reduce waste production. Composting is one of the best known processes for the biological stabilization of solid organic wastes. It involves the accelerated degradation of organic matter by microorganisms under controlled conditions, in which the organic material undergoes a characteristic thermophilic stage that allows for sanitization of the waste through the elimination of pathogenic microorganisms (Lung et al., 2001). For this reason, compost is considered to be an environmentally safe and agronomically advantageous organic amendment that stimulates soil microbial activity and crop growth (García et al., 1994; Pascual et al., 1997; Van-Camp et al., 2004). Changes in the properties of organic amendments after composting may directly affect the composition and activity of the soil microbial community (Bastida et al., 2008a; Pérez-Piqueres et al., 2006; Cross and Sohi, 2011). However, despite the fact that the restoration of arid and semiarid lands

with organic wastes has been a hot topic in recent years, the long-term impact of pruning wastes on the microbial community remains poorly understood.

Several microbial and biochemical indicators are commonly used for evaluating the effects of organic amendments on the soil microbial community. For instance, phospholipid-fatty acid analysis (PLFA) is often used as an indicator of microbial biomass and community structure in soil (Frostegard et al., 1993; Bastida et al., 2008a; Williams and Hedlund, 2013). Moreover, community-level physiological profiles (CLPP) are useful for detecting the functional responses of soil microbial communities to a variety of organic amendments (Bastida et al., 2013; Ng et al., 2014).

Soil enzymes play key roles in the biochemical functions of organic matter decomposition and nutrient cycling (Nannipieri et al., 1990; Waldrop and Firestone, 2004). Hence, it has been suggested that soil enzyme activity can be used as an indicator of soil fertility and microbial activity (Badiane et al., 2001) and for evaluating the influence of organic amendments on soil properties (Ng et al., 2014). A better understanding of the enzymes involved in carbon cycling is necessary to improve our knowledge concerning the processes leading soil restoration after application of organic amendments. Given the chemical complexity of soil organic matter contained in plant wastes, including polymeric carbon sources (i.e. cellulose, lignin, etc.), a suite of soil enzymes are involved in organic matter transformation and should be considered in restoration studies. For instance, polyphenol oxidase is involved in lignin degradation and plays an important role in soil C stabilization by favoring humic substance formation through the catalysis of polyphenol oxidation reactions (Weand et al, 2010). Cellulase breaks down cellulose into cellobiose, a sugar composed of two glucose units; and β-glucosidase, which hydrolyzes polymers of plant residues such as cellobiose.

In this experiment, vegetal pruning wastes and compost made from this material were applied as organic amendments in different doses (150 and 300 t ha^{-1}) to a semiarid soil five years ago. We aim to evaluate the long-term effects of these vegetal wastes at different doses and with distinct levels of stabilization (fresh or composted) on the chemical and microbiological properties of a semiarid soil. For this purpose, several indicators of microbial biomass and activity are used in this study. We hypothesized that compost would improve microbial activity and biomass more than fresh waste in the long-term because compost contains a more-stabilized organic matter. Moreover, we expected greater microbial biomass and activity in the plots amended with the highest dose of organic amendments. High doses would incorporate a higher amount

of organic carbon and nutrients in soil and promote a most-intense development of the microbial community.

2. MATERIAL AND METHODS

2.1. Study area, experimental design and soil sampling

This study was developed in an experimental field located in Santomera, in the Province of Murcia (southeast Spain) (38º 10' 91.5'' N; 1º 03' 79.8'' W), in an area that is greatly affected by soil degradation processes. The area is subjected to a semiarid climate with a mean annual rainfall of less than 300 mm and a mean annual temperature of 18ºC. The soil is classified as Haplic Calcisol (FAO, 2006) and its texture is clay-silt-loam. The pH of the soil is 8.5, its electric conductivity is 250 μScm^{-1}, its water holding capacity is 40.2 g $100g^{-1}$ and the bulk density is 2.57 g cm^{-3}. There was no vegetation growing on the soil at the beginning of this study. The soil was abandoned from agriculture use 20 years ago. This factor, together with inadequate climate conditions of the area and water scarcity, leads soil degradation processes in SE-Spain (López-Bermúdez and Albaladejo, 1990). These processes are strongly related to a loss of organic matter (García et al., 1994).

In November of 2008, 15 experimental plots (16 m^2 each) were established in the experimental area. The following treatments were established in replicate plots (n=3): 1) soil with fresh vegetal waste at 150 t ha^{-1} (VW150); 2) soil with fresh vegetal waste at 300 t ha^{-1} (VW300); 3) soil with composted vegetal waste at 150 t ha^{-1} (CVW150); 4) soil with composted vegetal waste at 300 t ha^{-1} (CVW300); and 5) soil without the addition of organic wastes (Control) (Fig. 4.1). The chemical properties of the organic amendments are presented in Table 4.1. Organic materials were incorporated into the first 15-20 cm of soil with a rotovator. The green waste originated mainly from pruning from urban parks. Compost was produced with this type of pruning waste mixed with pig slurry at 3:1 (w:v), for decreasing C/N ratio. For each soil sampling, six subsamples per plot were randomly collected with hand-driven probes to a depth of 15 cm in March of 2014. These subsamples were then mixed to constitute a single sample per plot. The samples were sieved to < 2 mm and stored at 4ºC until analysis. Samples were analyzed within one month after sampling.

Table 4.1. Characteristics of organic amendments added (VW: fresh vegetal waste; CVW: composted vegetal waste).

	VW	CVW
pH	7.90	8.88
Electrical conductivity (mS)	718.33	787.67
Carbohydrates (ppm)	1671.82	309.13
Water soluble C (ppm)	9601.19	2659.29
Total organic C (%)	34.22	19.32
Humic acids (mg kg^{-1})	1617.01	5241.57
Fulvic acids (mg kg^{-1})	2188.67	1527.34
Polyphenols (µg coumaric ac g^{-1} soil h^{-1})	1300.80	258.75
NH_4 (mg kg^{-1})	<2.5	<2.5
Available P (mg kg^{-1})	135.9	141.40
Total P (g $100g^{-1}$)	0.06	0.13
NO_3 (mg kg^{-1})	<5.0	<5.0
N (g $100g^{-1}$)	0.87	1.03
Available K (meq $100g^{-1}$)	4.65	14.13
Total K (g $100g^{-1}$)	0.38	0.75
Cd (mg kg^{-1})	<0.5	0.50
Cu (mg kg^{-1})	14.0	59.60
Cr (mg kg^{-1})	2.7	17.40
Ni (mg kg^{-1})	1.1	7.20
Pb (mg kg^{-1})	1.9	30.50
Zn (mg kg^{-1})	70.6	149.80
(Cellulose + Hemicellulose + Lignin) (g $100g^{-1}$)	35.15	6.76

Fig.4.1. Experimental plots

2.2. Chemical parameters, basal respiration and enzyme activities

Total organic carbon (TOC) was determined using a C analyzer (Thermo Finnigan Flash EA 1112). Water-soluble carbon (WSC) was determined through soil extraction (2 h shaking with a soil:distilled water ratio of 1:5), followed by centrifugation, filtration, and analysis of the extract solution on a C analyzer for liquid samples (Shimazdu 5050A). An aqueous solution 1:5 (w:v) was used to measure pH in a pH meter (Crison mod.2001, Barcelona, Spain).

Humic substances were extracted with a 0.1 M, pH 9.8 sodium pyrophosphate solution (w/v ratio=1:5) by mechanical shaking for 4 h. Then, the organic carbon concentration in the filtrated extract was estimated by reaction with $K_2Cr_2O_7$ and further measurement of absorbance at 590 nm as indicated by the method of Sims and Haby (1971).

Microbial respiration (CO_2 emission) was measured in 10 ml capped tubes containing 1 g of soil. Soil samples were humified with distilled water at 60% of their water-holding capacity. Vials were hermetically closed and incubated in the dark at 28 ºC for 11 days. The concentration of CO_2 was periodically analyzed with a gas chromatograph [Trace Ultra Thermo Scientific, Milan (Italy)] using a packed column [Trace PLOT TG-BOND Q GC, Trace Ultra Thermo Scientific, Milan (Italy)]. The CO_2 emitted per unit of organic carbon was then calculated (qCO_2).

Soil dehydrogenase activity was determined using 1 g of soil, and the reduction of *p*-iodonitrotetrazolium chloride (INT) to *p*-iodonitrotetrazolium formazan (INTF) was measured by the method of Trevors et al. (1982). Soil dehydrogenase activity was expressed as µg INTF g^{-1} soil h^{-1}. The urease activity in the soil was determined by the buffered method of Kandeler and Gerber (1988). In this procedure, 0.5 mL of a solution

of urea (0.48%) and 4 mL of borate buffer (pH 10) were added to 1 g of soil and then incubated for 2 h at 37 ºC. The ammonium concentration of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate to determine the amount of ammonium produced in the absence of added urea.

Phosphomonoesterase and β-glucosidase activities were determined following the methods described by Tabatabai and Bremner (1969) and a modification of Tabatabai's method (1982), respectively, by adding 2 mL of MUB (modified universal buffer) pH 11 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl phosphate (for phosphatase activity assay) or 2 mL of MUB pH 6 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl *β*-D-glucopiranoside (for *β*-glucosidase activity assay) to 0.5 g of soil. The mixtures were then incubated at 37 ºC for 1 h, after which time the enzymatic reactions were stopped by cooling on ice for 15 min. Then, 0.5 mL of 0.5 mol L^{-1} $CaCl_2$ and 2 mL of 0.5 mol L^{-1} NaOH (for phosphatase) or 2 mL of 0.1 mol L^{-1} tris(hydroxymethyl)aminomethane-sodium hydroxide (THAM-NaOH) of pH 12 (for *β*-glucosidase) were added. For the control test, the respective substrates were added before the addition of $CaCl_2$ and NaOH.

Polyphenol oxidase was determined by the method of Allison (2006) using 50 mM pyrogallol/50 mM EDTA as the substrate, and the absorbance of the supernatant from the reaction was determined directly at 460 nm after 1 h.

Lipase was measured according to the method of Margesin et al. (2002) by reaction with p-nitrophenyl butyrate 100 mM. Cellulase activity was determined following a modification of the method of Deng and Tabatabai (1994) using carboxymethylcellulose as substrate. Finally, catalase activity was determined by the method of Johnson and Temple (1964), using H_2O_2 as a substrate, which was shaken for 20 min. The filtrate was titrated with 0.1 M $KMnO_4$.

2.3. Phospholipid fatty acid analysis (PLFA)

Phospholipids were extracted from 6 g of fresh soil using chloroform-methanol extraction as described by Bligh & Dyer (1959) and fractionated and quantified using the procedure described by Frostegard et al. (1993). Phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al., 1985) and designated as described by Frostegard et al. (1993). The complete dried FAME fraction was dissolved in isooctane containing 0.23 mg ml^{-1} of 21:0 FAME as internal

standard. The analysis was perfomed using a Trace Ultra Thermo Scientific gas chromatograph fitted with a 60 m capillary column (Thermo TR-FAME 60m x 0.25 mm ID x 0.25 µm film), using helium as carrier gas (Fig. 4.2).

The following fatty acids are characteristic bacterial fatty acids and were chosen as bacterial biomarkers: i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c, 18:1ω9t, 10Me16:0 and 10Me18:0 (Frostegard et al., 1993; Dungait et al., 2011). The fatty acid 18:2ω6 was used as indicator of fungal biomass (Brant et al., 2006; Rinnan & Baath, 2009). The Gram-positive representative fatty acids used were i15:0, a15:0, i16:0, i17:0, 10Me16:0 and 10Me18:0; the Gram-negative fatty acids used were cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t (Frostegard et al., 1993; Dungait et al., 2011). 10Me-branched FAMES (10Me16:0 and 10Me18:0) were taken as specific actinobacterial biomarkers within Gram-positive bacteria (Dungait et al., 2011).

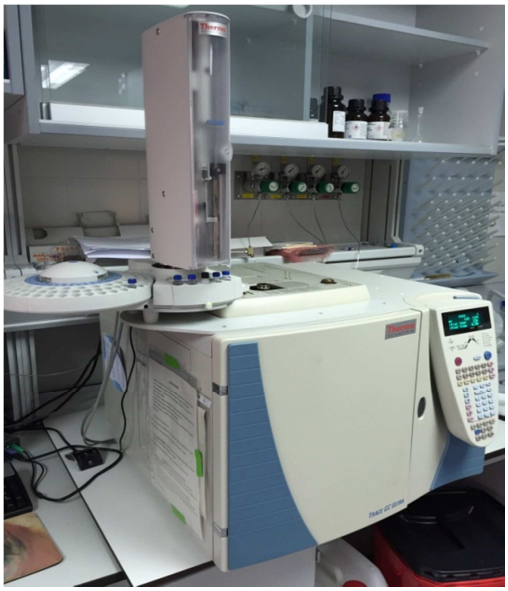


Fig.4.2. Chromatograph for PLFAs analysis.

2.4. Community-level physiological profiles (CLPPs) of the microbial community

Biolog EcoPlates (Biolog, Inc., Hayward, CA, USA), containing 31 different C sources and water were used to determine the microbial community-level physiological profiling based on carbon source utilization (Hitzl et al., 1997) (Fig. 4.3). The carbon sources belonged to the following groups: carbohydrates, polymers, carboxylic acids, aromatic compounds, phosphorylated chemicals, amino acids, esters and amines. Two-gram of

wet samples were shaken in 20 ml of sterile water at 150 rpm, for 15 min at 4 ºC. After incubation, a liquid extract was obtained by centrifuging at 2000 rpm for 10 min; 100 µl were inoculated on each Biolog plate well, and the plates were incubated at 28 ºC for 7 days. Microbial biomass of cell extracts was equalized by the use of PLFA content of each sample. This standardization prevents influences of the microbial biomass level in the oxidation of substrates and hence an adequate comparison of the functional community structures of each treatment. The rate of utilization was indicated by the reduction of tetrazolium, a redox indicator dye which changes from colorless to purple. Data were recorded for one week at 590 nm in an automated plate reader (Multiskan Ascen), until a plateau was reached. Microbial activity was expressed as average well color development (AWCD), as described by Garland (1996). The catabolic diversity (Hbio) for each sample was estimated using the Shannon-Weaver index, as calculated from the normalized AWCD data after 32 h of incubation in Biolog EcoPlates (Insam and Goberna, 2004).

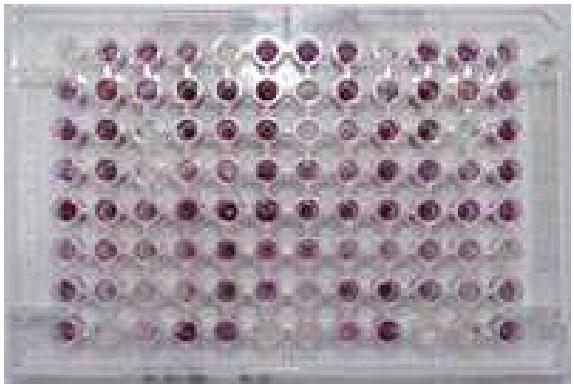


Fig.4.3. Biolog Ecoplate.

2.5. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. In order to determine significant differences between the means of the treatments, data was submitted to one-way ANOVA. ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference, at the 95% confidence interval in the lineal model). A factor analysis was carried out for relative quantity of PLFA and other for CLPP to explore possible differences between treatments. Correlation analysis was performed to evaluate the relationships between the analyzed variables.

3. RESULTS

3.1. Chemical, biochemical analysis and plant cover

After 5-years, plant cover was 77, 82, 85, 91 and 93% in control, VW150, VW300, CVW150, CVW300, respectively. Plant cover was slightly higher in amended plots than in control plots, but differences were not significant. Moreover, the structure of the plant community was similar in all treatments. The plant community was dominated in both treatments by *Carduus bourgeanus, Piptatherum miliaceum, Hedypnois cretica, Reichardia tingitana, Malva parviflora* and *Bromus Fasciculatus*.

TOC was higher in all treatments in comparison to the control (Table 4.2), particularly in the treatments consisting of fresh waste in comparison to the soil amended with compost. In contrast, WSC was higher in plots treated with composted vegetal wastes than in plots amended with fresh vegetal wastes. HS was higher in soil amended than in control soil, especially in the high dose. However, pH decreased significantly with organic additions except in soil amended with compost at low dose (Table 4.2).

Soil with fresh vegetal wastes showed significantly higher CO_2 emission than soil with composted green manure at both doses (P<0.05). The qCO_2 quotient was significantly lower than in the control (P<0.05) in all treatments (except in soil treated with a low dose of compost) (Table 4.2).

Fresh vegetal waste increased the overall enzyme activities in comparison to the control soil. Dehydrogenase and catalase are mainly intracellular activities. As was the case for extracellular enzymes, the activity of these intracellular enzymes was higher in the plots amended with fresh wastes than in the compost treatments (P<0.05) (Table 4.2). β-glucosidase and lipase activities were significantly higher in soil amended with fresh vegetal waste than in the control soil and soil amended with compost (P<0.05); there were no significant differences between doses. In contrast, cellulase activity was highly dependent on the applied dose, and high doses showed greater activity than low doses (P<0.05) (Table 4.2).

Phosphatase activity increased significantly for both amendments in comparison to the control soil (P<0.05). The activity of this enzyme, however, was significantly higher in soil amended with fresh vegetal waste than soil amended with compost. Urease activity was significantly higher in fresh and composted vegetal wastes than in the control soil (P<0.05).

Table 4.2. Chemical parameters. Enzyme activities and basal respiration of control soil; soil amended with fresh vegetal waste (VW150; VW300); and soil amended with composted vegetal waste (CVW150; CVW300).

	CONTROL	VW150	VW300	CVW150	CVW300
pH	8.58 b	8.09 a	8.13 a	8.40 ab	8.27 a
	(0.06)	(0.15)	(0.03)	(0.22)	(0.04)
TOC	1.10 a	4.63 b	8.37 c	2.27 a	4.44 b
	(0.06)	(0.11)	(0.63)	(0.51)	(0.22)
N	0.13 a	0.40 b	0.49 b	0.22 a	0.39 b
	(0.01)	(0.06)	(0.01)	(0.06)	(0.05)
C/N	8.41 a	11.57 b	17.19 c	10.15 ab	10.59 ab
	(1.12)	(1.31)	(1.62)	(0.65)	(0.57)
WSC	385.47 b	156.20 a	245.99 a	366.35 b	371.36 b
	(49.38)	(2.58)	(33.49)	(21.23)	(89.01)
Humic substances	4743.56 a	16120.34 c	23469.20 d	12369.64 b	22334.36 d
	(741.52)	(932.98)	(153.69)	(1803.62)	(280.22)
β-glucosidase	5.49 a	14.26 b	12.56 b	6.56 a	6.22 a
	(1.09)	(1.83)	(1.44)	(0.26)	(0.27)
Lipase	0.87 a	2.31 b	2.15 b	1.25 a	0.94 a
	(0.05)	(0.36)	(0.03)	(0.16)	(0.13)
Polyphenoloxidase	126.81 ab	132.03 b	127.42 ab	138.81 b	105.28 a
	(18.47)	(4.24)	(6.83)	(2.92)	(3.32)
Cellulase	101.91 a	457.46 c	632.78 d	189.40 ab	282.76 b
	(17.23)	(68.28)	(64.36)	(7.85)	(15.97)
Dehydrogenase	5.72 a	8.08 d	7.68 c	5.84 a	6.24 b
	(0.02)	(0.12)	(0.19)	(0.08)	(0.19)
Catalase	5.69 a	7.29 b	6.84 b	5.82 a	5.85 a
	(0.54)	(0.41)	(0.42)	(0.28)	(0.38)
Phosphatase	4.07 a	10.96 c	11.63 c	6.49 b	7.33 b
	(0.52)	(0.03)	(0.08)	(0.15)	(2.17)
Urease	1.47 a	2.31 c	1.78 b	1.75 b	1.99 b
	(0.06)	(0.10)	(0.02)	(0.05)	(0.18)
Basal Respiration	23.55 a	46.57 c	43.91 c	34.62 b	30.08 b
	(3.27)	(7.18)	(1.69)	(3.34)	(2.97)
qCO_2	21.36 c	10.04 b	5.30 a	13.61 b	6.82 a
	(0.95)	(1.30)	(0.75)	(3.20)	(1.08)
H	3.21 b	2.99 a	3.10 ab	3.18 b	3.03 a
	(0.01)	(0.10)	(0.01)	(0.02)	(0.01)

Standard deviations appear between brackets. TOC: Total Organic Carbon (g $100g^{-1}$); N (g $100g^{-1}$); WSC: Water Soluble Carbon (ppm); Humic substances (µg C g^{-1}); β-glucosidase (µmol PNF g^{-1} h^{-1}); lipase (µmol PNF g^{-1} min^{-1}); polyphenoloxidase (mM pyrogallol g^{-1} h^{-1}); cellulase (µg glucose g^{-1} h^{-1}); dehydrogenase (µg INTF g^{-1} h^{-1}); catalase (mmol H_2O_2 g^{-1} h^{-1}); phosphatase (µmol PNP g^{-1} h^{-1}); urease (µmol NH_4^+ g^{-1} h^{-1}); respiration (mg CO_2-C kg^{-1} day^{-1}); H: Shanon-Weaver index.

Polyphenol oxidase activity behaved differently than the rest of the enzyme activities. In this case, the activity was higher in treated soils than in control soil but there were not differences between treatments at high dose (Table 2). The polyphenol oxidase activity of CWV300 was the lowest (P<0.05).

3.2. Phospholipid fatty acid analysis (PLFA)

Bacterial PLFA content increased significantly in all treatments in comparison to control soil (P<0.05). Bacterial PLFA content was significantly higher in fresh than in composted vegetal waste (P<0.05), and VW300 presented the highest values (Table 4.3). A similar pattern was observed for the Gram-positive bacteria fatty acid content. However, the Gram-negative fatty acid content did not differ between doses in soil amended with fresh vegetal waste.

Soil amended with fresh waste showed significantly higher actinobacterial PLFA content than both soil amended with compost and control soil. High doses of all materials induced a higher actinobacterial PLFA content than low doses (P<0.05) (Table 4.3). As occurred in the case of bacterial PLFAs, the fungal PLFA content also increased to a greater extent in soils amended with fresh vegetal wastes in comparison to soils amended with stabilized wastes. VW300 showed the greatest values.

Both the ratio between the PLFA content of Gram-positive and Gram-negative bacteria and the ratio between fungal and bacterial PLFA were higher in the plots amended with fresh waste than those treated with composted waste. The ratio between the PLFA contents of Gram-positive and Gram-negative bacteria was greater in the high-dose treatments than in the corresponding low-dose applications and the control. In contrast, the ratio of fungal to bacterial PLFA did not show higher values in the high dose treatments in comparison to the treatments with the corresponding material at a low dose.

In order to estimate the structure of the microbial community, a principal component analysis of the relative abundance of fatty acids was performed. Factor 1 explained 55.20% of the variance of the results and Factor 2 explained 37.52% (Fig. 4.4). Differences in the community structure appeared. According to Factor 1, the structure of the microbial community of control soil and CVW150 differed from the rest of the treatments. According to Factor 2, two groups could be established: one made up of all treatments except CVW300 and another made up of this last treatment. *Actinobacteria* and Gram-negative bacteria received a high loading score in Factor 1. In contrast,

fungal and Gram-positive biomarkers received high loading scores in Factor 2 (Fig. 4.4).

Table 4.3. Microbial biomass measured by PLFA analysis (nmol g soil^{-1}) and ratios of control soil; soil amended with fresh vegetal waste (VW150; VW300); and soil amended with composted vegetal waste (CVW150; CVW300).

	CONTROL	VW150	VW300	CVW150	CVW300
Bacteria	42.32 a (3.39)	97.06 c (2.52)	116.20 d (9.55)	56.06 b (5.98)	66.48 b (5.86)
Fungi	5.68 a (0.24)	12.24 b (0.92)	16.50 c (0.11)	5.71 a (1.46)	4.20 a (0.09)
Gram-positive	25.10 a (2.17)	60.79 c (0.58)	77.78 d (6.38)	33.15 a (3.96)	42.29 b (3.41)
Gram-negative	17.21 a (1.25)	36.26 c (2.12)	38.42 c (4.43)	22.90 ab (2.08)	24.19 b (2.51)
Actinobacteria	0.79 a (0.09)	2.06 c (0.09)	2.86 d (0.17)	1.01 a (0.13)	1.32 b (0.16)
Gram+/Gram-	1.46 a (0.04)	1.68 ab (0.09)	2.04 c (0.21)	1.45 a (0.06)	1.75 b (0.06)
Fungi/Bacteria	0.13c (0.01)	0.13 c (0.01)	0.14 c (0.01)	0.10 b (0.02)	0.06 a (0.0)

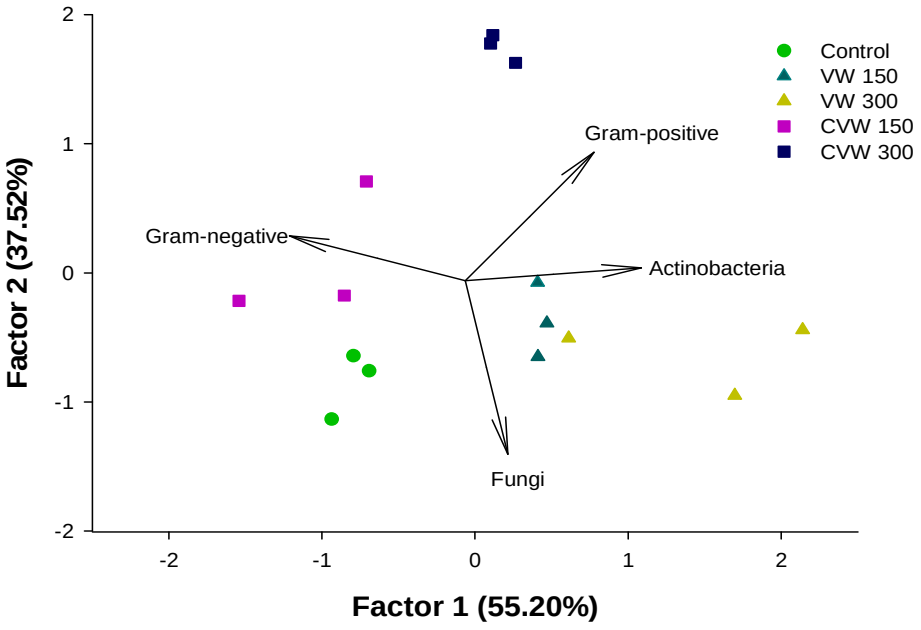


Fig.4.4. Principal Component Analysis of PLFAs in control soil, soil amended with fresh green waste (VW150, VW300) and soil with composted green waste (CVW150, CVW300).

3.3. Community-level physiological profiles (CLPPs) of the microbial community

The functional diversity and functional structure of each microbial community were analyzed using Biolog EcoPlates. Based on carbon utilization patterns, the functional catabolic diversity was calculated using the Shannon-Weaver index. The catabolic diversity remained equal to the control in soil treated with composted vegetal waste at a low dose, yet was significantly lower in the rest of treatments (P<0.05) (Table 4.2).

The functional structure of the microbial communities was evaluated using a factor analysis of the normalized AWCD values for each single substrate. Factor 1 accounted for 45.47% of the total system variance and Factor 2 accounted for 25.30% (Fig. 4.5). Factor 1 separated the treatments into three groups: control soil and CVW150; another group formed by fresh pruning waste in low dose and composted vegetal waste in high doses; and finally, another group with VW300. With respect to Factor 2, soil samples were separated into three groups: one made up of VW300 and CVW300; another made up of control soil and CVW150; and a final group made up of VW150. Polymers, aromatic compounds and phosphorylated chemicals received a high loading score in Factor 1. In the other hand, amines and amino acids received a high loading score in Factor 2.

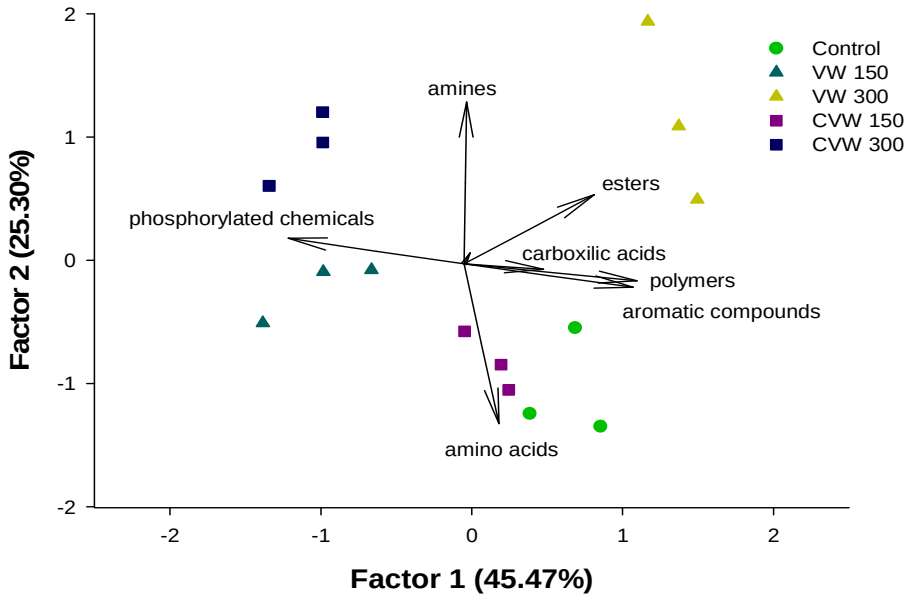


Fig.4.5. Principal Component Analysis of CLPPs in control soil, soil amended with fresh green waste (VW150, VW300) and soil with composted green waste (CVW150, CVW300).

3.4. Correlations between variable

A correlation matrix was generated to evaluate the relationships between the variables analyzed (Table A4.3, annex). The amount of TOC positively correlated with all enzyme activities studied except for polyphenol oxidase and urease activities. Furthermore, significant positive correlation coefficients were observed between TOC and the PLFA content of all microbial groups studied (P<0.05).

Significant positive correlation coefficients were also observed between the C of humic substances and the PLFA content of Gram-positive bacteria, Gram-negative bacteria and *Actinobacteria*, but this was not true for fungal PLFAs.

Negative correlation coefficients were observed between the WSC and all enzymatic activities (except polyphenol) and with the PLFA content of various microbial groups. Basal respiration was positively correlated with both TOC and PLFA content (P<0.05). pH was negatively correlated with all variables except for total N and polyphenol oxidase activity. However, pH was positively correlated with the functional diversity. Correlations were not observed between plant cover and the rest of variables.

4. DISCUSSION

The maintenance of a vegetal cover is fundamental for soil fertility and helps to preserve a basic level of microbial activity and biomass in semiarid ecosystems (García et al., 1994; Bastida et al., 2006b). In this study, scarce differences in the plant cover or community composition were observed between control plots and plots that received amendments 5 years ago. It is likely that initial plowing activities together with organic amendments at the time the experiment was established favored the development of opportunistic plant cover.

4.1. Carbon fractions and microbial activity

The addition of organic waste of plant origin (pruning waste), both fresh and stabilized through composting, increased the soil TOC even 5 years after application. This increase was found to depend on the degree of stabilization of the material used and the dose applied, as other authors have described (Elfstrand et al., 2007; Crecchio et al., 2004; Bastida et al., 2008a). The increase in organic C and nutrients in the soil resulting from the initial addition of waste caused a greater increase in the basal respiration of soils treated with amendments than in the control soil, as other authors have also observed (Dube et al., 2009; Iqbal et al., 2010). In general, the parallel

increase in basal respiration and hydrolase activities, especially those activities related to the C cycle, reflects the relationship between the degradation of organic matter at the extracellular level and gains in energy at the intracellular level through respiration.

However, considering that the water-soluble C fraction contains labile substrates that are capable of sustaining microbial growth (De Luca and Keeney, 1993; García et al., 2002), it would be expected that the microbial activity would be higher in plots receiving compost treatments (in which WSC values were higher even 5 years after amendment) (Bastida et al., 2008a). On the contrary, basal respiration was higher in the plots amended with fresh waste than in plots amended with compost. One possible explanation for this finding is the greater amount of microbial biomass in fresh waste-treated soils (Martens et al., 1992; Goyal et al., 1993). This result might be explained by the high values of organic carbon and nitrogen observed in the fresh-waste treated plots in comparison to other treatments (Marschner et al., 2003). Moreover, this explanation is supported by positive correlation between carbon and nitrogen, and PLFA contents. Enzymatic activities 5 years post-treatment were more dependent on the degree of stabilization of the amendment than on the dose applied. As other authors have noted, this may be due to the existence of a threshold beyond which enzyme activity does not increase in conjunction with increases in the dose of exogenous organic material applied (Crecchio et al., 2004; Bastida et al., 2007). In fact, the dose could even cause an inhibitory effect on some enzymes, as several authors have described (Marcote et al., 2001; Xue and Huang, 2013). In our case, polyphenol oxydase activity was lower in amended soils than in the control. Despite the fact that the behavior of polyphenol oxydase is not entirely clear, since it often does not correlate with hydrolase activity (Sinsabaugh, 2010), some authors have demonstrated that this enzyme may either decrease in quantity or be inhibited by the addition of exogenous organic matter or as a result of the N content of the soil (Sinsabaugh et al., 2005; Stursova and Sinsabaugh, 2008). These observations are consistent with our results.

In comparison to the overall increase in enzymatic and microbial activity in soils amended with fresh material, soils treated with compost produced no clear increase in the majority of activities related to the carbon cycle (i.e. cellulase, β-glucosidase and lipase activities) compared to the control soil, although a number of authors have shown an increase in these activities after several years of compost treatments (Guisquiani et al., 1994, 1995; Crecchio et al., 2001). It is likely that the distinct chemical nature of the added materials was primarily responsible for the observed

long-term response of enzyme activities. In fact, considering the differences in carbon quality of the added materials, the higher level of stabilization of compost may still influence results and could under-stimulate the response of enzyme activities involved in C-cycling. The lack of increase in enzyme activity in compost-amended soil could thus be due to the fact that compost components are more difficult to degrade compared to fresh pruning waste because of the stabilization process (Katterer et al., 2014). Furthermore, the compost treatment seems to induce a lower microbial biomass capable of generating such enzymes than the fresh green waste treatment.

Contrary to what was observed for enzyme activities related to the C cycle, the activity of hydrolase enzymes involved in the P cycle (phosphatase) and N cycle (urease) increased in the compost-amended soil compared to the control. The increase in the phosphatase activity, especially in fresh green waste treatments, may be related to the fact that the activity of this enzyme is closely linked to the amount of microbial biomass rather than to the availability of P (Bowles et al., 2014).

4.2. Biomass, microbial community structure and catabolic diversity

The application of different organic amendments caused differences in the microbial biomass and community structure. Such differences in the structure of the microbial community may be related to the chemical nature of the materials used as amendments (i.e. fresh vs composted) (Saetre et al., 2000; Ng et al., 2014). This postulation is consistent with previous studies that have shown that composting alters carbon quality and microbial community (Pérez-Piqueres et al., 2006; Bastida et al., 2008a; Cross and Sohi, 2011).

Microbial biomass was influenced by the dose of the material applied and the type of material (Table 4.3). This could be due to a positive correlation between microbial biomass and the TOC and N content in a soil, all of which are dependent on the dose (Marschner et al., 2003). The microbial biomass increased to a greater extent in soil amended with fresh green waste over the long term, particularly at the highest dose. Some studies have found that microbial biomass is positively related to the TOC and WSC content of amended soils (Marschner et al., 2003; Carrasco et al., 2010). In our study, we found positive correlation between the amount of TOC and the C/N ratio, but this was not the case with WSC. Moreover, there were significant and negative correlations between pH and PLFA groups. This result may indicate that pH exerts a control on the microbial community (Fierer and Jackson, 2006; Lauber et al., 2009). At the bacterial level, the Gram-positive/Gram-negative ratio and C/N ratio were higher in

VW300 than in the other treatments. This could be due to the fact that Gram-positive bacteria have a greater ability to degrade complex carbon substrates (Paul and Clark, 1989; Halverson et al., 2000), which could result from the high cellulose content present in fresh, non-composted waste.

Although fungi are characterized as being related to the production of polyphenol oxidase and therefore the formation of humic substances (Allison and Jastrow, 2006; Sinsabaugh, 2010; Weand et al., 2010), this microbial group showed no correlation with either of these variables in our study. As has been observed in other studies, polyphenol oxidase activity can be produced by bacteria and by fungi (Bugg et al., 2011; Ng et al., 2014). This is consistent with our data, as the fungi showed an increase in biomass compared to the control soil with the addition of fresh waste pruning waste, although there was a much greater increase in Gram-positive bacteria. Furthermore, bacterial PLFA content positively correlated with humic substance carbon. In fact, within the Gram-positive bacteria group, the fatty acid content representative of *Actinobacteria* was greater in the soil amended with fresh waste than in the composted or control soils. The Gram-positive bacteria group is associated with lignin decomposition since it is able to use lignin-derived compounds (Kirby, 2006); its increase is therefore not surprising subsequent to the addition of fresh pruning waste.

The changes in microbial biomass described above may be related to changes in the structure of the microbial community in the amended soils. For this reason, the microbial community structure was studied through a principal component analysis of the fatty acids. The microbial community structure in soil amended with fresh green waste and in CVW300 varied with respect to that of control soil. Moreover, the structure community in soil amended with CVW300 was different respect to soil amended with fresh green waste (as regards of the Factor 2). Nevertheless, the structure was similar in both control soil and CVW150. These results indicate that the dose of compost (but not fresh material) and the stabilization level impacted the structure of soil microbial community. The addition of structured and stabilized organic matter (compost) at the lowest dose can provide the most similar organic matter to that of the soil itself and therefore induce less changes in the microbial community structure than high dose.

The change in microbial community structure was mainly due to *Actinobacteria* and Gram-negative bacteria. This fact was not surprising because green residues formed by complex polymers of carbon such as cellulose or lignin and some Gram-positive bacteria (including *Actinobacteria*) are capable of degrading complex substrates of carbon (Paul and Clark, 1989; Halverson et al., 2000). Gram-negative bacteria, on the

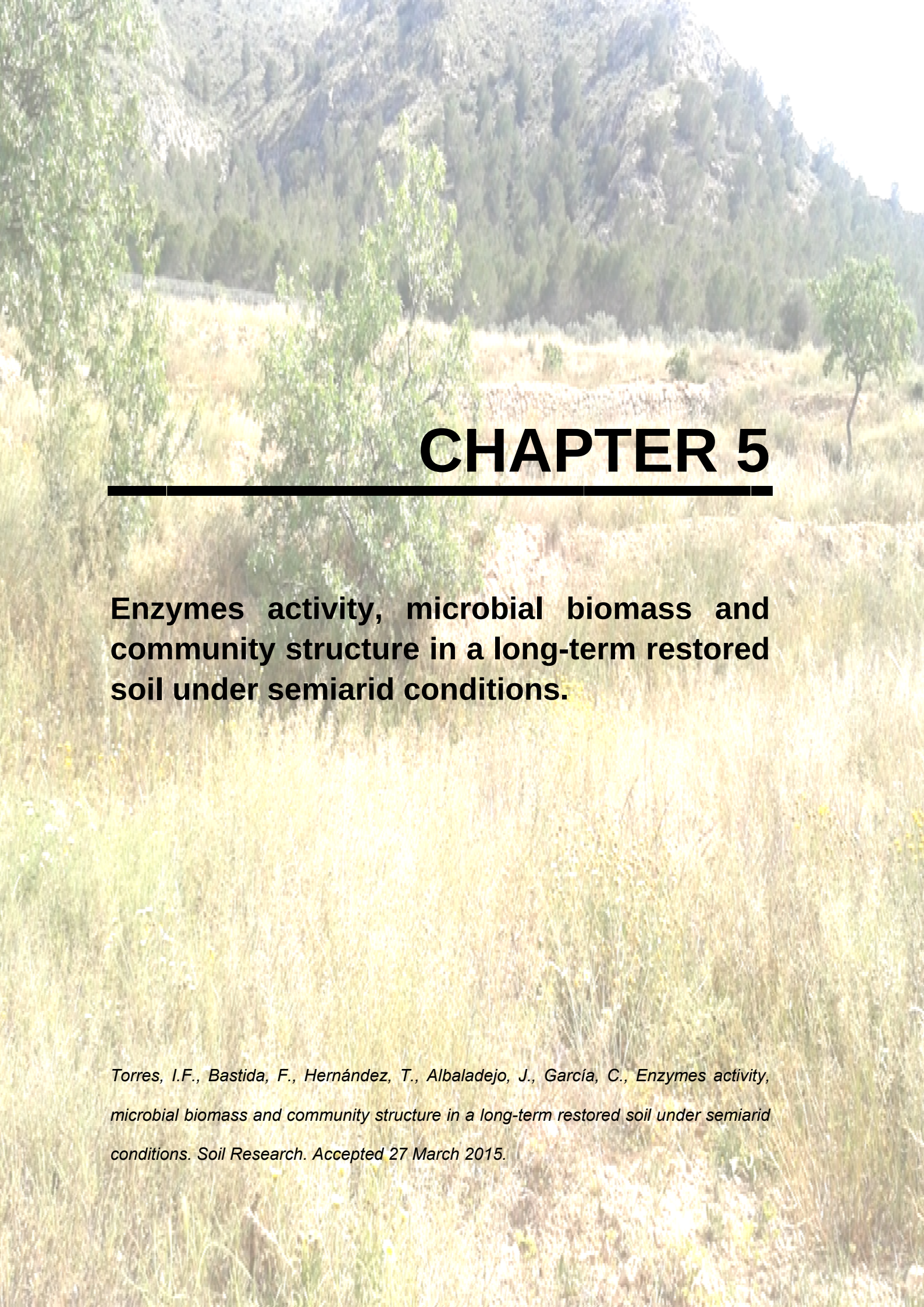
other hand, are characterized by the fact that they mainly utilize simple carbon forms (Holding, 1960).

Variations in the microbial community structure (assessed by PLFAs) were associated with a shift at the catabolic level (CLPPs). As in the case of PLFAs, the catabolic structures of CVW150 and the control soil were similar, but they were different compared to the other treatments. Catabolic structures of the microbial community were different depending on the dose and stabilization level of the added material. This fact reflects a long-term influence of amendment in the catabolic functionality of the soil microbial community.

Similarly, the control soil and CVW150 showed greater catabolic diversity than the other treatments. In comparison to results in short-term studies that have shown an increase in catabolic diversity with the addition of organic amendments (Gómez et al., 2006; Ros et al., 2006; Hu et al., 2011), other authors have observed that, in the long term, catabolic diversity decreases with the application of high doses of municipal waste (Bastida et al., 2013).

5. CONCLUSIONS

The degree of stabilization of the added organic material (5 years ago) had a persistent effect on the microbial community of a semiarid soil. The addition of fresh pruning waste promoted an increase in microbial biomass and more notable activity than the addition of composted waste. However, the dose of vegetal waste applied did not have a notable effect on microbial activity but impacted the functional structure of the microbial community. We may conclude that it is not necessary to compost green waste in order to improve the microbiological quality of semiarid soils in the medium-long term.



CHAPTER 5

Enzymes activity, microbial biomass and community structure in a long-term restored soil under semiarid conditions.

Torres, I.F., Bastida, F., Hernández, T., Albaladejo, J., García, C., Enzymes activity, microbial biomass and community structure in a long-term restored soil under semiarid conditions. Soil Research. Accepted 27 March 2015.

ABSTRACT

Our aim was to evaluate the long-term influences of urban organic amendments on the enzymes involved in carbon cycle under semiarid conditions, including the changes in the biomass and structure of the microbial community. A soil was restored 24 years ago with an organic amendment based on domestic organic waste. Organic amendment was applied to soil in order to increase the content of total organic carbon (TOC) by 0.5 and 1.5% with respect to the original TOC content. Enzyme isoforms composition was studied using zymographic techniques based on protein extraction, separation by gel electrophoresis and further enzyme-specific, in-gel staining. The total cellulose and β-glucosidase activities, the microbial biomass estimated by phospholipid-fatty acid analysis (PLFAs) and the number of isoforms of each enzyme showed increases related to the initial amount of organic amendment and the consequent development of vegetation. The information obtained by the enzyme activity assays may be improved by the use of zymographic techniques that allow the investigation of the variety of isoforms of each enzyme. This information could improve the understanding of the relation between the microbial community and the carbon cycling in restored areas.

1. INTRODUCTION

In semiarid areas, soil degradation processes are mainly due to human activity (over-exploitation of soils, abandonment of soils from agriculture) and the unfavourable environmental conditions. These factors are linked to a reduction of plant growth and soil organic matter (García et al. 1997). Soil organic amendment is one of the most-effective strategies to combat degradation processes and to restore soil fertility. Besides, these amendments allow the development of a natural plant cover that helps to protect the soil against erosion (Bastida et al., 2007; Albaladejo et al., 2008; Bastida et al., 2008a). Physical and chemical properties have been used traditionally to evaluate the successful of soil restoration experiments (Parr and Papendick, 1997). In contrast, the use of microbial and biochemical indicators is less well developed, although they are very sensitive and provide immediate and precise information on small changes occurring in soil (Bending et al., 2004). Indeed, microbial activity has a direct influence on the stability and fertility of ecosystems through extracellular enzymes (Dick and Tabatabai, 1993, Smith and Papendick, 1993; Sinsabaugh, 1994; Schimel and Bennet, 2004). However, some authors consider that the use of enzymes as indicators of soil quality is not adequate when soil degradation brought about by land use (Trasar-Cepeda et al., 2008). Soil enzymes play key roles in biochemical functions such as organic matter decomposition (Burns 1983; Sinsabaugh et al., 1991; Dick et al., 1994).

Cellulase and β-glucosidase are important enzymes related with the C cycle since cellulose is one of the most abundant organic compounds in the biosphere (Falkowski et al., 2000; Criquet, 2002). It consists of long polymers of β-1,4-linked glucose units that are degraded by cellulolytic enzymes to glucose, including β-glucosidase that constitutes the final step in the degradation of cellulose, providing simple sugars for the soil microbial community. In this way, β-glucosidase plays an important role in the degradation of soil organic matter and plant residues. Increased activity, often associated with increased soil microbial biomass, would reflect a soil's ability to break down plant residues and improve the availability of nutrients for subsequent crops (Stott et al., 2010).

Generally, the biochemical information retrieved to evaluate soil restoration has been restricted to the measurement of enzyme activities (Acosta-Martínez, 2007; Bastida et al., 2008a). However, scarce information into the variety of specific enzyme isoforms long-term after organic matter addition is available. The variety of isoforms in soil could

show the capabilities of the microbial community to degrade carbon substrates (Bending et al., 2004; Carpenter-Boggs et al., 1988).

The study of enzyme diversity, understood as the set of different isoforms of a particular enzyme can be accomplished by zymography. The zymographic technique can reveal enzymes by their activities after separation by electrophoresis in gel in the presence of sodium dodecyl sulphate and polyacrilamide (SDS-PAGE); and the enzyme renaturation (Khalili et al., 2011). The advantage of zymography is that it identifies individual proteins in a mixture of extracted enzymes and gives a rapid estimation of enzyme molecular weights (Lacks and Springhorn, 1980). Moreover, zymography allows isoform activities to be compared with the total enzyme activity. This technique has been applied for years to the study of variety of enzyme isoforms in cell-free cultures (Imam et al., 1993). For instance, Dutta et al. (2008) suggested that different isoforms of cellulases function in a distinctive, coordinated fashion in order to hydrolyse the polymeric cellulose to soluble, monomeric glucose in cultures of *Penicillium citrinum*. However, zymographic studies in soil are innovative (Cañizares et al., 2011; Khalili et al., 2011) and the long-term effects of soil restoration on isoenzymes have not been explored so far. In this study our purpose is the study of variety of enzyme isoforms involved in the carbon cycling of restored soils after organic amendment at long-term.

In addition to zymography, phospholipids fatty acids (PLFAs) were measured and used to estimate changes in biomass and structure of the soil microbial community. PLFA are components of the cell membranes from living biomass and some of them are specific to or dominant in certain microbial groups, such as Gram-positive, Gram-negative or fungi (Grayston and Prescott, 2005). The analysis of PLFAs helps to ascertain if changes in variety of isoforms are related to variations in microbial biomass and/or community structure.

We hypothesize that addition of organic matter at long-term could impact on enzymes which are responsible for carbon cycling. To test this hypothesis, we will use a well-established soil restoration experiment that was started 24 years ago (Albaladejo et al., 2000; Bastida et al., 2008a).

The aims of this study were: i) the evaluation of the long-term influence of urban organic amendments on activity of enzymes involved in carbon cycle under semiarid conditions by zymographic analysis; and ii) the evaluation of changes in microbial biomass and structure of microbial community associated with long-term different doses of organic waste addition and their relation to the variety of isoforms.

2. MATERIAL AND METHODS

2.1. Study area and experimental design

The experimental site was located in Murcia, southeast Spain. This area is greatly affected by soil degradation processes such as erosion, agricultural abandonment and low organic matter content, which are typical in arid conditions. The site was under semiarid climate where the mean annual rainfall was lower than 300 mm and mean annual temperature was over 20ºC.

The soil is poorly developed, with an ochric epipedon as the sole diagnostic horizon, and is classified as a Xeric Torriorthent (Soil Survey Satff,1998). In October 1988, the organic fraction of a domestic organic waste was subjected to a pre-composting process by turning (twice in 20 days) in order to obtain a minimal stabilization of this organic material. The properties of added material are showed in Table 5.1. This organic waste was used for soil amendment in 6x5 m field plots. The plots were established in replicates (n=3). Three treatments were established in order to raise the TOC content of the soil: i) 0.5% (65 t ha^{-1}) (low dose amendment, D1); ii) 1.5 % (195 t ha^{-1}) (high dose amendment, D2). Additionally, three plots of control soil without organic amendment were established (C) (Fig. 5.1).

Fig.5.1. Experimental plots.

Twenty-four years after the amendment, in October 2011 the plots were sampled. Six subsamples per plot were collected randomly with hand-driven probes, to a depth of 15 cm, and then mixed to constitute a single sample per plot with initial moisture content between 6.5-7.5%. The samples were sieved to < 2 mm and stored in plastic bags for up to two weeks at 4ºC.

Table 5.1. Characteristics of the organic fraction of the municipal solid waste added to soil. [a]Values based on dry weight, [b]Standard deviation.

Parameter	Mean[a]	St. Dev.[b]
pH	6.50	0.12
Electrical conductivity (dS m^{-1})	4.00	0.27
Total organic C (g kg^{-1})	242.00	19.83
Humic substances C (g kg^{-1})	2.60	0.11
Total N (g kg^{-1})	13.00	1.09
Total P (g kg^{-1})	5.50	0.28
Available P (g kg^{-1})	0.60	0.01
Total K (g kg^{-1})	4.20	0.23
Available K (g kg^{-1})	3.00	0.05
Cu (mg kg^{-1})	237.21	19.28
Zn (mg kg^{-1})	650.17	37.54
Cr (mg kg^{-1})	365.29	18.01
Cd (mg kg^{-1})	2.00	0.11
Ni (mg kg^{-1})	328.21	19.20
Pb (mg kg^{-1})	235.87	17.82

2.2. Chemical properties

Humic substances were extracted by adding 40 ml of 0.1 M, pH 9.8 sodium pyrophosphate solution to 2 g of soil and shaken for 4 h. Then, the organic carbon concentration in the filtered extract (filter paper ashless, 110 mm Ø, Albet LabScience) was estimated by reaction with 0.098g of $K_2Cr_2O_7$ and further measurement of absorbance at 590 nm (Sims and Haby, 1971).

Water-soluble carbon (WSC) was determined after extraction from (2 h shaking with a soil, 0.1 g soil ml^{-1}) followed by centrifugation at 15000 rpm for 15 minutes, filtration, and analysis of the extract solution on a C analyzer for liquid samples (Shimazdu 5050A). Soil total organic carbon (TOC) was determined using a C analyzer (Thermo Finnigan Flash EA 1112) (Fig. 5.2).

Fig.5.2. C analyzer for WSC measurement.

2.3. Microbial and biochemical parameters assay

Enzyme activities were determined using the following methods. Soil dehydrogenase activity was determined using 1 g of soil, and the reduction of *p*- iodonitrotetrazolium chloride (INT) to *p*-iodonitrotetrazolium formazan (INTF) was measured by the method of Trevors et al. (1982). Soil dehydrogenase activity was expressed as μg INTF g^{-1} soil h^{-1}.

Polyphenoloxidase was determined by the method of Allison (2006) using 50 mM pyrogallol/50 mM EDTA as substrate, and the absorbance of the supernatant from the reaction was determined directly at 460 nm after 1 h.

β-glucosidase activity was determined following a modification of the method of Tabatabai (1982) by adding 2 mL of MUB pH 6 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl *β*-D-glucopiranoside to 0.5 g of soil. The mixture was then incubated at 37ºC for 1 h, after which time the enzymatic reaction were stopped by cooling on ice for 15 min. Then, 0.5 mL of 0.5 mol L^{-1} $CaCl_2$ and 2 mL of 0.1 mol L^{-1} tris(hydroxymethyl)aminomethane-sodium hydroxide (THAM-NaOH) of pH 12 were added. For the control test, the respective substrate was added before the addition of $CaCl_2$ and THAM-NaOH.

Cellulase activity was determined following a modification of the method of Deng and Tabatabai (1994). 5 g of soil and 20 ml of sodim-acetate buffer with 2% of carboxymethylcellulose as substrate and 0.5 ml of toluene were incubated at 37ºC during 24 hours. Then, the samples were centrifugated and the activity was measured by the spectrophotometric method of Somogyi-Nelson (1944).

Invertase activity was measured by the method of Hoffmann and Pallauf (1965) modified by García-Álvarez and Ibañez (1994). Finally, catalase activity was determined by the method of Johnson and Temple (1964), using H_2O_2 as substrate, shaked for 20 min and the filtrate was titrated with 0.1 M $KMnO_4$.

Soil respiration was analyzed by placing 20 g of soil moistened at 40%–50% of its water holding capacity, in hermetically sealed flasks and incubating for 27 days at 28°C. The CO_2 released was periodically measured (every day for the first four days and then weekly) using an infrared gas analyzer (CheckmateII, PBI Dansensor, Denmark) and the flasks were opened after each measurement to avoid the accumulation of CO_2. The data were summed to give a cumulative amount of CO_2 released after 27 days of incubation, and basal soil respiration was expressed as mg CO_2–C kg^{-1} soil per day.

2.4. Phospholipid fatty acids (PLFA*)*

Phospholipids were extracted from 6 g of soil using a chloroform-methanol extraction based on Bligh and Dyer (1959), fractionated, and quantified using the procedure described by Frostegard et al. (1993) and Bardgett et al. (1996). The analysis of fatty acids methyl ester (FAME) was performed as described by Lucas-Borja et al. (2012).

PLFA molecular structures are described using standard nomenclature. The first number refers to the total number of C-atoms and the number after the colon refers to the number of double bonds. A number following a “w” is the location of the first double bond relative to the aliphatic end of the molecule. Notations “Me”, “OH” and “cy” are, respectively, methyl, hydroxyl, ciclopropane groups and notations “i” and “a”, respectively, are iso- and anteiso-branched fatty acids.

The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1w7c, 16:1w7t, 18:1w9c y 18:1w9t were chosen to represent bacterial biomass (Frostegard et al., 1993; Dungait et al., 2011), where i15:0, a15:0, 15:0, i16:0, i17:0 were Gram-positive specific and cy17:0, cy19:0, 16:1w7c, 16:1w7t, 18:1w9c y 18:1w9t were Gram negative specific. 18:2w6 was taken to indicate fungal biomass (Rinnan and Baath, 2009).

2.5. Separation of proteins by gel electrophoresis

Cellulase isoforms were determined by the method of Khalili et al. (2011) with slight modifications. 3 g of soil were mixed with 4 ml of Tris-HCl buffer (50 mM, pH 7) containing 1% (w/v) sodium-dodecyl-sulphate (SDS). After incubation with agitation for

4 hours at 20ºC, 2.2 ml of Tris-HCl (50 mM, pH 7) were added and the extract was shaken for 30 minutes at 20ºC. Samples were then centrifuged at 17226 g for 10 min at 4ºC and the supernatant was collected as the source of extractable proteins. Then, proteins were precipitated by addition of 8 ml of chilled 100% acetone (-20 ºC), the mix was incubated for 30 min at -20 ºC and then centrifugated at 20217 g during 15 min. After centrifugation acetone was discarded and the pellet was resuspended in 50 ml of loading buffer (0.5 M Tris-HCl, pH 6.8, 10% (v/v) glycerol and 10% (w/v) SDS). 30 µl of each sample were separated by SDS-PAGE, using 1% carboxymethylcellulose at 70V. Enzymes were re-natured by 5 washing steps with a buffer prepared with sodium-acetate 50 mM pH 5.5 and β-mercaptoethanol 5mM. The last washing was performed with sodium acetate buffer 50 mM pH 5.5 for 16 hours. Then, gels were colorimetrically revealed by following the method of Khalili et al. (2011). For this purpose, 0.1% solution of Congo Red was applied to gel for 15 min and then washed with NaCl 1M.

β-glucosidase was extracted from 5 g of soil. 12.5 ml of Tris-HCl 50 mM pH 7 was added and mixed with a vortex for 1 minute. Then, the mix was shaken for 30 minutes and sonicated for 2 min. Samples were centrifuged at 20217 g for 10 min at 4ºC and the supernatant was collected. The supernatant was filtered first in 3 KDa-centrifugal filters (15 ml, Amicon Ultra. Millipore) at 5000 g for 30 min at 4ºC and then with 10 KDa-centrifugal filters (0.5 ml, Amicon Ultra. Millipore) at 14000 g for 30 min at 4ºC. Finally, 200 µl of Tris-HCl were added at the extract and centrifuged for 15 min in order to remove salts from soil samples.

The electrophoresis was performed using a polyacrylamide gel containing 7.5%-4% acrylamide/Bisacrylamide, without SDS at 70V (Fig. 5.3). The gel was washed with succinic acid 50 mM pH 4 and the in-gel enzyme activity was revealed with 0.5 mM MUG (4-methylumbelliferyl glucuronide) for 20 min., as described by Kim et al. (2007). The activity was analyzed by a UV transilluminator (Bio-Rad Chemidoc XRS System, Segrate (Milan), Italy). The amount of protein loaded into gels was not standardized in order to compare the actual activity presented in each treatment.

Fig. 5.3. Protein electrophoresis.

2.6. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. In order to determine significant differences between the means of the treatments, data was submitted to one-way ANOVA. ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference, at the 95% confidence interval in the lineal model). A factor analysis was carried out for enzymatic activities and other for relative quantity of specific PLFA to explore possible differences between treatments.

3. RESULTS

3.1. Plant cover and vegetal community composition

The amended soil showed a higher percentage of plant cover than the control after 24 years of the single amendment application. The plant cover was: 20%, 35% and 70% in control soil, soil with low dose amendment and soil with high dose amendment, respectively. The composition of vegetal community of the three plots was similar with the following predominant species: *Lygeum spartum*, *Moricandia arvensis*, *Asparagus horridus*, *Suaeda vera*, *Piptatherum miliaceum*, and *Salsola genistoides*.

3.2. Chemical properties

The values of chemical properties of soil such as organic carbon, N, humic substances and water-soluble C increased with increasing amount of organic matter added. Total N and humic substances C increased significantly with applied dose (P<0.05). The content of total organic carbon was higher significantly in D2 treatment than in C and

D1; and D1 was significantly higher than C soil too (P<0.05). WSC increased with organic matter dose, D2 was significantly higher respect to C soil (Table 5.2).

Table 5.2. Chemical parameters in control and amended soils.

	Control		D1		D2	
	Mean	**St.Dev**	**Mean**	**St. Dev**	**Mean**	**St. Dev**
TOC ($g100g^{-1}$)	0.63 a	0.23	1.00b	0.20	1.68 c	0.07
N ($g100g^{-1}$)	0.07 a	0.01	0.15 b	0.04	0.24 c	0.04
HS ($g\ kg^{-1}$)	2.56. a	0.33	6.09 b	0.62	7.85 c	0.20
WSC ($g\ kg^{-1}$)	0.14 a	0.02	0.19 ab	0.03	0.21 b	0.03
Soil respiration ($g\ C\text{-}CO_2\ dia^{-1}\ kg^{-1}$)	0.13 a	0.01	0.24 b	0.03	0.42 c	0.03

TOC (total organic carbon), N (total nitrogen), HS (humic substances carbon), WSC (water-soluble carbon). For each parameter, data followed by the same letter are not significantly different (P<0.05). D1 (Low-dose, 65 t ha^{-1}), D2 (High-dose, 195 t ha^{-1}).

3.3. Microbial and biochemical parameters

Cellulase activity was significantly higher in D2 treatment than D1 and C soil (P<0.05). Catalase activity increased significantly with quantity of added amendment (P<0.05). In contrast, polyphenoloxidase activity had a higher activity in C and D1 than D2 treatment. Finally, dehydrogenase, invertase and β-glucosidase showed greater activity in D2 and D1 treatments than C, however, the differences between the D1 and D2 treatments were not significant (P<0.05) (Fig.5.4).

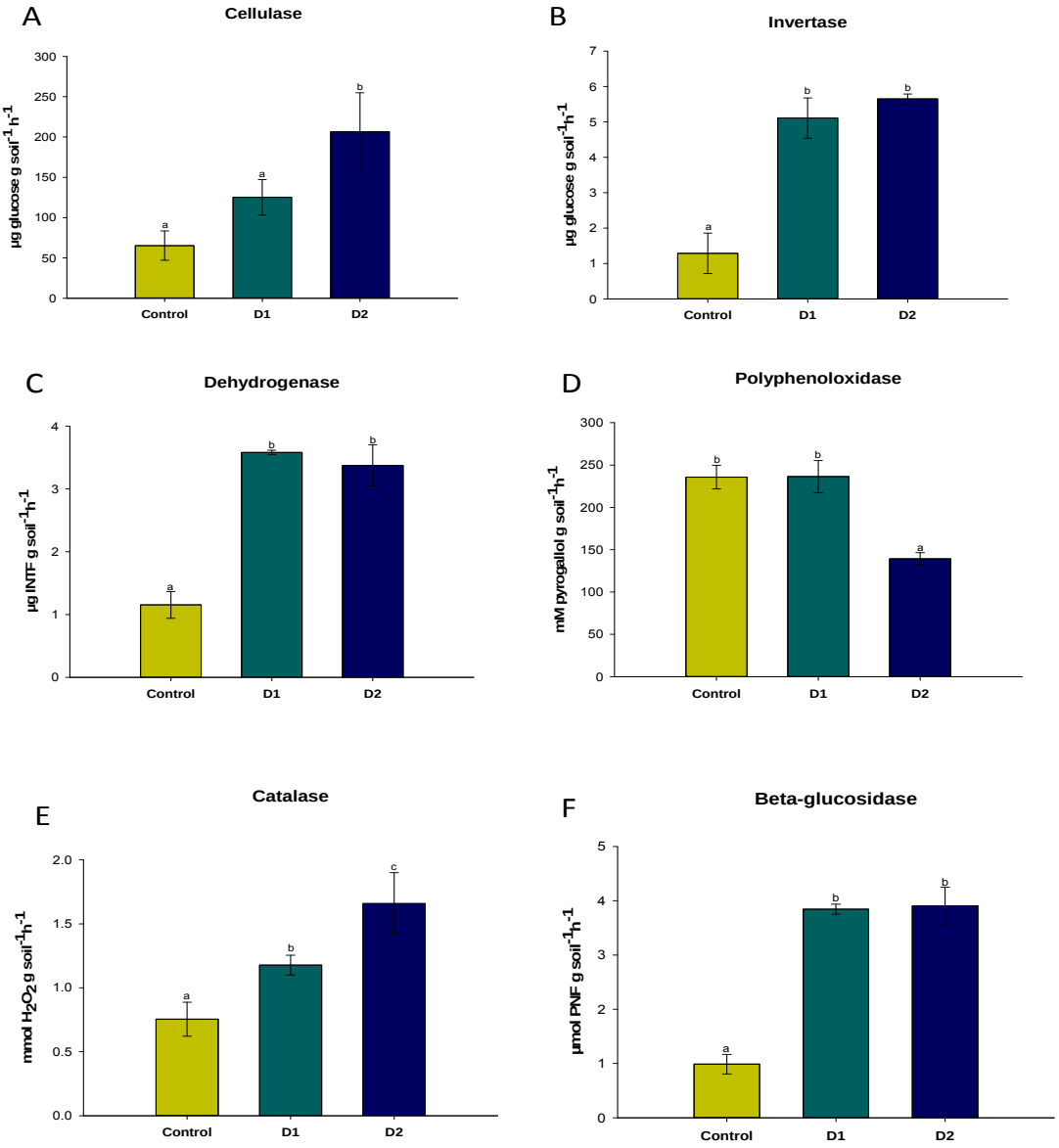


Fig.5.4. Total enzymatic activities for Abanilla soil after long-term organic amendment. Data followed by the same letter are not significantly different (P<0.05). D1 (Low-dose, 65 t ha^{-1}), D2 (High-dose, 195 t ha^{-1}).

Multivariate factor analysis performed with the values of enzyme activities revealed that factor 1 explained 81.05% of the variance, while factor 2 explained 14.02%. For factor 1, the ANOVA analysis established two sample groups, one comprised by the control, and another consisting of the D1 and D2 treatments. For factor 2, two sample groups were found too, one comprised by control and D1, and another consisting of D2 (Fig. 5.5).

Soil respiration was significantly higher in the D2 treatments than D1 and control, and was significantly higher in D1 than in control soil too. (P<0.05) (Table 5.2).

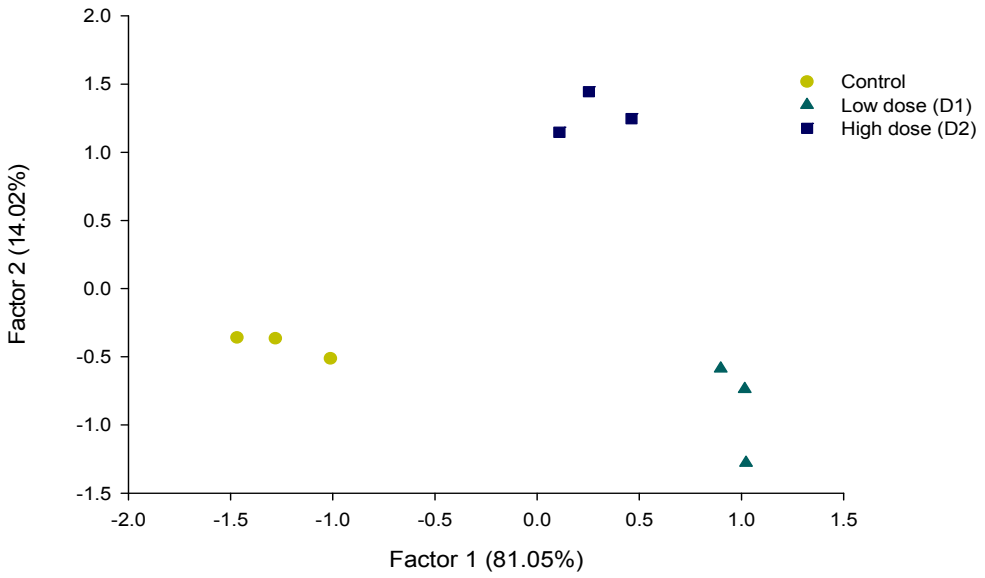


Fig.5.5. Multifactorial analysis made with enzyme activities in control soil (C), low dose (D1) and high dose (D2) added into soil.

3.4. Gel electrophoresis and zymography

The number of isoforms of cellulase and β-glucosidase increased with the amount of organic amendment compared to control soil. In the case of cellulase, one isoenzyme of high molecular weight appeared at the top of the gel for all three treatments (C, D1, D2) (Fig. 5.6A). For the restored soils (D1 and D2), we detected two additional isoforms of lower molecular weight (Fig. 5.6A).

In the case of β-glucosidase, a band with high molecular weight also appeared for the control, D1 and D2 soils (Fig. 5.6B). For D1, two additional bands with lower molecular weight were observed and three additional bands occurred for treatment D2 (Fig. 5.6B).

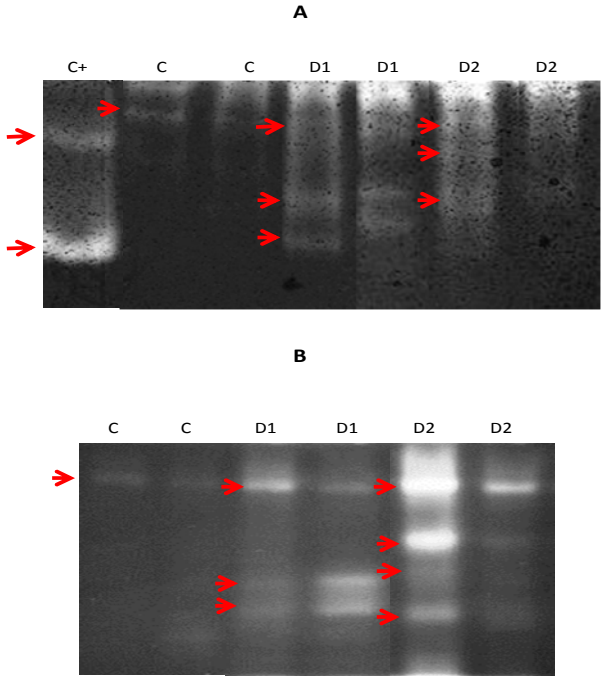


Fig.5.6. Zymogram analysis of β-glucosidase and cellulase. A) Zymogram of cellulase isoenzyme. B) Zymogram of β-glucosidase isoenzyme. In zymograms of cellulase and β-glucosidase, the samples were used in duplicates (control soil (C), soil with low dose amendment (D1), soil with high dose amendment (D2)). Arrows point out different isoenzymes that were present in each sample. (C+: control positive).

3.5. Phospholipid fatty acids (PLFA)

PLFAs analysis revealed a significant increase in the biomass of the soil microbial community 24 years after the addition of organic waste. The biomass of fungi and Gram-negative communities was significantly higher in D2 treatment than in C and D1 treatment (P<0.05). Gram-positive bacteria biomass was significantly higher in D2 treatment than in C (P<0.05) (Table 5.3).

In order to estimate the changes in the structure of the microbial community, a principal component analysis was carried out with the relative abundances of the PLFAs. Factor 1 explained 61.4% of the variance, and factor 2 explained 20.4%. For factor 1, the ANOVA analysis established two sample groups that differed significantly (P<0.05), one comprised by control and D1, and another consisting of the D2 treatment. For factor 2, no differences between treatments were found; the ANOVA only established a group containing control, D1 and D2 doses (Fig. 5.7).

Table 5.3. PLFAs in control and amended soils.

	Control		D1		D2	
	Mean	**St.Dev**	**Mean**	**St. Dev**	**Mean**	**St. Dev**
Bacteria (nmol g^{-1})	2.84 a	0.97	4.35 b	1.27	7.54 c	1.77
Fungi (nmol g^{-1})	0.24 a	0.02	0.35 b	0.03	0.49 c	0.04
G + (nmol g^{-1})	1.51 a	0.64	2.28 ab	0.74	4.04 b	0.98
G – (nmol g^{-1})	1.33 a	0.34	2.07 a	0.54	3.50 b	0.80
G +/G-	1.10 a	0.22	1.09 a	0.10	1.16 a	0.02
F/B	0.09 a	0.03	0.08 a	0.02	0.07 a	0.01

G + (Gram-positive), G – (Gram-negative), G+/G- (Gram-positive- Gram-negative ratio), F/B (Fungi-Bacteria ratio). For each parameter, data followed by the same letter are not significantly different (P<0.05). D1 (Low-dose, 65 t ha^{-1}), D2 (High-dose, 195 t ha^{-1}).

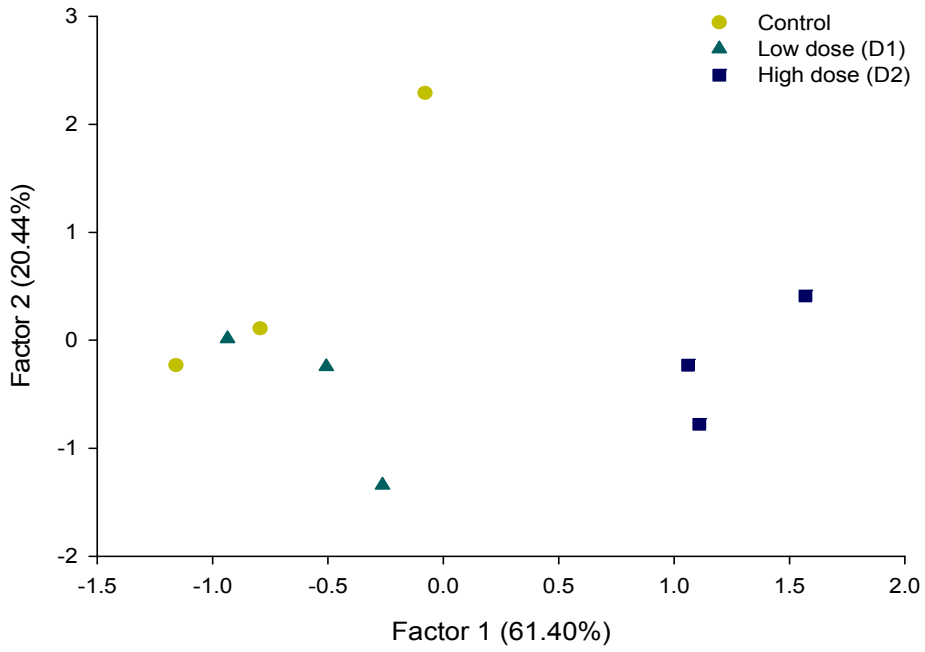


Fig.5.7. Multifactorial analysis made with relative amount of fatty acids in control soil (C), low dose (D1) and high dose (D2) of organic amendment after 24 years of organic matter addition.

4. DISCUSSION

The addition of organic wastes stimulated vegetation development; in turn, this improved plant development thus providing ongoing organic carbon inputs to the soil. The development of plant cover in degraded soils is an important factor for soil quality because it protects soils against erosive processes and supplies organic C as energy source for the microorganisms through root exudates and plant remains (Balloni and Favilli, 1987; García et al., 1994; Brockway et al., 1998). Addition of organic matter in the past had caused an increase of organic carbon, water-soluble carbon and humic substances. Water-soluble carbon is considered a labile fraction that is easily degradable by soil microorganisms (De Luca and Keeney, 1993). The activities of enzymes involved in carbon cycling, except polyphenoloxidase, increased due to increase in soil organic matter caused both directly by organic amendments two decades previously and the consequent stimulation of vegetation, as observed by Harris et al. (1996). Others authors have observed increases in enzyme activity and development of soils subjected to organic restoration (Ros et al., 2003; Liang et al., 2005; Bastida et al., 2008a). However, the behaviour of polyphenoloxidase is not entirely clear. As observed in our study, this enzyme is commonly not correlated to hydrolase activities (Sinsabaugh, 2010). Some authors report that polyphenoloxidase activity decreased with an increase in soil organic matter concentration (Sinsabaugh et al., 2005; Stursova and Sinsabaugh, 2008). However, it is not clear whether the decrease of polyphenoloxidase activity is due to a decrease in the amount of enzyme or an inhibition caused by the higher organic carbon content in restored soils.

Given the abundance of cellulose as organic matter of plant origin and its impact in soil, we focused our study in the enzymes involved in cellulose degradation (cellulase and β-glucosidase enzymes) that are implicated in carbon cycling. Cellulose activity was higher in the amended plots than in the control soil and these variations were parallel to the organic matter content and plant development after long-term amendment. In comparison to control, the higher vegetation cover in restored soils would release higher amount of plant debris into soil that contains substrate for the cellulose activity (García et al., 1994). The β-glucosidase activity was also higher in the amended plots than in the control soil but no differences were shown between treatments (D1 and D2), as in the case of water soluble carbon (Table 5.2). In this sense, cellulase activity showed a closer relationship with vegetation cover while β-glucosidase did not responded to vegetation cover when different doses were applied. Some activities such as β-glucosidase, invertase and dehydrogenase seemed to reveal a threshold for the

addition of high quantities of organic matter. In this way, these activities did not increase proportionally with the organic matter addition as could be previously observed in other studies (Bastida et al., 2008a). It is possible that for this reason PCA analysis of enzymatic activities revealed a change between control soil and soil where organic amendment was applied with slightly differences between both doses of amendment.

The development of microbial biomass was related to the dose of organic amendment and the parallel increase of vegetation cover. Indeed, the plant cover was: 20%, 35% and 70% in control soil, soil with low dose amendment and soil with high dose amendment, respectively. In this sense, Bastida et al. (2008a) observed that the quantitative changes in the plant inputs due to the organic amendments could imply the presence of a greater amount of organic inputs into the soil and hence better microbial development. The increase in microbial biomass and the parallel change in the community structure, as observed by PCA analysis of fatty acids, could explain the higher microbial respiration in the amended soil, as well as variations in enzyme activities and zymography patterns (Criquet et al., 2002; Di Nardo et al., 2004; Pramanik and Chung, 2011). In any case, we cannot ruled out that the amount of certain isoforms in the control would be below the detection limit for each enzyme as a consequence of the low metabolic activity and microbial biomass compared to treated soils.

The structure of microbial community of D2 treatment was significantly different to C and D1 treatment, as demonstrated by the multivariate analysis of PLFAs (Fig.4). A change in the structure of microbial community after long-term organic amendment has been noticed also in other studies (Petersen et al., 2003; Bastida et al., 2013) and may partially explain changes in the enzyme activity and expression of some enzymes (Perissol et al., 1993). For instance, the behavior of polyphenoloxidase reflects the structure of microbial community structure, i.e. the activity was the lowest in D2 and the community structure was also distinct in such treatment. Polypyhenoloxidase is dominant in a limited number of microbial groups (i.e. Fungi; *Actinobacteria*) and therefore it could be quite responsive to changes in community structure (Schimel, 1995).

Knight and Dick (2004) showed by kinetic studies that β-glucosidase isoenzymes present higher K_m (substrate affinity) for managed than unmanaged soil. This fact suggested that β-glucosidase may have different isoforms depending on the land management, soil substrate and environmental conditions. Similarly, Nazir et al. (2010)

showed that cellulose and β-glucosidase enzymes may also have different isoforms due to soil substrate or strain of microorganisms (Sonia et al., 2008).

In conclusion, the addition of organic amendments to semiarid soil leads to the long-term development of the microbial biomass and consequently an increase in microbial activity. In addition, this input of organic matter leads to the expression of a larger variety of enzyme isoforms involved in carbon cycling that would benefit the biochemical potential of restored soils in semiarid environments.

CONCLUSIONES

La presente Tesis Doctoral tiene como Objetivo mejorar el conocimiento sobre el ciclo de la materia orgánica en suelos semiáridos con un elevado grado de degradación biológica y muy escasa cobertura vegetal con el fin de poder elaborar estrategias para su restauración. Para ello nos hemos centrado particularmente en la capacidad que presenta este tipo de suelo para responder a fuentes de carbono exógenas derivadas de residuos vegetales, el papel que desempeña la comunidad microbiana en la degradación de estas fuentes de carbono, y su relación con los ciclos biogeoquímicos de los elementos importantes (C, N y P), con particular incidencia en el del carbono como elemento clave en la sostenibilidad de estos ecosistemas. Se maneja como Hipótesis general de trabajo de esta Tesis Doctoral el hecho de que las comunidades microbianas de suelos áridos y semiáridos tienen una limitada capacidad para el procesamiento de sustratos carbonados, a consecuencia del escaso contenido en materia orgánica que presentan habitualmente estos suelos.

Para conseguir los objetivos que permitan la mejora de conocimientos señalados con anterioridad, se han realizado experimentos tanto a nivel de laboratorio como de campo, en los que se ha adicionado al suelo degradado materia orgánica de origen vegetal, intentando de este modo conocer su efecto en las propiedades químicas, bioquímicas y microbiológicas de suelos semiáridos. La utilización de técnicas isotópicas nos ha permitido conocer de manera detallada los procesos bioquímicos y microbianos que gobiernan la dinámica del carbono en suelos semiáridos.

Como **conclusión general** se puede indicar que la adición de materia orgánica de origen vegetal a suelos semiáridos proporciona una mayor calidad al suelo y una mejoría en las propiedades biológicas del mismo. Este impacto depende del tipo concreto de suelo (de su textura, de sus propiedades químicas, etc.), del nivel de degradación del mismo, y por supuesto, de la complejidad y grado de estabilización de la materia orgánica exógena adicionada. Con ello se quiere indicar que dependerá de sus opciones para mineralizarse, para introducirse en procesos de humificación, o para fijarse en el suelo sobre los coloides minerales de dicho suelo. Notoriamente, los suelos semiáridos no pierden su potencial para responder a inputs orgánicos a pesar de estar desprovistos de vegetación y no recibir materia orgánica exógena durante un largo período de tiempo. Además, los suelos con alto nivel de degradación presentan una mayor predisposición y sensibilidad a la adición de ciertos sustratos orgánicos relacionados con residuos vegetales.

Como **Conclusiones específicas** podemos señalar los siguientes aspectos:

- La materia orgánica exógena, añadida a suelos semiáridos sometidos a un elevado nivel de degradación, no es mineralizada totalmente por la comunidad microbiana contenida en esos suelos (p.ej: glucosa, celulosa y lignina). Este hecho es debido a que dichos sustratos son parcialmente protegidos física y químicamente por las partículas del suelo, y asimilados por la biomasa microbiana. Además, la persistencia de estas moléculas en el suelo es dependiente de la naturaleza química de las mismas, siendo la lignina la más resistente a la degradación por las poblaciones microbianas existentes en suelos semiáridos.

- La adición de celulosa y lignina no estimuló consistentemente el desarrollo microbiano en suelos semiáridos. Sin embargo, la glucosa, una molécula mucho más simple, sí que fue utilizada como fuente de carbono y energía para el aumento de la biomasa microbiana. A pesar de que sólo una pequeña fracción de la comunidad microbiana fue capaz de asimilar los sustratos más complejos tipo celulosa y lignina, dicha fracción es fundamental para el ciclo del carbono y la sostenibilidad de suelos semiáridos.

- Las características del suelo, y en particular su textura, así como su nivel de degradación, influyen en el tiempo de respuesta de la comunidad microbiana a la adición de moléculas orgánicas de origen vegetal, y por tanto en su mineralización y en los diferentes grupos microbianos que contribuyen a la degradación de cada tipo de molécula. Además, la mineralización del sustrato y la contribución de los grupos microbianos en la misma son más dependientes del tipo de suelo que de la naturaleza química de la molécula añadida al suelo.

- La utilización de técnicas isotópicas ha resultado ser muy útil para este tipo de ensayos. El marcaje de moléculas orgánicas con ^{13}C ha permitido observar que la adición de materia orgánica exógena a suelos semiáridos conlleva una activación de la comunidad microbiana y, de forma paralela, una mineralización de la materia orgánica del propio suelo conocida como “priming effect”. Esta degradación de la materia orgánica del suelo es llevada a cabo por grupos microbianos que difieren de los degradadores de los sustratos orgánicos exógenos adicionados al mismo.

- Se ha estudiado la adición de restos vegetales a suelos semiáridos, en condiciones reales de campo, como estrategia para conseguir la restauración de los mismos. Dichos restos vegetales se introdujeron en el suelo con dos diferentes niveles de estabilización: “frescos”, y “estabilizados” después de someterlos a un proceso

bioxidativo de compostaje (restos vegetales compostados). La adición de residuos vegetales “frescos” provocó un efecto mayor en la comunidad microbiana que la adición de dichos residuos un su forma compostada. La estabilización de los residuos vegetales conforma una nueva estructura de materia orgánica, con menor capacidad de actuar como sustrato para las poblaciones microbianas existentes.

- La dosis de la materia orgánica adicionada al suelo no tiene un efecto notable a largo plazo sobre la actividad microbiana debido a que puede existir una “dosis umbral” de materia orgánica exógena, a partir de la cual no se incentiva su uso por las poblaciones microbianas existentes en suelos semiáridos. Sin embargo, la dosis sí que ejerce un efecto notable en la estructura funcional de la comunidad microbiana presente en el suelo.
- La adición de carbono a suelos semiáridos afecta a la bioquímica de los ciclos de otros elementos importantes (N y P) en el suelo. Este hecho ha sido demostrado por el efecto de los sustratos orgánicos ensayados (procedentes de restos vegetales), sobre las actividades enzimáticas implicadas en los mencionados ciclos de elementos.
- La adición de enmiendas orgánicas a largo plazo permite la expresión de una mayor variedad de isoenzimas involucradas en el ciclo del carbono, pudiendo beneficiar el potencial bioquímico de suelos semiáridos restaurados.

CONCLUSIONS

The aim of this Doctoral Dissertation was to expand to knowledge on the cycling of organic matter in semiarid soils with a high biological degradation and scarce plant cover, and the development of strategies for the soil restoration. To reach this goal we have focused on the following topics in particular: the ability of this type of soil to respond to exogenous sources of carbon derived from plants; the role of the soil microbial community in the processing of organic carbon; and the relationship of these carbon sources with the biogeochemical cycles of elements (C, N and P), with a particular focus on carbon as a key element in the sustainability of these ecosystems. The general working Hypothesis of this Doctoral Thesis is that the microbial communities in arid and semiarid soils have a limited capacity for processing carbonaceous substrates as a result of the low content of organic matter usually found in these soils.

To achieve the goals that would enable us to improve our knowledge of the topics described above, we carried out experiments both in the laboratory and in the field. In these experiments, we added plant-derived organic matter to degraded soils in order to ascertain its effects on the chemical, biochemical and microbiological properties of semiarid soils. The use of isotopic techniques gave us a detailed understanding of the biochemical and microbial processes that govern carbon dynamics in semiarid soils.

The **overall conclusion** of this Doctoral Dissertation is that the addition of organic matter of plant origin to semiarid soils improves the quality and biological properties of the soil. The effects of organic matter depend on the particular soil type (its texture, chemical properties, etc.); the degree of degradation of the soil; and, of course, the complexity and degree of stabilization of the exogenous organic matter added. In other words, the impact of an organic amendment depends on its options for being mineralised, for undergoing humification and for being fixed in the soil on the soil mineral colloids. Interestingly, semiarid soils do not lose their potential to respond to organic input despite the fact they are devoid of vegetation and have not received exogenous organic matter for a long time. Furthermore, soils with a high level of degradation are particularly predisposed and sensitive to the addition of certain plant-derived organic substrates.

Furthermore, we can highlight the following **specific conclusions**:

- The added organic matter that simulates plant-derived input (glucose, cellulose and lignin) is not completely mineralised by the microbial communities in semiarid soils. This is mainly because these substrates are partially physically and chemically

protected by the soil particles and assimilated by the soil microbial biomass. Furthermore, the persistence of these molecules in the soil depends on their chemical nature, with lignin being the most resistant of the three to degradation by the microbial populations found in semiarid soils.

- The addition of cellulose and lignin did not consistently stimulate microbial growth in semiarid soils. Nevertheless, glucose, a much simpler molecule, was in fact used as a carbon and energy source for increasing microbial biomass. Although only a small fraction of the microbial community was able to assimilate the cellulose and lignin, the more complex substrates, this fraction was crucial for carbon cycling and ultimately for sustainability in semiarid soils.

- The characteristics of a soil, particularly its texture and level of degradation, influence the response time of the microbial community to the addition of plant-derived organic molecules. As a result, these characteristics also affect the mineralisation of such molecules as well as the different microbial groups that contribute to the degradation of each type of molecule.

- The use of isotopic techniques was highly useful in the evaluation of the chemical and microbiological fate of carbon in semiarid soils. The use of isotopically (^{13}C) labelled organic molecules in our experiments made it possible to observe that the addition of exogenous organic matter to semiarid soils activates the microbial community and, in parallel, leads to the mineralisation of the soil organic matter, known as the “priming effect”. This degradation of the soil organic matter is carried out by microbial groups that are different from those that degrade the exogenous organic substrates added to the soil.

- We studied the addition of plant waste to semiarid soils in real field conditions as a strategy to restore such soils. The plant waste used was introduced into the soil with different levels of stabilisation, i.e., it was either “fresh” or stabilised after being subjected to a bio-oxidative composting process (composted plant waste). The addition of “fresh” prunning waste had a greater effect on the microbial community than the addition of the same waste in composted form. Stabilised (composted) prunning waste constitutes a structured of organic matter, with less capacity to act as a substrate for the existing microbial populations.

- The dose of the organic matter added to the soil does not have a considerable effect in the long term on the soil microbial activity due to fact that there may be a "threshold dose" of exogenous organic matter, beyond which there is no stimulus for

the microbial populations in semiarid soils to use this organic matter. Nevertheless, the dose was found to have a noticeable effect on the functional structure of the microbial community in the soil.

- Adding carbon to semiarid soils affects the biochemistry of the cycles of other important elements (N and P) in the soil. This fact was proven by the effect of the organic substrates assayed (from prunning waste) on the enzyme activities involved in the cycles of these different elements.
- The addition of organic amendments leads to the expression of a greater variety of isoenzymes involved in the carbon cycle in the long term and may benefit the biochemical potential of the semiarid soils restored.

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ANNEX 1

Supporting information of Chapter 1: Can the labile carbon contribute to carbon inmobilization in semiarid soils? Priming effects and microbial community dynamics.

THE THREE INCUBATIONS ARE COMPARABLE

A preliminary experiment (without isotope addition) was done in order to test if the different incubations were comparable. Control soil and soil amended with natural abundance glucose at two doses (75 µg C g^{-1} soil and 300 µg C g^{-1}, LD and HD respectively) were subjected to incubation in the three different systems: Experiment 1 (in plastic containers with 100 g of soil), Experiment 2 (in 12 ml Labco tubes with 1 g of soil), and Experiment 3 (Petri dishes with 25 g of soil).

The moisture was controlled gravimetrically by adjusting the water-holding capacity to 60% every 2 days. The incubation was performed during 17 days at 28 ºC. These conditions were the same than in the experiments corresponding to the results showed in the article.

In order to proof that the three experiments were comparable, a sub-sample of 1g from each experiment was taken at 1, 4 and 17 days. These subsamples were incubated in 12 ml tubes during 23 days and the total amount of CO_2 was registered by a gas-chromatograph (Shimadzu GC-14, Kyoto, Japan) equipped with a thermal conductivity detector (TCD).

Soil respiration was similar and no significant differences (P<0.05) appeared between treatments of the different experiments at the same incubation time. These results point to the fact that the experiment conditions did not influence the obtained results (Table A1.1).

Table A1.1. Soil respiration (µg CO_2-C g^{-1} soil) for subsamples taken from each experiment and incubated during 25 days in 12ml tubes.

		Day 1	Day 4	Day 17
Experiment 1	**Control**	99	59	15
	LD	161	61	16
	HD	371	128	17
Experiment 2	**Control**	101	61	15
	LD	160	62	15
	HD	369	133	17
Experiment 3	**Control**	102	62	16
	LD	155	65	16
	HD	370	131	17

Moreover, we measured dehydrogenase activity as a general indicator of microbial activity in subsamples from 1, 4 and 17 days of incubation. Results were not significant differences (P<0.05) between treatments of the different experiments at the same incubation times (Table A1.2). These results also support the absence of significant effect of incubation conditions in the obtained results. Additionally, dehydrogenase activity was not significantly different (P<0.05) for samples incubated during 60 days in different conditions.

Table A1.2. Dehydrogenase activity (µg INTF g^{-1} soil h^{-1}) for subsamples taken from each experiment**.**

		Day 1	Day 4	Day 17
Experiment 1	**Control**	0.78	0.48	0.23
	LD	1.81	0.52	0.24
	HD	3.17	1.28	0.27
Experiment 2	**Control**	0.81	0.55	0.28
	LD	1.67	0.60	0.28
	HD	3.25	1.31	0.27
Experiment 3	**Control**	0.85	0.52	0.30
	LD	1.77	0.57	0.29
	HD	3.08	1.27	0.30

The method described by García et al. (1997) was used to measure dehydrogenase activity, reducing INT (2-p-iodophenyl-3-p-nitro-phenyl-5-phenyltetrazolium chloride) to INTF (iodonitrophenyl-formazan), which was measured in a spectrophotometer at 490 nm.

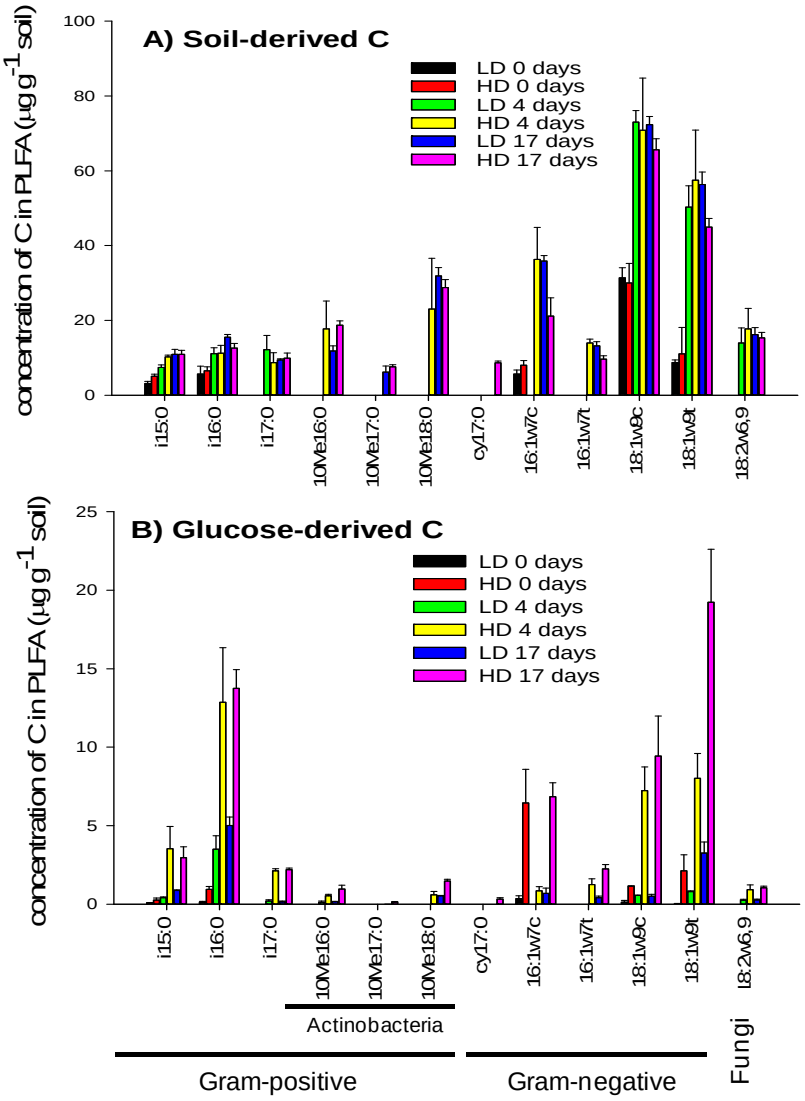
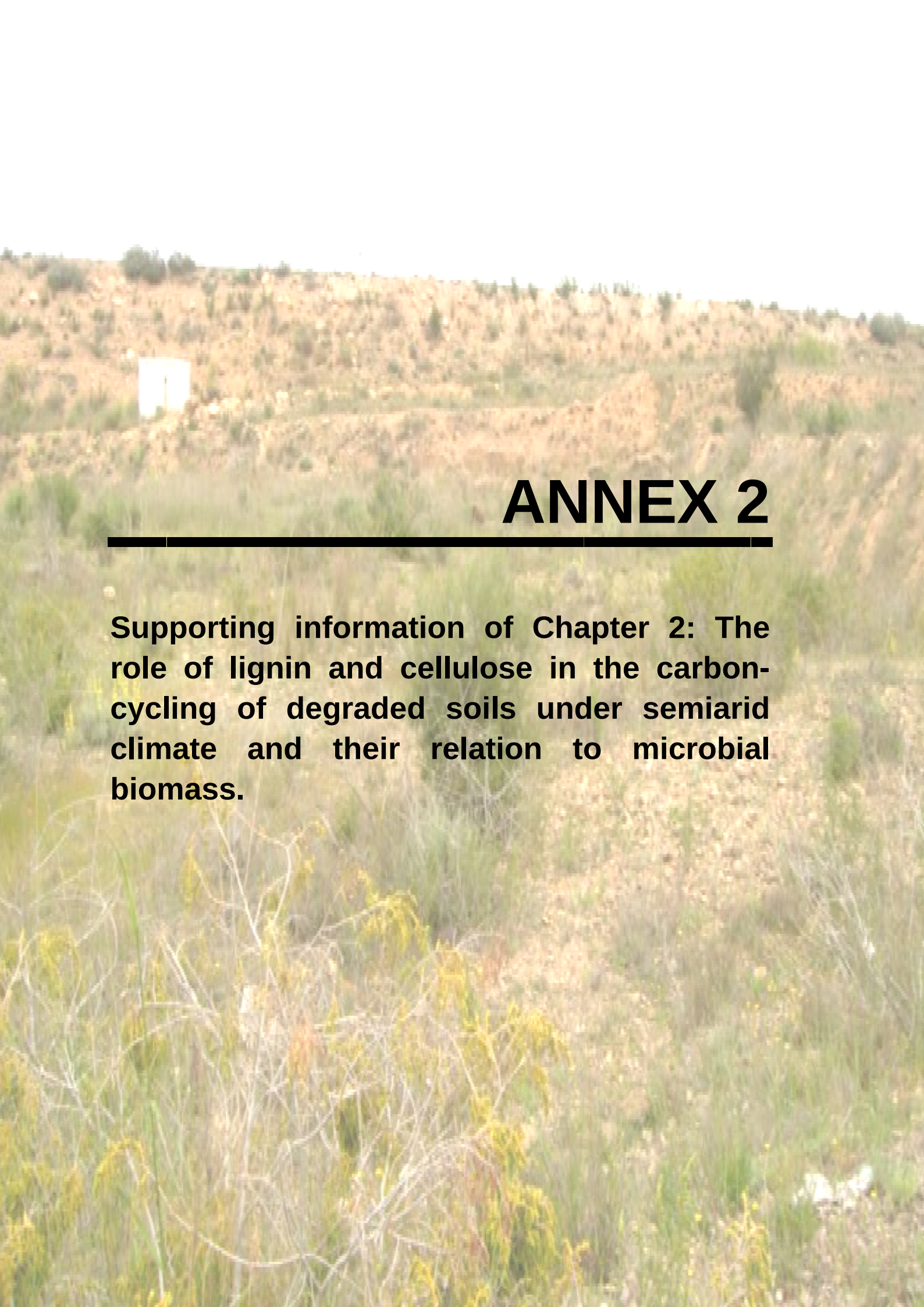


Fig. A1.1. Concentration of A) soil-derived C and B) glucose-derived C within PLFAs in the different treatments.

Table A1.3. ^{13}C-enrichment in FAMES (atom %) from ^{13}C-glucose treatments at different incubation times.

	Gram-positive bacteria[3]						Gram-negative bacteria						Fungi
	i15:0	**i16:0**	**i17:0**	**10me16:0**	**10me17:0**	**10me18:0**	**cy17:0**	**cy19:0**	**16:1w7c**	**16:1w7t**	**18:1w9c**	**18:1w9t**	**18:2w6,9**
1 day													
LD[1]	**3.72**	**5.02**	**3.44**	*	*	*	*	*	**6.36**	*	**1.48**	**1.35**	**n.e.**
	1.12	*1.27*	*0.39*	*	*	*	*	*	*1.56*	*	*0.31*	*0.25*	*
HD[2]	**6.90**	**14.85**	**7.18**	**n.e.**	**n.e.**	**n.e.**	*	*	**43.53**	*	**4.60**	**15.80**	**n.e.**
	1.50	*6.14*	*0.46*	*n.e*	*n.e.*	*n.e.*	*	*	*12.53*	*	*0.53*	*4.20*	*
4 days													
LD	**6.11**	**23.37**	**2.62**	**1.40**	*	**n.e**	*	*	**n.e.**	**n.e.**	**1.80**	**2.52**	**2.69**
	0.18	*4.53*	*0.51*	*0.04*	*	***n.e.***	*	*	*	*	*0.02*	*0.04*	*0.25*
HD	**25.04**	**50.02**	**20.35**	**3.63**	*	**3.63**	*	*	**3.50**	**8.60**	**9.74**	**12.43**	**5.83**
	1.86	*2.78*	*4.25*	*0.87*	*	*1.32*	*	*	*1.24*	*4.12*	*0.59*	*3.22*	*1.39*
17 days													
LD	**7.96**	**24.24**	**2.58**	**2.46**	**1.22**	**2.53**	*	*	**2.90**	**3.89**	**1.74**	**6.34**	**2.51**
	0.04	*1.71*	*0.56*	*0.20*	*0.01*	*0.48*	*	*	*0.98*	*0.98*	*0.50*	*1.97*	*0.30*
HD	**21.50**	**50.96**	**18.53**	**5.62**	**2.49**	**5.75**	**4.12**	*	**22.94**	**18.97**	**12.93**	**29.45**	**7.04**
	6.94	*4.35*	*1.81*	*2.05*	*1.05*	*0.34*	*0.84*	*	*4.76*	*3.46*	*3.56*	*9.50*	*0.14*

[1] LD (low-dose); [2] HD (High-dose); [3]10Me-branched FAMES are taken as actinobacterial biomarkers within Gram-positive bacteria. Mean values are showed in bold letters; Standard deviation values are showed in italics. Asterix indicates that the ^{13}C-enrichment could not be determined because the concentration was too low to analyze the stable carbon isotope ratio using GC-C-IRMS or GC-MS mass spectra; n.e.: not ^{13}C-enriched.



ANNEX 2

Supporting information of Chapter 2: The role of lignin and cellulose in the carbon-cycling of degraded soils under semiarid climate and their relation to microbial biomass.

Table A2.1. ANOVA of repeated measures with molecule type and soil type as main factors.

	WSC		SP		SR		SM		PE	
	F	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Molecule	3.81	0.05	88.91	<0.001	6.48	0.01	7.19	0.03	33.85	<0.001
Soil	586.28	<0.001	27434.55	<0.001	841.11	<0.001	203.36	<0.001	17.03	0.003
Mol x soil	2.36	0.14	70.71	<0.001	3.70	0.06	5.77	0.04	70.02	<0.001
	TOC		**Bacteria**		**Gram-positive**		**Gram-negative**		**Fungi**	
	F	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Molecule	0.67	0.53	25.17	<0.001	13.83	0.001	13.13	0.001	5.65	0.02
Soil	1059.23	<0.001	1525.90	<0.001	664.87	<0.001	904.06	<0.001	539.62	<0.001
Mol x soil	0.02	0.98	12.83	0.001	10.48	0.002	4.52	0.03	10.67	0.002
	F/B		**G+/G-**		**^{13}C Bacteria**		**^{13}C Gram-positive**		**^{13}C Gram-negative**	
	F	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Molecule	3.90	0.05	0.47	0.64	18.74	0.003	85.18	<0.001	4.59	0.07
Soil	0.001	0.98	4.54	0.05	52.47	<0.001	320.51	<0.001	14.30	0.005
Mol x soil	2.63	0.11	0.20	0.82	2.03	0.19	18.12	0.003	8.82	0.02
	^{13}C Fungi		**^{13}C BS**							
	F	**P**	**F**	**P**						
Molecule	2.83	0.13	217.98	<0.001						
Soil	35.31	<0.001	14.35	0.005						
Mol x soil	52.40	<0.001	2.02	0.19						

WSC (water soluble carbon fraction); SP (carbon fraction extracted by sodium-pyrophosphate); SR (soil respiration); SM (substrate mineralization); PE (priming effect); TOC (total organic carbon); F/B (Fungi to Bacteria ratio); G+/G- (ratio of Gram-positive bacteria to Gram-negative bacteria); BS (bulk soil).

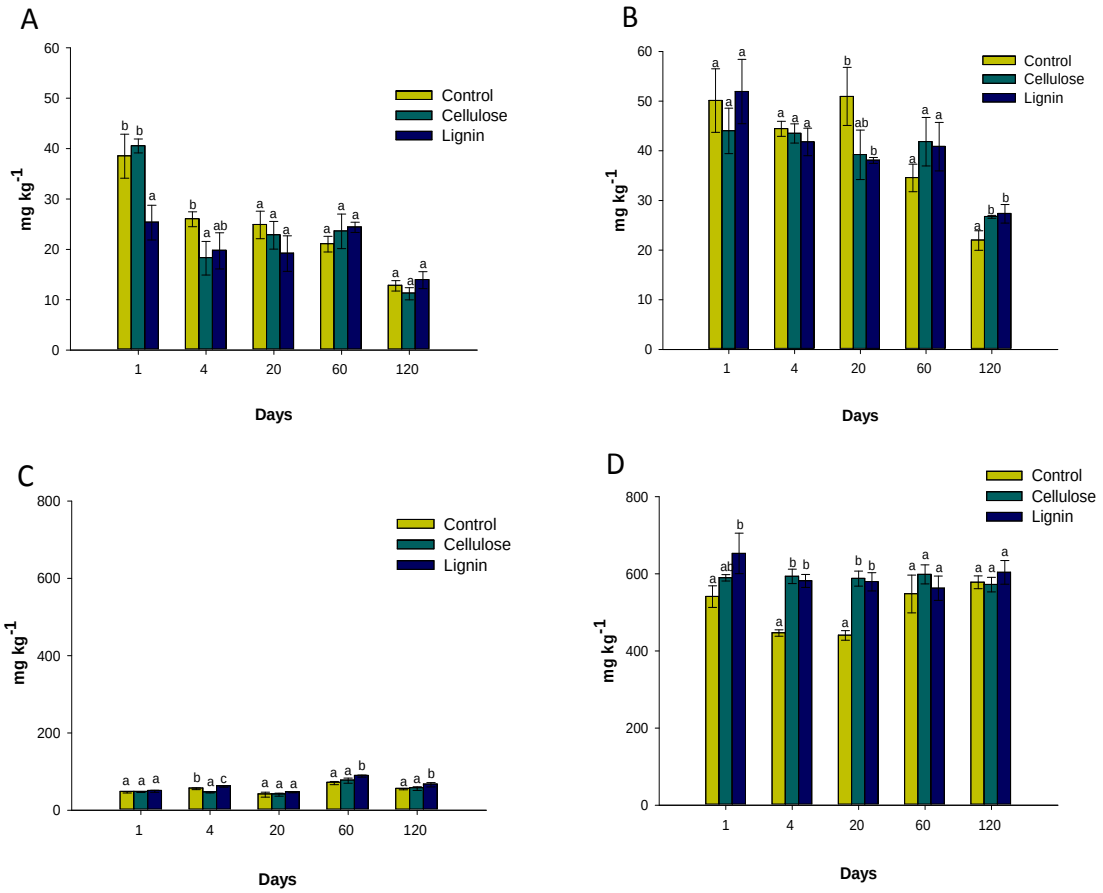


Fig.A2.1. Water soluble carbon fraction (A and B) and carbon fraction extracted by sodium pyrophosphate (C and D) in control, soil amended with cellulose and soil amended with lignin. For each incubation time, data followed by the same letter are not significantly different (P<0.05).

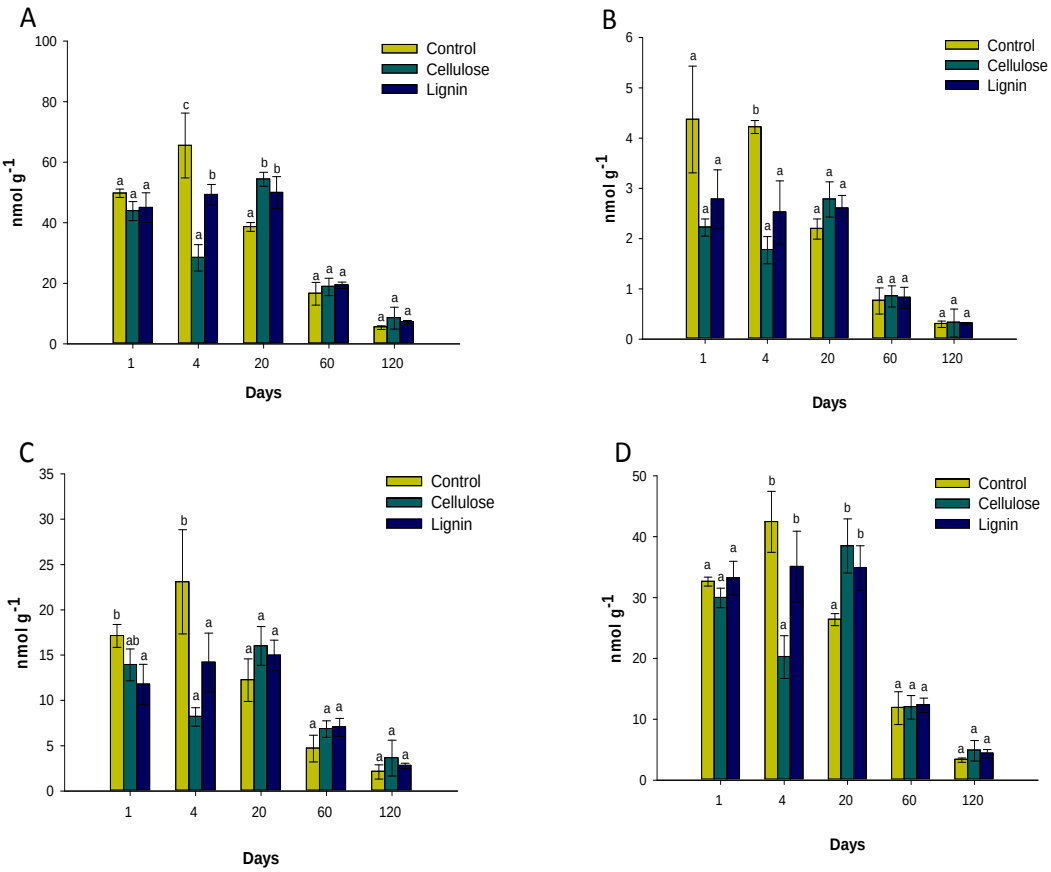


Fig.A2.2. Concentration of carbon bound to bacteria (A), fungi (B), Gram-positive (C) and Gram-negative (D) PLFA of control soil, soil amended with cellulose and soil amended with lignin for Abanilla soil. For each incubation time, data followed by the same letter are not significantly different (P<0.05).

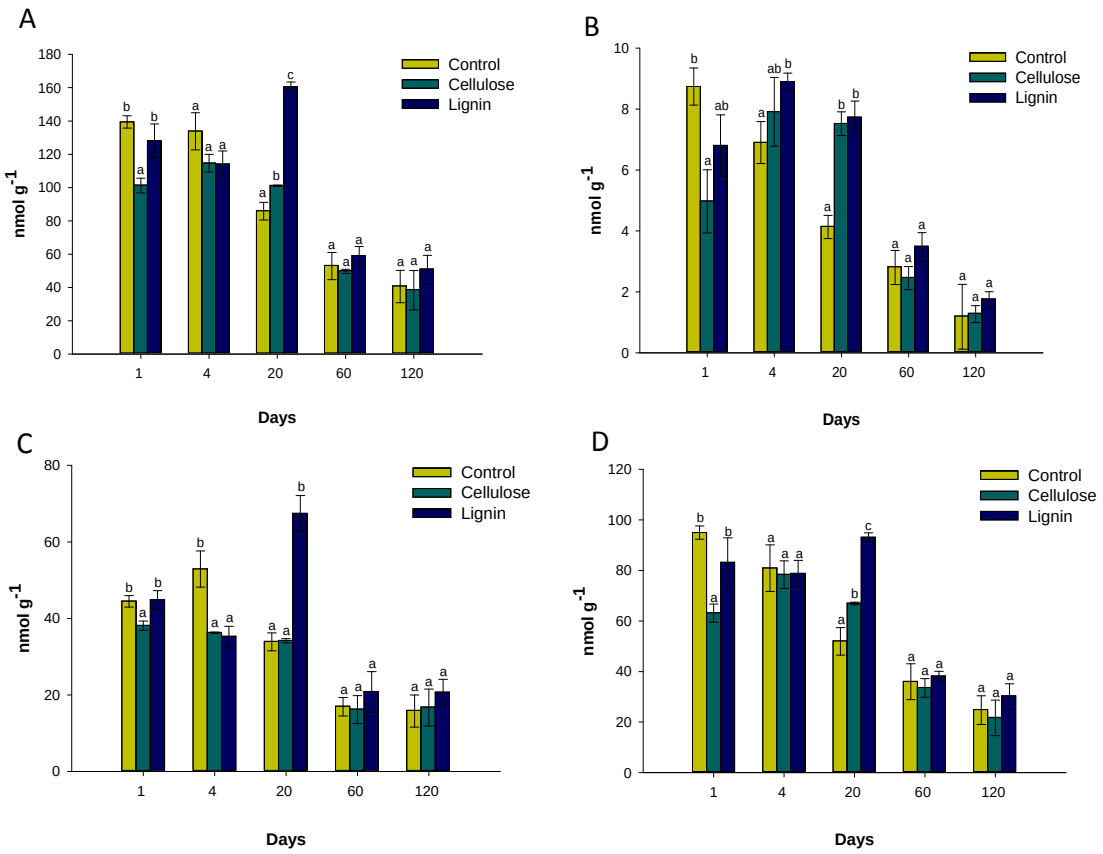


Fig.A2.3. Concentration of carbon bound to bacteria (A), fungi (B), Gram-positive (C) and Gram-negative (D) PLFA of control soil, soil amended with cellulose and soil amended with lignin for Santomera soil. For each incubation time, data followed by the same letter are not significantly different (P<0.05).

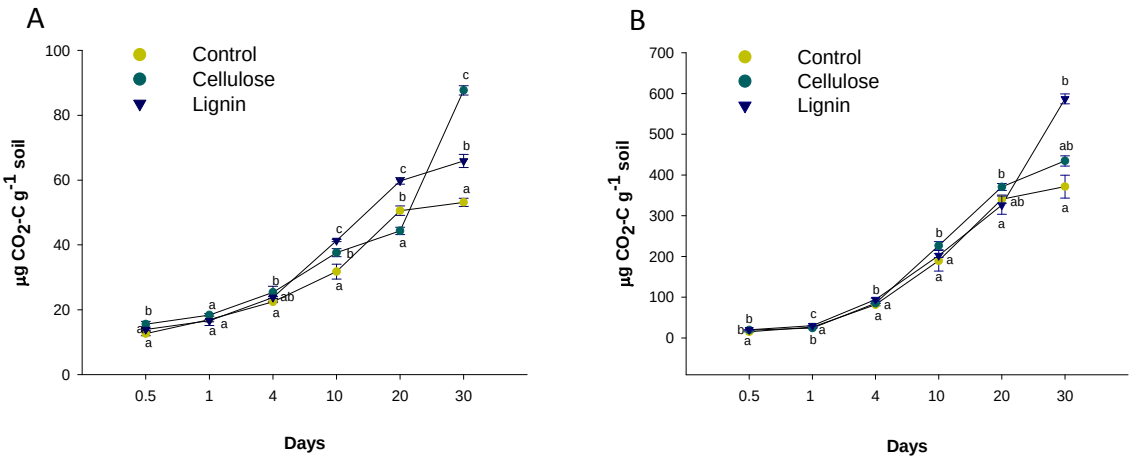
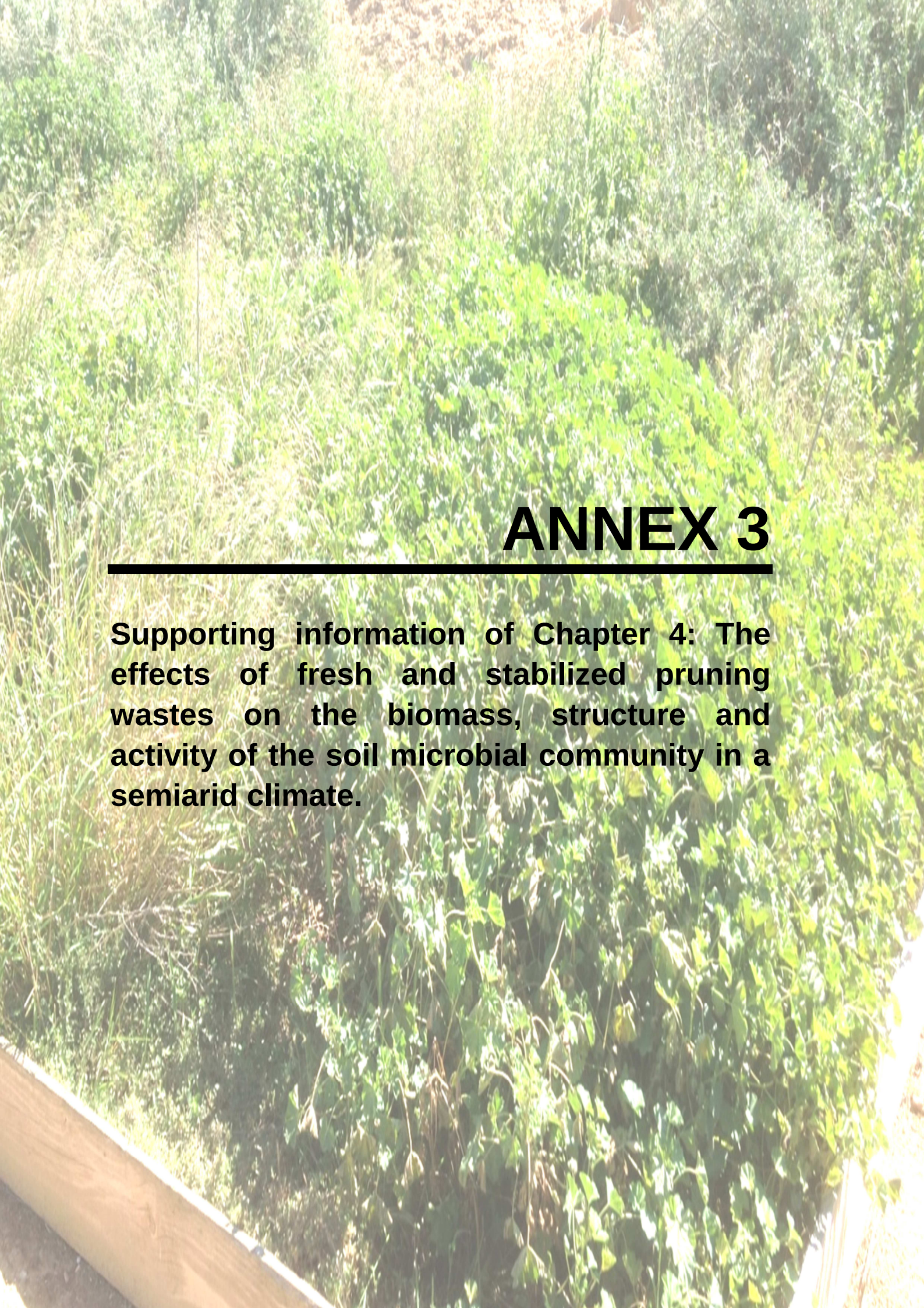


Fig.A2.4. Soil respiration in Abanilla (A) and Santomera (B). For each incubation time, data followed by the same letter are not significantly different (P<0.05).



ANNEX 3

Supporting information of Chapter 4: The effects of fresh and stabilized pruning wastes on the biomass, structure and activity of the soil microbial community in a semiarid climate.

Table A4.1. Coverage and structure of the plant community in control soil, soil amended with fresh green manure (VW) and soil treated with composted green manure (CVW), 6 and 18 months after organic amendment addition.

	6 months			18 months		
	Control	**VW**	**CVW**	**Control**	**VW**	**CVW**
% coverage	**25**	**15**	**45**	**55**	**77**	**78**
Diplotaxis erucoides	presence	presence	presence	presence	presence	presence
Piptatherum miliaceum				presence	presence	presence
Reichardia tingitana					presence	presence
Malva parviflora	presence	presence	presence			
Atriplex halimus	presence	presence	presence	presence		presence
Anagallis arvensis		presence	presence			
Beta maritima	presence	presence	presence	presence	presence	presence
Solanum nigrum		presence	presence			
Convolvulus althaeoides				presence	presence	presence
Lavatera cretica				presence	presence	presence

Table A4.2. Coverage and structure of the vegetation in control soil, soil amended with fresh green manure (VW150 and VW300), and soil treated with composted green manure (CVW150 and CVW300) 60 months after organic amendment addition.

	Control	**VW150**	**VW300**	**CVW150**	**CVW300**
% coverage	**77**	**82**	**85**	**91**	**93**
Hedypnois cretica	presence	presence		presence	presence
Bromus fasciculatus	presence	presence	presence	presence	presence
Diplotaxis erucoides	presence		presence		presence
Piptatherum miliaceum	presence	presence		presence	presence
Reichardia tingitana	presence		presence	presence	presence
Carduus bourgeanus		presence	presence	presence	presence
Malva parviflora	presence	presence	presence	presence	presence
Atriplex halimus	presence			presence	presence
Anagallis arvensis	presence				

Table A4.3. Correlations between variables. * signification *P*<0.05. ** signification *P*<0.01.

	TOC	N	WSC	HS	*β*-GL	Lip	Ppo	Cel	DH	Cat	Phos	URA	Resp	Bact	F	G+	G-	Act	H	pH
TOC																				
N	0.86**																			
WSC	-0.50	-0.3																		
HS	0.86**	0.66**	-0.35																	
***β*-GL**	0.67**	0.55*	-0.89**	0.44																
Lip	0.68**	0.54*	-0.85**	0.42	0.97**															
Ppo	-0.16	0.25	-0.14	-0.35	0.16	0.24														
Cel	0.93**	0.83**	-0.71**	0.75**	0.86**	0.85**	-0.07													
DH	0.76**	0.61*	-0.86**	0.55*	0.95**	0.94**	-0.01	0.88**												
Cat	0.60**	0.42	-0.87**	0.38	0.89**	0.89**	0.12	0.73**	0.89**											
Ph	0.83**	0.61*	-0.85**	0.72**	0.87**	0.88**	0.05	0.92**	0.90**	0.84**										
URA	0.35	0.03	-0.70**	0.50	0.61*	0.57*	-0.09	0.47	0.67**	0.64**	0.69**									
Resp	0.73**	0.54*	-0.81**	0.63*	0.84**	0.88**	0.16	0.84**	0.84**	0.79**	0.93**	0.65**								
Bact	0.92**	0.80**	-0.73**	0.72**	0.86**	0.88**	-0.71	0.96**	0.92**	0.80**	0.93**	0.52*	0.88**							
F	0.79**	0.77**	-0.73**	0.45	0.86**	0.89**	0.10	0.90**	0.86**	0.77**	0.83**	0.28	0.80**							
G+	0.94**	0.81**	-0.70**	0.74**	0.83**	0.85**	-0.09	0.96**	0.90**	0.77**	0.92**	0.49	0.86**	0.91**	0.91**					
G-	0.85**	0.74**	-0.77**	0.66**	0.90**	0.92**	-0.03	0.93**	0.94**	0.84**	0.93**	0.59*	0.90**	0.98**	0.89**	0.96**				
Act	0.93**	0.83**	-0.69**	0.71**	0.82**	0.83**	-0.05	0.96**	0.88**	0.69**	0.90**	0.41	0.82**	0.96**	0.93**	0.97**	0.91**			
H	-0.48	-0.26	0.54*	0.61*	-0.52*	0.43	0.35	-0.51	-0.65**	-0.47	-0.59*	-0.85**	-0.49	-0.53*	-0.27	-0.53*	-0.54*	-0.48		
pH	-0.73**	-0.49	-0.69**	-0.67**	-0.79**	-0.80**	0.12	-0.80**	-0.80**	-0.74**	-0.81**	-0.68**	-0.81**	-0.82**	-0.67**	-0.80**	-0.85**	-0.75**	0.61*	

β-GL: β-glucosidase; Lip: lipase; Ppo: polyphenol oxidase; Cel: cellulase; DH: dehydrogenase; Cat: catalase; Phos: phosphatase; URA: urease; Resp: respiration; Bact: bacteria; F: fungi; G+: Gram-positive; G-: Gram-negative; Act: actinobacteria; H: Shanon-Weaver index.

ANNEX 4

Published articles

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Can the labile carbon contribute to carbon immobilization in semiarid soils? Priming effects and microbial community dynamics

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ABSTRACT

Tracer experiments with isotopic-enriched carbon compounds can provide information regarding the carbon cycling in semiarid soils. We studied priming effects and microbial utilization of glucose as an example of bioavailable labile molecule in the carbon cycle of a semiarid soil. The soil, which has low content of total organic carbon (5.0 g kg^{-1}), was amended with U^{13}C-glucose (99 atom %) at concentration of 75 μg C g^{-1} soil (LD) or 300 μg C g^{-1} soil (HD). Glucose-derived carbon remained in soil after two months of incubation. The percentage of residual carbon stabilized was greater in LD with 40% of the initial ^{13}C added compared to 30% of the initial ^{13}C added in the HD. Comparison of ^{13}C content in water- and sodium-pyrophosphate extracts pointed to a significant humification of up to 2.4% of the initial ^{13}C-glucose. Glucose was subjected to an intense mineralization in the first 17-days of 22.8% and 40.94% for the LD and HD, respectively. The stable isotope probing (SIP) of phospholipid fatty acids (PLFAs) by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) showed that bacteria dominated glucose metabolism in comparison to fungi. Gram-negative populations were initially more involved in glucose assimilation than Gram-positive bacteria. In the fatty acids fraction, up to 95% of the ^{13}C was predominantly found in fatty acids typical for Gram-negative bacteria. However, after 4 and 17 days the ^{13}C-enrichment in Gram-positive biomarkers increased. The mineralization of soil organic matter triggered by glucose additions was more intense in HD (3.6% of soil TOC) than LD (1.0% of soil TOC) and reached the highest level after 4 days in HD. Priming was controlled by Gram-negative populations but fungi and, particularly actinobacteria played an important role in latter steps. Our data indicated that the intense metabolism of SOM due to priming phenomena compromises the potential carbon sequestration in this semiarid soil amended with glucose.

1. Introduction

Soils developed in arid and semiarid conditions are characterized by very low organic matter content (OM) from the scarce plant biomass (García et al., 1994) and it has been proposed that these soils have a big potential for carbon sequestration (Lal, 2004). However, such carbon sequestration would depend on the type of organic substrates, soil type and microbial community. Within organic carbon, labile compounds such glucose are quickly assimilated by microbial biomass (Perelo and Munch, 2005; Fischer et al., 2010). In boreal and temperate climates, isotopic evidences found in experiments with ^{13}C- or ^{14}C-labeled glucose indicate that carbon added to soils is not completely mineralized (Blagodatskaya et al., 2007; Schneckenberger et al., 2008; Fischer et al., 2010). However, the extension of glucose mineralization in semiarid soils has not been fully examined yet.

While a major part of the released CO_2 after organic amendment is supposed to derive from the mineralization of the exogenous organic matter added to soil, an additional CO_2 fraction may come from the mineralization of autochthonous soil organic matter as a consequence of increased microbial activity. This last mechanism, which is called “priming effect” (Kuzyakov, 2006), has been proved in temperate and boreal soils with high OM content and even in soils with low total carbon content (Hoyle et al., 2008), but not in soils from semiarid areas with a very low OM content in a pre-desertic state. In such environments a reduction of soil organic matter as a consequence of priming may have ecological significance.

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Carbon fluxes and microbial community function can be tracked by stable isotope probing (SIP) methodologies which are mainly based on the incubation of an environmental sample with a ^{13}C-labeled substrate, followed by the analysis of ^{13}C-incorporation into biomarkers (Neufeld et al., 2007). In particular, the use of ^{13}C-labeled substrates in combination with phospholipid fatty acids analysis referred as PLFA-SIP (Boschker et al., 1998) can be useful to examine how soil microbial community transforms carbon compounds (Waldrop and Firestone, 2004) and its relation to priming phenomena (Nottingham et al., 2009). Whether or not microbial populations in such semiarid, pre-desertic, environments are able to mineralize an additional part of SOM remains unknown, but it has been suggested that priming effects are related to the dynamics of microbial community (Fontaine et al., 2003). We hypothesized that the addition of glucose to a semiarid soil will stimulate priming effect and hence constraint the potential carbon sequestration at short term.

To trace the fate of labile-carbon in a pre-desertic area, semiarid-soil samples were incubated with ^{13}C-labeled glucose. The persistence of glucose derived carbon was analyzed in bulk soil, and in carbon extracted with water and sodium-pyrophosphate. Furthermore, the conversion of glucose-carbon into carbon dioxide and microbial biomass was assessed by analysis of the isotope enrichment of CO_2 and fatty acids, respectively. The aims of the study are: i) to evaluate the fate of glucose and the short-term accumulation of glucose derived carbon in a semiarid soil, ii) to elucidate the extent of short-term mineralization of glucose and priming effect in relation to the dose of glucose, and iii) to analyze the anabolic conversion of ^{13}C-glucose into microbial biomass.

2. Material and methods

2.1. Study area and soil sampling

An area of 100 m^2 was selected for soil sampling in Abanilla, Province of Murcia (SE Spain, semiarid climate). This area was agriculturally abandoned since 1980. The soil in this area represents a typical marsh lithology. The mean annual rainfall is lower than 300 mm. Soil was selected due to its low organic carbon, microbial biomass content and microbial activity. Abanilla soil represents a highly degraded soil with no vegetation growing on it and it is therefore an adequate model to study processes of carbon transformation in pre-desertic conditions. The main physico-chemical characteristics of Abanilla soil are: pH (7.77), electrical conductivity (2.65 dS m^{-1}), clay-loam texture, total N (1.3 g kg^{-1}), total C (40 g kg^{-1}) and total organic C (5.0 g kg^{-1}). A detailed description of this soil is provided by Bastida et al. (2006). Within this area, three plots were selected. In September-2010, 8 subsamples were taken from the top 15 cm of soil and pooled to obtain one composite sample per plot. Soil samples were sieved by <2 mm and stored in lab conditions during 1 week until the beginning of the incubation experiments.

2.2. Experimental design and soil incubations

Three independent incubation experiments were performed. In all experiments, an aqueous solution of U^{13}C-glucose (99 atom %) (Cambridge Isotope Laboratories, Andover, MA) (^{13}C-glucose) was prepared. In order to compare the effect of glucose-dose, 75 μg C g^{-1} soil (low-dose treatment, LD) or 300 μg C g^{-1} soil (high-dose treatment, HD) were supplied. For a non-labeled control experiment, the same treatments but using glucose with natural isotopic abundance were prepared. The doses correspond approximately to the same amount of microbial biomass C (around 80 μ C g^{-1} soil) in the case of LD or four-times the microbial biomass C content, in the case of HD. These amounts are similar to the doses of organic amendments applied in field restoration experiments in semiarid environments (Bastida et al., 2012). Furthermore, a control experiment soil without glucose was set-up using the same conditions than glucose-treatments. The water-holding capacity was gravimetrically controlled at 60% during incubation for all treatments. All treatments were performed in triplicates.

The first phase aimed to evaluate the amount and form of the added carbon remaining in the soil after a two months incubation period. This experiment was carried out in plastic containers with 100 g of soil. Each treatment was prepared in independent triplicates for each incubation time (1 day, 30 days and 60 days). Incubation was performed in chambers at 28 °C in darkness. Total organic carbon, water-soluble carbon and sodium-pyrophosphate extracted carbon were analyzed for its total content and isotopic ratio.

The second incubation was devoted for the study of glucose mineralization and priming effects and was carried out in 12 ml-capped glass vials (Labco Limited, Lampeter, UK) containing 1 g of soil. The amount and the isotopic ratio of the CO_2 produced by cumulative mineralization were analyzed at each time as indicated below. Independent triplicates of each treatment were incubated for 1, 4, 7, 11, 14 and 17 days at 28 °C in darkness. A higher time-resolution at the initial time points was designed in this experiment since it has been described that glucose mineralization is usually more intense at the initial stages (Schneckenberger et al., 2008).

The third incubation was dedicated to the study of microbial community dynamics related to glucose transformation. This assay was performed in Petri dishes with 25 g of soil. Assay was performed in chambers at 28 °C in darkness. Each treatment was prepared in independent triplicates for each incubation time (1 day, 4 days and 17 days). Soil samples were used for analysis of phospholipid fatty acids and PLFA-SIP.

Preliminary analysis showed that the three experimental incubations (see below) were comparable (Tables S1 and S2, Supplementary material).

2.3. Carbon analyses

The total organic carbon (TOC) of soil samples was determined after hydrolysis with 2N HCl in a Leco Truspec CN elemental analyzer (St. Joseph, MI). Analysis of carbon isotope ratios of bulk soil as well as water-soluble and sodium-pyrophosphate extracts were performed at the Stable Isotope Facility (University of California, Davis).

Bulk soil was analyzed for carbon isotope ratios using a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000 °C in a reactor packed with chromium oxide and silvered cobaltous/cobaltic oxide. N_2 and CO_2 were separated on a Carbosieve GC column (65 °C, 65 ml/min) before entering the IRMS. The final delta values used for ^{13}C-content calculations were expressed relative to international standards V-PDB (Vienna PeeDee Belemnite) (Coplen et al., 2006) according to:

$$\delta[(‰)] = \left(\frac{R_{\text{sample}}}{R_{\text{reference}}} - 1\right) \times 1000 \quad (1)$$

where R_{sample} and $R_{\text{reference}}$ are the ratios of the heavy isotope to the light isotope (^{13}C/^{12}C) in the sample and in the international standard, respectively. Delta units were converted to atom fraction of ^{13}C expressed as atom %:

$$\text{atom \% } {}^{13}\text{C} = \frac{100}{\frac{1}{\left(\frac{\delta}{1000}+1\right)R_{\text{PDB}}}+1} \quad (2)$$

where δ is the measured $\delta^{13}C$ (‰) of the sample using the delta notation and $R_{\text{V-PDB}}$ is the isotope ratio of V-PDB = 0.0112372 (Slater et al., 2001).

Water-soluble carbon was extracted with distilled water (1:5, solid:liquid) by shaking for 2 h at 50 °C. The carbon was quantified with a Shimadzu TOC5050A Total Organic Carbon Analyzer after filtering. Sodium-pyrophosphate extractable carbon was extracted with 0.1 M sodium-pyrophosphate pH 9.8 (1:5 solid:liquid) by shaking for 4 h (Stevenson, 1982; Lucas-Borja et al., 2012). Subsequently, sodium pyrophosphate extracts were centrifuged at 13,000 rpm for 10 min and the carbon content was determined using a Shimadzu TOC5050A Total Organic Carbon Analyzer. Hot-water and sodium-pyrophosphate extractions were performed in parallel samples, not sequentially. Water-soluble carbon represents the easy available carbon for microorganisms, while carbon contained in sodium pyrophosphate extracts represents both labile and a more stable carbon presented in humic substances (Stevenson, 1982).

Of each extract, an aliquot of 8 ml was transferred to a 50 ml vial and was diluted with 22 ml of bi-distilled water. One ml of 30 mM sodium-azide was added to inhibit microbial growth. Samples were acidified and purged with helium off-line to remove all dissolved inorganic carbon (DIC). Depending on sample concentration, 1- to 4-ml aliquots were transferred to digestion vessel heated before and reacted with sodium persulfate to convert DOC into CO_2. The CO_2 was carried in a helium flow to the isotope ratio mass spectrometer where isotope ratios were measured. The analytical measurement was carried out by an Aurora 1030 TOC Analyzer (OI Analytical, College Station, TX) coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) coupled to a GD-100 Gas Trap Interface (Graden Instruments).

2.4. Analysis of CO_2, glucose mineralization and calculations of priming effects

Four milliliters of headspace gas from each vial from the mineralization experiment were transferred to pre-evacuated glass vials (Labco Limited, UK) for analysis of the amount and isotopic composition of total CO_2. The isotope ratio of CO_2 was determined by gas chromatography–isotope ratio mass spectrometry as described elsewhere (Herrmann et al., 2010).

The isotopic ratio of CO_2 was used for the calculation of the percentage of CO_2–C derived from the added glucose or from SOM, as described by Waldrop and Firestone (2004). Priming effect was defined as the increase or decrease in the soil organic matter mineralization following substrate addition. Priming effect was calculated as the increase in total soil respiration following substrate addition minus the amount of carbon respired from the added substrate and from control soil without amendment (Blagodatskaya et al., 2007; Equation (3)). This was expressed as a percentage where 100% represents a doubling of the SOM-C respiration (Waldrop and Firestone, 2004; Brant et al., 2006).

$$\text{Priming effect} = \underbrace{(\text{total CO}_2 - \text{Glucose derived CO}_2)}_{\text{Soil amended with glucose}} - \underbrace{\text{total CO}_2}_{\text{Unamended soil}} \quad (3)$$

2.5. Phospholipid fatty acids analysis (PLFA)

Phospholipids were extracted from 8 g of soil using a chloroform–methanol extraction as described by Bligh and Dyer (1959), fractionated and quantified using the procedure described by Frostegård et al. (1993). Phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al., 1985) and designated as described by Frostegård et al. (1993). The complete dried FAME fraction was dissolved in *n*-hexane containing 20.06 mg ml^{-1} of 21:0 FAME as internal standard. Mass spectrometric analyses of FAMEs were carried out as described previously (Bastida et al., 2011). The absolute and relative amounts of FAMEs in the samples were determined according to the concentration of the internal standard added.

The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t are characteristic bacterial fatty acids and were chosen as bacterial biomarkers (Frostegård et al., 1993; Dungait et al., 2011). The 18:2ω6 was taken as indicator of fungal biomass (Rinnan and Baath, 2009). The ratio of bacterial to fungi PLFA was taken as an indicator of the dynamics between bacteria and fungi. The Gram-positive specific fatty acids i15:0, a15:0, i16:0, and i17:0 and the Gram-negative specific fatty acids cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t (Frostegård et al., 1993; Dungait et al., 2011) were taken as indicators for the ratio between Gram-positive and Gram-negative bacteria. 10Me-branched FAMES (10Me16:0, 10Me17:0 and 10Me18:0) were taken as specific actinobacterial biomarkers within Gram-positive bacteria (Dungait et al., 2011).

2.6. Isotopic analysis of fatty acids

Carbon isotope composition of FAMEs extracted from control and non-enriched glucose treatments were analyzed using a gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS) system as described previously (Bastida et al., 2011). The $\delta^{13}C$ values of the fatty acids reported were corrected for the carbon introduced during derivatization (Abraham et al., 1998) and converted to atom % following Equation (2). Samples were measured at least in triplicate with an analytical error smaller than ±0.5‰ standard deviation.

The incorporation of ^{13}C in FAMEs extracted from high dose treatments resulted in isotope ratios out of linearity limit of GC-C-IRMS. Because the isotope composition of the highly enriched ^{13}C-fatty acids could not be analyzed accurately using GC-combustion-isotope ratio mass MS, a 7890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used for the determination of isotope enrichment into fatty acids in the following way: the ^{13}C-incorporation into fatty acids caused the appearance of a series of isotopomers besides the natural molecular ion (M^+) (Annweiler et al., 2000; Fang et al., 2004). According to the distribution of these isotopomers, the percentage of labeled carbon (atom %) in the fatty acids could be calculated as described by Bombach et al. (2010).

Amount of ^{13}C incorporated into fatty acids was calculated as described by Boschker (2004). The percentage of ^{13}C incorporated in specific Gram-positive (i15:0, i16:0, i17:0, 10Me16:0, 10Me17:0 and 10Me18:0), Gram-negative (cy17:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t), actinobacteria (10Me16:0, 10Me17:0 and 10Me18:0) and fungi (18:2ω6,9) relative to the total amount of ^{13}C in those fatty acids was used as an indication of the flow of ^{13}C in the different compartments of the microbial community.

2.7. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. The data were submitted to ANOVA of repeated measures. In the case of PLFA analysis, the intra-subject factor (time) was set up with 3 different levels and in the case of CO_2 analysis the intra-subject factor (time) was set up with 6 different levels. The inter-subject was defined as the treatment (dose). ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference at the 95% confidence interval in the lineal model). In order to determine significant differences among treatments at the same time, the data were subjected additionally to one-way ANOVA. Changes in the structure of the microbial community structure were evaluated by factor analysis using the relative abundances of all identified FAMES.

3. Results

3.1. Carbon storage in soil

The fate of glucose was evaluated by analysis of total carbon and isotopic ratio in bulk soil as well as in water- and sodium-pyrophosphate extracts. The TOC in the low-dose treatments (LD) and high-dose treatments (HD) was higher compared to the control receiving no glucose at the initial time (1 day) (Fig. 1A). After 60 days of incubation, no significant differences were observed for the TOC content in soil with and without glucose.

At the beginning of the incubation, water-soluble carbon content was in mean 3.5-fold higher in the HD treatment in comparison to the control and LD treatment (Fig. 1B). After 2 months of incubation, there were not significant differences in the content of extractable C with water and sodium-pyrophosphate (Fig. 1B and C).

The persistence of ^{13}C derived from glucose was analyzed by means of ^{13}C enrichment in total organic carbon, water-soluble and sodium pyrophosphate extractable fractions (Fig. 2A). The percentage of ^{13}C in bulk soil was significantly higher in the LD treatment than in the HD treatment ($P < 0.05$) and tended to decrease during incubation. After 2 months, 29 μg ^{13}C g^{-1} which were equivalent to 40% of the initial ^{13}C amount added and 83 μg ^{13}C g^{-1} which were equivalent to 30% of the initial ^{13}C amount added still remained in soil for LD and HD, respectively (Fig. 2A).

The patterns of ^{13}C derived from glucose recovered with hot-water or sodium pyrophosphate were similar. In both cases, a higher percentage of ^{13}C added to soil was found in the HD treatment compared to the LD treatment after 24 h of incubation. These patterns were opposite to the percentage of ^{13}C in bulk soil. The amount of ^{13}C obtained by both extraction techniques (hot-water and sodium-pyrophosphate) was similar at the beginning of the incubation experiment. However, the ^{13}C content in the sodium-pyrophosphate extraction after 2 months was around 4-times higher (up to 2.4% of the initial ^{13}C-glucose) than the amount of ^{13}C extracted with water (Fig. 2C). In particular, the total ^{13}C content of the sodium pyrophosphate extract was higher in HD (3.95 ± 0.58 μg ^{13}C g^{-1}) than LD (1.83 ± 0.04 μg ^{13}C g^{-1}).

The ^{13}C-content of the non-extractable carbon fraction was calculated by subtracting the amount of ^{13}C found in the sodium pyrophosphate-extract from the amount of ^{13}C found in bulk soil. In absolute values, the non-extractable ^{13}C was always significantly higher ($P < 0.05$) in HD than LD (78.89 ± 6.77 μg ^{13}C g^{-1} soil and

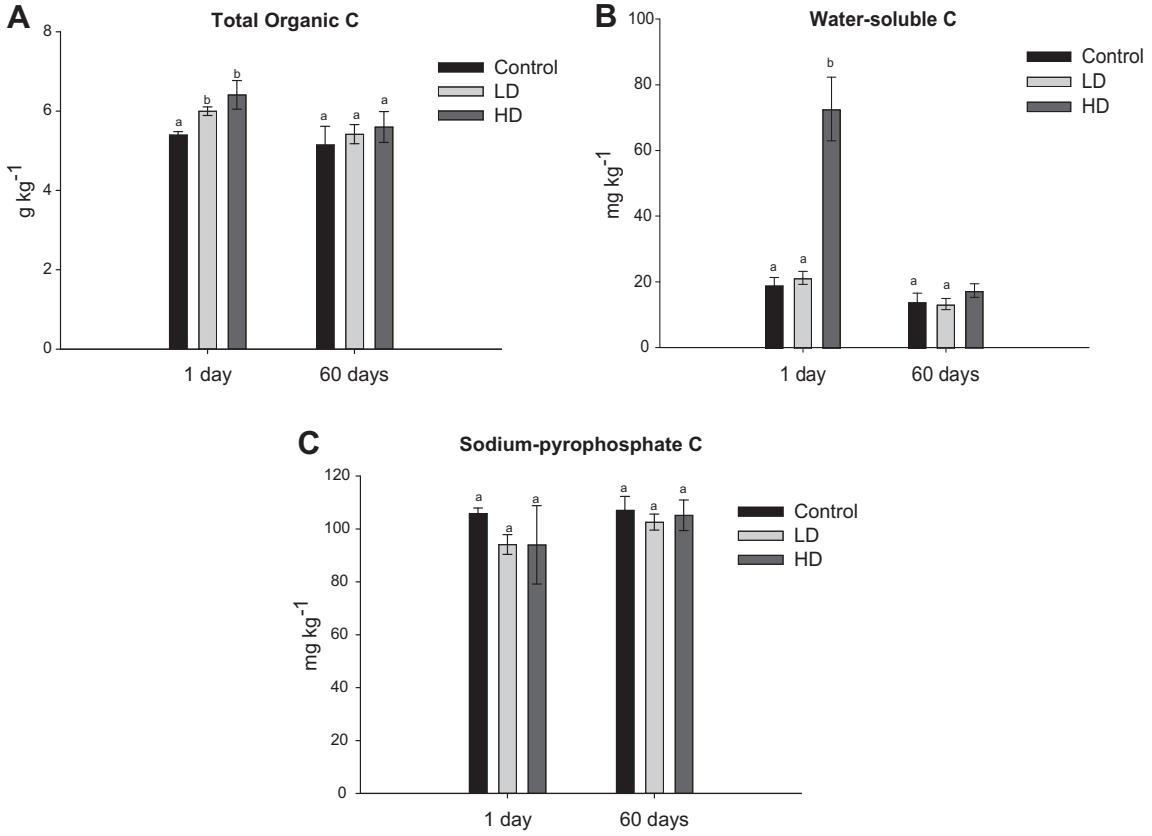

Fig. 1. Total organic carbon (TOC) (A) and carbon content in water-soluble (B) and sodium pyrophosphate (C) in control and glucose-amended soils. LD (low-dose), HD (high-dose). For each incubation time, data followed by the same letter are not significantly different ($P < 0.05$).

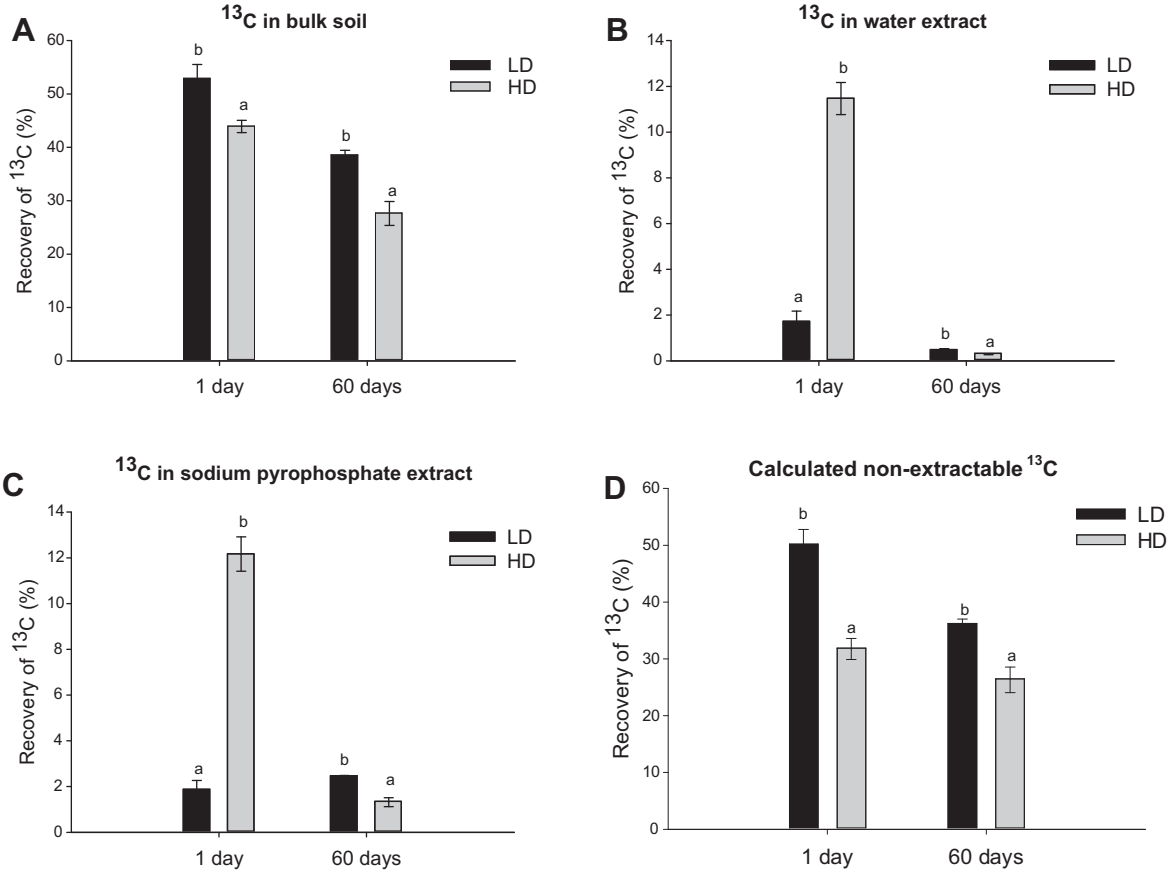

Fig. 2. Recovery of ^{13}C in bulk soil (A), water soluble (B) and sodium pyrophosphate (C) extracts and calculated non-extractable ^{13}C (D) after addition of labeled glucose. LD (low-dose), HD (high-dose). For each incubation time, data followed by the same letter are not significantly different ($P < 0.05$).

27.06 ± 0.70 μg ^{13}C g^{-1} soil, respectively). However, the percentage of immobilization of added ^{13}C-glucose was significantly higher in the LD than HD (Fig. 2D).

3.2. Glucose mineralization and priming effects

Soil respiration, glucose mineralization and priming effect were significantly influenced by treatment and time ($P < 0.05$) (Table 1). After 17 days of incubation, total respiration reached 113, 161 and 368 μg CO_2–C g^{-1} soil in control, LD and HD, respectively (Fig. 3A). Mineralization of the ^{13}C-glucose resulted in an enrichment of the ^{13}C within the CO_2 which was already detectable after 1 day of incubation. From the 4 days until the end of the incubation, glucose mineralization was always significantly higher in HD than LD treatments ($P < 0.05$) (Fig. 3B). In the course of the incubation, the evolution of $^{13}CO_2$ increased. After 17 days of incubation, 22.8% and 40.9% of the initially added glucose was mineralized in the LD and HD treatments.

With the exception of day 1, priming effect was significantly higher ($P < 0.05$) in HD than LD treatment in the course of the incubation (Fig. 3C). After 1 day, priming reached a maximum of 59.8% in the LD treatment, while in the HD treatment the highest

Table 1
Results of ANOVA of CO_2 and PLFA analysis.

Parameter[a]	SR		GM		PR		PR %		Bacteria		Fungi	
	F[b]	*P*[c]	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Factors												
Treatment (Tr)	2994.96	<0.001	802.79	<0.001	153.30	<0.001	723.79	<0.001	39.97	<0.001	2.03	0.21
Time (Ti)	3445.32	<0.001	888.48	<0.001	24.29	0.008	2029.00	<0.001	433.39	<0.001	168.12	<0.001
Tr × Ti	484.81	<0.001	138.00	<0.001	30.40	0.06	1238.17	<0.001	1.67	0.26	0.45	0.65

	G+		G−		Act		F/B		G+/G−	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment (Tr)	10.07	0.012	9.26	0.015	2.51	0.16	12.35	0.007	5.95	0.038
Time (Ti)	309.01	<0.001	239.25	<0.001	3503.21	<0.001	22.52	0.03	61.07	<0.001
Tr × Ti	2.04	0.21	0.98	0.43	13.22	0.06	11.66	0.009	4.84	0.056

[a] SR (Soil respiration); GM (Glucose mineralization); PR (Priming effect, absolute values); PR % (Priming effect, %); G+ (Gram-positive bacteria); G− (Gram-negative bacteria); Act (Actinobacteria); F/B (Fungi to bacterial ratio); G+/G− (Gram-positive to Gram-negative ratio).
[b] *F*-ratio.
[c] *P* values.

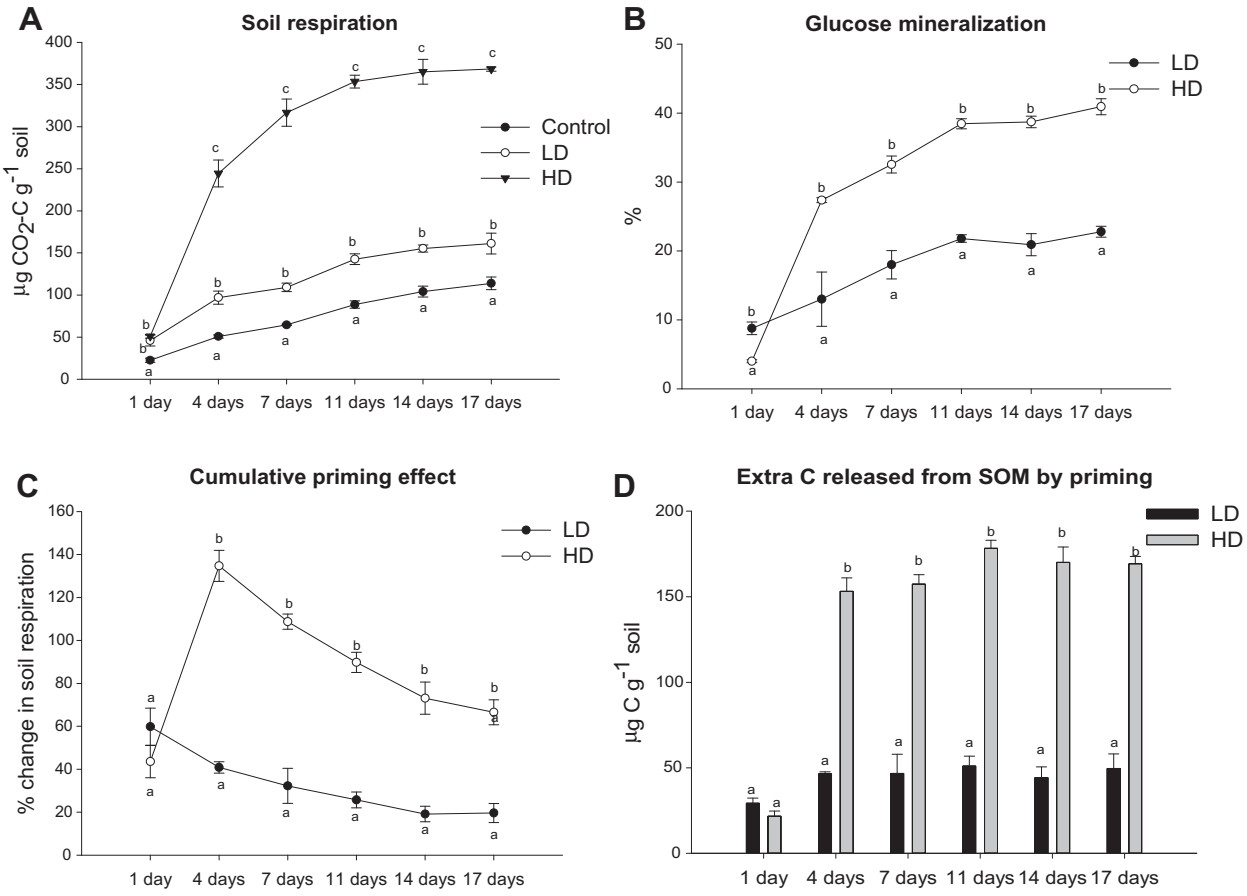

Fig. 3. Soil respiration (A), glucose mineralization (B), cumulative priming (C), and carbon amount released by priming (D). LD (low-dose), HD (high-dose). For each incubation time, data followed by the same letter are not significantly different ($P < 0.05$).

priming occurred after 4 days of incubation (134.7%). As a consequence of such priming effect, nearly 50 and 180 μg of autochthonous carbon per gram of soil were released in LD and HD treatments (Fig. 3D). These amounts corresponded to a mineralization of 1.0 and 3.6% SOM, respectively.

3.3. PLFAs and community structure

Bacterial PLFA content, including Gram-positive and Gram-negative populations, were significantly influenced by both glucose-dose and incubation time ($P < 0.05$) (Table 1). Glucose treatment lead an increase in bacterial PLFA from the beginning of the experiment to the day 4th and a further slightly increase in the course of incubation. HD showed significant higher values of Gram-positive PLFA content than LD, and LD showed higher values than control along the incubation ($P < 0.05$) (Table 2). Gram-negative PLFA content was significantly higher in HD and LD compared to the control at 1 day (Table 2).

The ratio of Gram-positive to Gram-negative PLFA content was higher ($P < 0.05$) in the soil with glucose than in the control. Fungi and actinobacteria were significantly ($P < 0.05$) influenced by incubation time with a higher PLFA content after 4 and 17 days of incubation compared to the experiment at day 1. However, the glucose dose did not significantly influence the content of fungal and actinobacterial fatty acids (Table 1). The fungi to bacteria ratio was significantly lower in the HD treatment compared to the control and LD treatment at day 1 but no shifts in the ratios occurred during the course of incubation.

For estimating the effect of glucose treatment on whole microbial community structure, multifactorial analysis with the relative abundance of fatty acids was performed (Fig. 4). Factor 1 explained the 36.8% of the variability of the results and Factor 2 explained 19%. At day 1, PLFA data of all three different treatments grouped closely together demonstrating a similar microbial community structure. Clear shifts in the community structure appeared at day 4. Regarding factor 1, the microbial community of HD after 4 days of incubation clustered in the same group as all samples after 17 days of incubation (Fig. 4).

3.4. ^{13}C incorporation into PLFA (PLFA-SIP)

Fatty acids extracted from control and ^{12}C-glucose amended samples showed natural isotope of 1.08 ± 0.002 atom %. In soil amended with ^{13}C-glucose, a fast ^{13}C-labeling of PLFA occurred within 1 day of incubation. When comparing the degree of ^{13}C-labeling, two different statements can be made: (i) HD treatment showed always a significant higher ^{13}C-enrichment in each fatty acid compared to the LD treatment. (ii) Fatty acid representatives of Gram-positive bacteria were more ^{13}C-enriched with values up to 50.96 atom % (i16:0) than fatty acids of Gram-negative fatty acids (43.53 atom %, 16,1ω7c) (Table S3).

Total amount of ^{13}C found in the different compartments of the microbial community followed a common trend for both LD and HD. Out of the total ^{13}C in fatty acids, 63% (LD) and 95% (HD) were presented in Gram-negative fatty acids at first day of incubation and the rest was in Gram-positive bacteria (Fig. 5). No enrichment was found

Table 2
Changes in bacterial and fungal PLFAs (nmol g^{-1}), ratio of fungi to bacterial PLFAs (F/B) and ratio of Gram-positive to Gram-negative bacterial PLFAs (G+/G−) in control and glucose-treatments during the incubation period of 17 days.

	1 day						4 days						17 days					
	Control		LD		HD		Control		LD		HD		Control		LD		HD	
	Mean	SD[a]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bacterial (B)	2.38a	0.27	4.03b	0.43	9.61c	2.14	16.80a	1.91	19.57a	3.56	21.97	3.48b	17.10a	1.77	20.61b	0.10	22.99	1.70b
Fungi (F)	0.25a	0.07	0.29a	0.06	0.29a	0.11	0.72a	0.09	0.79a	0.22	1.04a	0.30	0.78a	0.09	0.92a	0.10	0.91a	0.08
Gram-positive (G+)	0.40a	0.08	0.92a	0.31	2.55b	1.10	5.30a	0.76	6.45a	1.99	7.70b	2.18	4.74a	0.57	6.05b	0.03	8.31c	0.33
Gram-negative (G−)	1.98a	0.19	3.11b	0.19	4.09c	1.36	9.37a	0.96	10.54a	0.43	12.42a	1.79	8.96a	1.16	11.65a	0.16	11.3a	1.46
Actinobacteria	n.d.		n.d.		0.57b	0.11	2.13a	0.21	2.59a	1.60	2.87a	0.35	3.41b	0.15	2.91a	0.21	3.37b	0.13
F/B	0.10b	0.02	0.07b	0.01	0.03a	0.02	0.04a	0.00	0.04a	0.02	0.04a	0.01	0.05a	0.00	0.04a	0.00	0.04a	0.00
G+/G−	0.20a	0.02	0.30a	0.10	0.63b	0.16	0.56a	0.03	0.61a	0.19	0.62a	0.15	0.53a	0.01	0.52a	0.01	0.74b	0.07

For each incubation time, data followed by the same letter are not significantly different ($P < 0.05$).
[a] SD (standard deviation). LD (low-dose), HD (high-dose). n.d.: non-detected.

in actinobacteria and fungi at this time. However, after 4 days the ^{13}C content in Gram-positive increased compared to that level found in fatty acids representatives of Gram-negative at 1 day and reached higher values than typical Gram-negative bacterial fatty acids in the following period of the experiment (Fig. 5). 2.05 and 3.02% of ^{13}C bound to the total ^{13}C Gram-positive fatty acid biomarkers were found in fatty acid representatives of actinobacteria. Furthermore, a minimal portion of isotope labeling appeared in fungi fatty acids (4 and 2%, respectively for LD and HD). After 17 days, the amount of ^{13}C in Gram-positive decreased in comparison to 4th day of incubation whilst the amount in Gram-negative increased. The amount of ^{13}C in actinobacteria was 5.85 and 4.22% of the total ^{13}C found within the representative fatty acids.

3.5. Soil-derived carbon incorporation into PLFA

Total amount of soil-derived carbon found in the different compartments of the microbial community followed a similar trend for both LD and HD. Overall, the absolute amount of soil-derived C in fatty acids increased with the incubation time. This amount was especially higher in some Gram-negative representatives (18:1ω9c and 18:1ω9t) than Gram-positive typical fatty acids (i15:0, i16:0, i17:0) (Fig. S1, Supplementary material). Out of the total ^{13}C in fatty acids, the amount of soil-derived C was higher in Gram-negative than Gram-positive and fungi fatty acids for both LD and HD treatments during the experiment (Fig. 6.). However, this amount decreased during incubation. Conversely, the amount of soil-derived C found in Gram-positive fatty acids increased during the incubation (Fig. 6.). Out of the total amount in Gram-positive bacteria, up to 18 and 22% of the soil-derived carbon was found within actinobacterial PLFAs after 17 days of incubation, respectively for LD and HD. The maximum amount of soil-derived C in fungi (8% and 7%, respectively for LD and HD) was reached after 4 days of incubation (Fig. 6).

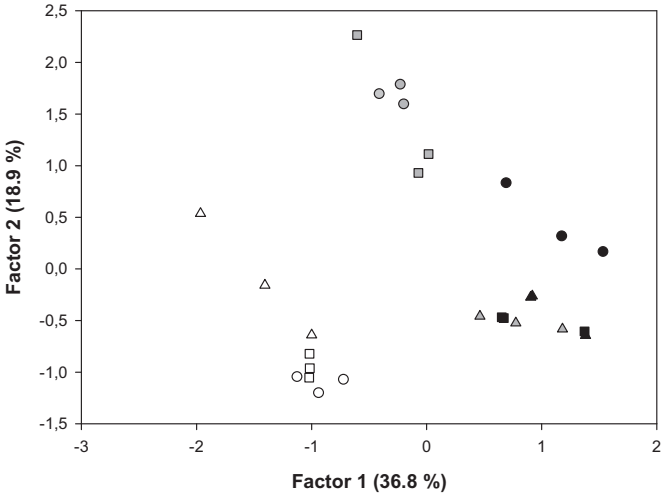

Fig. 4. Community structure analysis by PLFAs of control and glucose amended samples at different incubation times. Legend: ○ (Control), □ (Low-dose, LD), △ (High-dose, HD). Greyscale indicates incubation time: Open symbols (1 day), Gray symbols (4 days), Black symbols (17 days).

4. Discussion

4.1. Carbon storage in soil

Glucose can be immobilized in microbial biomass (Perelo and Munch, 2005; Hoyle et al., 2008). Fischer et al. (2010) demonstrated that microbial immobilization of glucose out-competes physico-chemical sorption in soil. These authors observed that up to 59.1% of glucose-carbon can be stored in microbial biomass while only 6.7% was adsorbed in soil matrix. Glucose uptake by microbial cells can take 1–2 min (Hill et al., 2008; Fischer et al., 2010). However, in soils with low biomass, microbial immobilization of glucose can be limited or, at least, delayed. Hoyle et al. (2008) indicated that only 2.7% of added carbon was found within the microbial biomass after incubation for 2500 h in a soil with a microbial biomass C of 121 μg C g^{-1}. The remainder glucose-carbon was assumed to be retained within the soil organic matter pool. Accordingly, the low initial microbial biomass in Abanilla soil (80 μg C g^{-1}, Bastida et al., 2006) might not be enough to quickly assimilate a big portion of added carbon that can be physically protected by soil particles (Jones and Edwards, 1998). In fact, a great part of added glucose-carbon still remained in soil after two months of incubation. The higher percentage of ^{13}C recovered in liquid extracts in HD than LD at first day can be due to the need of a longer period for microbial assimilation and physical stabilization in soil particles when added carbon was abundant.

Carbon extracted with water includes the more labile fractions (Cook and Allan, 1992), while extractions with alkaline solutions, such sodium pyrophosphate, additionally include also more stable carbon pools (Stevenson, 1982). After 2 months of incubation, the total amount of ^{13}C was higher in the sodium-pyrophosphate extracts than in the water-soluble extracts for both LD and HD. This data suggests a minor but significant humification of carbon derived from glucose whereas an important part might be physically protected in mineral colloids during the incubation (Derrien et al., 2006; Ekkschmitt et al., 2008; Schmidt et al., 2011). Several authors have indicated out a chemical stabilization (i.e. humification) of carbohydrates (Gleixner et al., 1999; Piccolo et al., 1999).

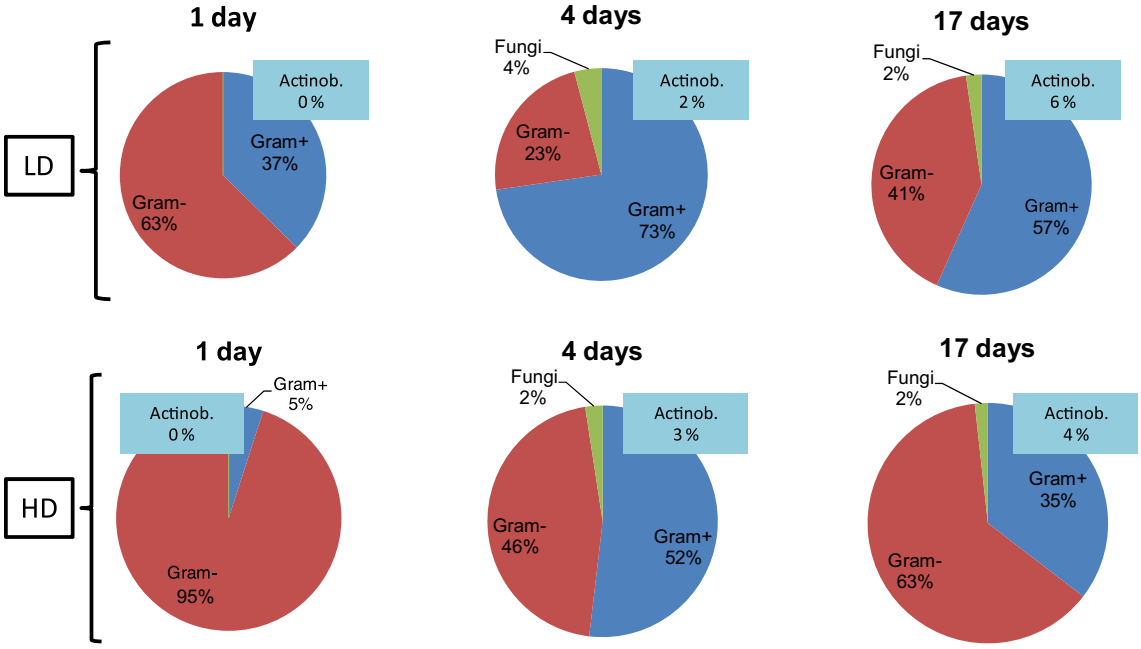

Fig. 5. Percentage of ^{13}C incorporated into PLFA which is shared by specific groups of the community. LD (low-dose), HD (high-dose). The percentage of ^{13}C in actinobacteria out of the total Gram+ pool is showed in rectangles.

Contrarily, Schmidt et al. (2011) concluded that this neo-formation of stable organic matter is not quantitatively relevant for humus formation in soil.

4.2. Glucose mineralization and priming effects

Several authors have pointed out an increase in mineralization after glucose addition (Wu et al., 1993; Schneckenberger et al., 2008). Nevertheless, the increase in glucose mineralization was not proportional to the applied dose of glucose that was 4 times higher in HD than in LD. Accordingly, Bremer and Kuikman (1994) proposed the existence of an upper limit of glucose mineralization above which further addition do not lead to increasing mineralization rate. These results point to an inability of the microbial community for further metabolisation of high amounts of labile carbon in the HD treatment at short-

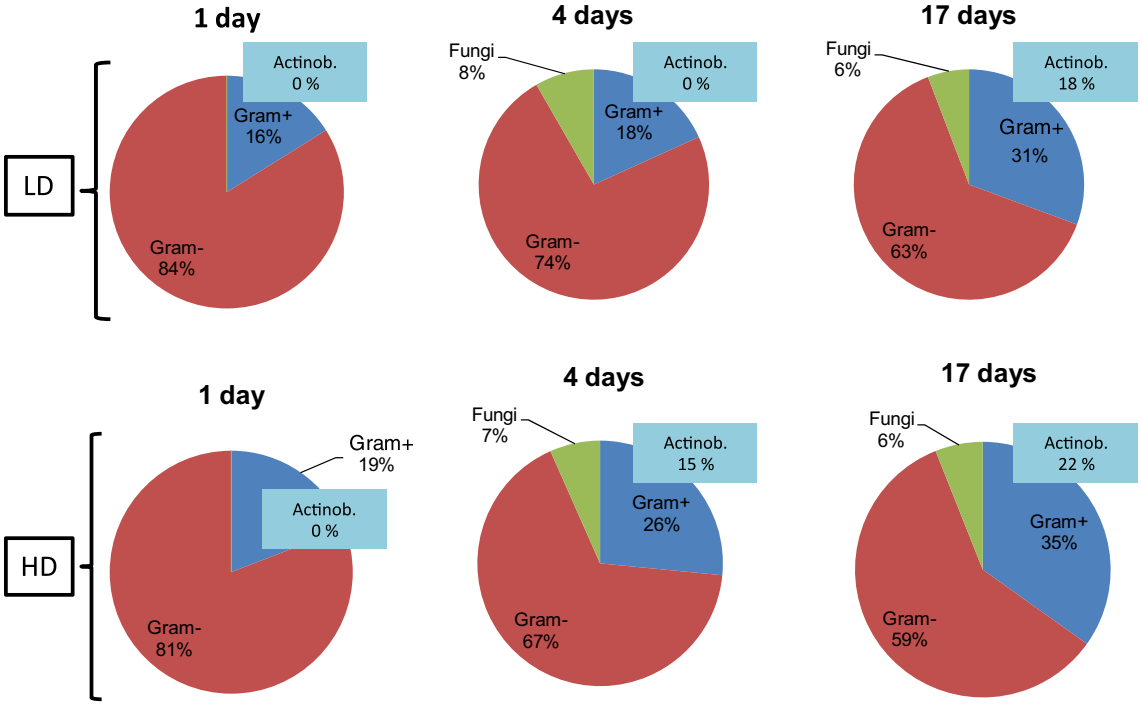

Fig. 6. Percentage of soil-derived C incorporated into PLFA which is shared by specific groups of the community. LD (low-dose), HD (high-dose). The percentage of ^{13}C in actinobacteria out of the total Gram+ pool is showed in rectangles.

time. However, glucose mineralization increased during incubation by the growing microbial community in the next days (Blagodatskaya et al., 2009).

Noteworthy, the released CO_2 is almost equally originated from glucose and primed SOM mineralization, as described above. The input of easily available organic substances in soil may change the turnover of native soil organic matter and cause priming effects (Kuzyakov et al., 2000; Blagodatskaya et al., 2007). An accelerated CO_2 lost as a consequence of intensified microbial metabolism could be of paramount ecological importance in pre-desertic areas with low OM content as SE-Spain. Two types of priming effects have been proposed: i) *apparent priming* which is due to the increased turnover of microbial biomass, and ii) *real priming* which is a consequence of the SOM mineralization (Blagodatskaya and Kuzyakov, 2008). As in the case of García-Pausas and Paterson (2011) and Brant et al. (2006), the intensity of priming effect was highest at a short term (after 4 days of incubation), especially in the high dose treatment. It has been suggested that apparent priming effect can be related to a non-increase in microbial biomass due to the accelerate turnover of microorganisms (Blagodatskaya et al., 2007). However, our study provides evidence that supports a real priming mechanism: (i) priming coincides with a noticeable microbial growth partially based on both ^{13}C-incorporation and soil-derived C into PLFAs (Nottingham et al., 2009), (ii) carbon released as a consequence of priming (up to 180 μg C g^{-1} soil) exceeds the original microbial biomass carbon of this soil of about 80 μg C g^{-1} (Bastida et al., 2006).

4.3. Microbial dynamics in relation to glucose mineralization and priming effects

In some previous studies, no significant changes in community structure occurred after addition of glucose leading to the hypothesis that glucose is ubiquitously present in soil environments and do not preferentially support specific groups of the microbial community (Falchini et al., 2003; Nannipieri et al., 2003; Rinnan and Baath, 2009). Conversely, specific soil microbial populations which were unable to use the existing but hardly available soil organic carbon particularly in such a poor soil switched from their starving state and began to grow when an easily available substrate was provided (*r*-strategists) (Fontaine et al., 2003). It has been proposed that those changes might be associated with a change in the community structure (Schneckenberger et al., 2008). In our study, multivariate analysis of FAMEs revealed that the only variation in microbial community structure compared to control was observed in the HD treatment after 4 days of incubation. Possibly (i) an excess of glucose induces the growth of specific components of the microbial populations (*r*-strategists) that change the community structure; and/or (ii) *K*-strategists populations begin to grow when energy-rich compounds are already exhausted (Fontaine et al., 2003) or become unavailable for microbes (Schmidt et al., 2011).

In relation to microbial biomass, several authors did not find changes in the concentration of PLFAs after glucose addition (Rinnan and Baath, 2009). In other cases, the responses were different and depended on soil type and biomass (Dungait et al., 2011). In our study, incubation conditions (i.e. moisture) influenced PLFA content as stated by the evolution of control treatment during the incubation. In any case, the addition of glucose to an extremely poor soil had an important impact in the microbial biomass in comparison to control treatment at each specific incubation time. It is noteworthy to mention that this semiarid soil has almost no vegetal cover and hence soil is not adapted to carbon inputs.

The low ^{13}C-enrichment into fungi and actinobacteria representative fatty acids may suggest that those groups are marginally competitors for glucose or low-intense cross-feeding phenomena over bacteria. It is noteworthy to mention that this conclusion is valid for the studied desert soil with low microbial biomass and presumably low proportion of fungal biomass. Gram-negative bacteria played a more important role than Gram-positive bacteria in the initial biodegradation of glucose, as previously highlighted (Brant et al., 2006; García-Pausas and Paterson, 2011). Contrarily, these results disagree with the findings of Dungait et al. (2011). After 4 days of incubation the higher ^{13}C bound in Gram-positive than Gram-negative bacterial fatty acids despite its lower PLFA content indicates a high but delayed efficiency of Gram-positive bacteria in the use ^{13}C-derived carbon (Brant et al., 2006; Fontaine et al., 2003).

Fontaine and Barot (2005) proposed that real priming effect is caused by the increase in microbial biomass of *K*-strategists when energy-rich compounds are unavailable. In our study, the significant increase of microbial biomass during the experiment that courses with an increase of soil-derived C within certain PLFA biomarkers supports a real priming effect (Nottingham et al., 2009). Overall, Gram-negative populations were particularly important in metabolizing soil organic carbon in comparison to Gram-positive bacteria. Similar results were found by other authors (Waldrop and Firestone, 2004; Nottingham et al., 2009). However, the importance of Gram-positive bacteria in relation to priming effect increased during incubation time. As in the case of glucose-mineralization, Gram-positive bacteria showed a delayed response in the mineralization of SOM. Moreover, within Gram-positive, actinobacteria played an important role in the mineralization of SOM. In the case of HD treatment, the peak of priming coincided with the highest increase of soil-derived C in actinobacterial and fungal PLFAs. These results suggest that these populations act as *K*-strategists in the degradation of SOM. Nevertheless, from the high content of ^{13}C found in Gram-positive bacterial PLFAs, it is obvious that the so-called *K*-strategists populations are also using ^{13}C-derived carbon from glucose itself, glucose metabolites or glucose-derived biomass.

The use of isotope-based methodologies allowed a deep understanding of carbon-cycling in a semiarid soil subjected to a labile carbon amendment in form of glucose. According to the proposed objectives, we conclude:

i) After 2 months, up to 40% of glucose-derived carbon remained in soil.
ii) Gram-negative bacteria initially drove glucose mineralization and they were actively followed by Gram-positive bacteria, while fungi populations play only if at all a minor role in glucose transformation in the tested soil.
iii) Priming effects induced by glucose seem to be controlled by Gram-negative populations but fungi and, particularly actinobacteria played an important role in latter steps.
iv) Soil organic matter represents a major substrate for priming effect in semiarid areas when microbial community is stimulated with high doses of glucose. Indeed, the importance of SOM in the total budget of mineralization was quantitatively comparable to the amount of mineralized glucose. Up to 180 μg C of soil organic matter was mineralized as a consequence of priming, while up to 120 μg of CO_2–C evolved from glucose.

At short-term, the potential for carbon sequestration derived from glucose is strongly affected by the metabolism of autochthonous SOM in a semiarid soil. However, further long-term studies are

necessary to assess the impact of labile compounds in the carbon stocks of soil and the possibility of the formation of stable humic substances. From the practical point of view, a possible SOM degradation should be considered when fresh organic amendments are used for soil restoration in semiarid areas.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2012.10.037.

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The role of lignin and cellulose in the carbon-cycling of degraded soils under semiarid climate and their relation to microbial biomass

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ABSTRACT

A high level of biological degradation is usually observed in soils under semiarid climate where the low inputs of vegetal debris constraint the development of microbiota. Among vegetal inputs, cellulose and lignin are dominant substrates but their assimilation by the microbial community of semiarid soils is yet not understood. In the present study, ^{13}C-labeled cellulose and ^{13}C-labeled lignin (75 μg ^{13}C g^{-1} soil) were added to two semiarid soils with different properties and degradation level. Abanilla soil is a bare, highly degraded soil without plant cover growing on it and a total organic C content of 5.0 g kg^{-1}; Santomera soil is covered by plants (20% coverage) based on xerophytic shrubs and has a total organic C content of 12.0 g kg^{-1}. The fate of added carbon was evaluated by analysis of the carbon isotope signature of bulk soil-derived carbon and extractable carbon fractions (water and sodium-pyrophosphate extracts). At long-term (120 days), we observed that the stability of cellulose- and lignin-derived carbon was dependent on their chemical nature. The contribution of lignin-derived carbon to the pool of humic substances was higher than that of cellulose. However, at short-term (30 days), the mineralization of the added substrates was more related to the degradation level of soils (i.e. microbial biomass). Stable isotope probing (SIP) of phospholipid fatty acids (PLFA-SIP) analysis revealed that just a minor part of the microbial community assimilated the carbon derived from cellulose and lignin. Moreover, the relative contribution of each microbial group to the assimilation of lignin-derived carbon was different in each soil.

1. Introduction

Poor vegetal cover, inadequate texture and the high salt content in arid and semiarid environments constrain the development of soil microbial communities (Albadalejo and Díaz, 1990; García et al., 1994) and cause severe biological degradation of soils, which affects the carbon cycling of these environments (Bastida et al., 2006). In this scenario, the scarce and dormant microbial biomass must efficiently deal with low inputs from the poor above-ground plant communities in order to obtain energy for its maintenance. Among such vegetal inputs, cellulose and lignin are dominant components. Lignin is a cross-linked polyphenol macromolecule with molecular masses exceeding 10.000 amu. It is relatively hydrophobic and aromatic in nature. Cellulose is a polysaccharide consisting of a linear chain of several hundred to over ten thousand β(1→4) linked D-glucose units (Updegraf, 1969). Recent evidence suggests that the stability and low degradability of lignins in soils seems to be overestimated and that their contribution to humus is exaggerated (Stevenson, 1982; Thevenot et al., 2010). Indeed, several authors have concluded that lignin is not stabilized in mineral soil horizons (Rumpel et al., 2004; Vancampenhout et al., 2012) and observed a weak contribution of lignin to the stable carbon pool (Thevenot et al., 2010). In contrast, Hoffman et al. (2009) found a relatively high persistence of lignin in soil. In any case, both soil characteristics (i.e., texture, clay content, etc.) and biotic environment can influence the stabilization of carbon in soil (Rumpel et al., 2004).

Despite the dynamics of these compounds are fundamental for terrestrial carbon cycling (Rodriguez et al., 1997; Eichorst and Kuske, 2012), the fate of lignin and cellulose are not fully understood particularly in carbon-limited soil ecosystems, i.e. semiarid climates. Previous studies have highlighted a key role for fungi in the biodegradation of cellulose (Fontaine et al., 2011). However,

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other authors have suggested an ecological succession driven firstly by bacteria and secondly by fungi (Hu & van Bruggen, 1997; Schutter and Dick, 2001). Conversely, Snajdr et al. (2010) and Schutter and Dick (2001) affirmed that fungi played a less important role than expected in lignin degradation.

In this study, the fate of cellulose and lignin was traced in degraded soils under semiarid climate conditions using stable isotope probing (SIP) methodologies. For these purposes, ^{13}C-labeled-cellulase and ^{13}C-labeled-lignin were added to two soils with different level of degradation and distinct characteristics such as texture, electrical conductivity and level of microbial biomass. The combination of SIP experiments with phospholipid fatty acids analysis (PLFA-SIP) allows to gain quantitative information on the microbial assimilation of lignin- and cellulose-derived carbon and to trace the microbial groups involved in the degradation (Boschker et al., 1998; Waldrop and Firestone, 2004; Rinnan and Baath, 2009).

We aim to shed light on the fate of cellulose and lignin in degraded soils under semiarid climate conditions and the role of the soil microbial community on these processes. The specific objectives of this study were: i) to evaluate the stability and humification of cellulose and lignin in semiarid soils with different levels of degradation at long-term; ii) to evaluate the mineralization of these substrates at short-term, and iii) to identify the microbial groups responsible for the transformation of both compounds.

We hypothesize that the fate of cellulose- and lignin-derived carbon is related to the level of degradation of both soils. Furthermore, we expect that mineralization and microbial assimilation of carbon derived from both substrates will be higher in the soil with a lower degradation level due to its initially greater microbial biomass. Moreover, the relative contribution of each particular microbial group to the assimilation of substrates is expected to be similar in both soils and dependent on the substrate type.

2. Material and methods

2.1. Study area and soil sampling

Two different soils were chosen in the Province of Murcia located in the South-East of Spain. Both soils are subjected to a semiarid climate with a mean annual rainfall of less than 300 mm and a mean annual temperature of 18 °C. Both soils were agriculturally used in the past and were subjected to natural degradation processes due to the adverse climate conditions of South-East Spain. In the basis of total organic C, nitrogen content, vegetal cover and microbial biomass and its activity, the two soils have different levels of degradation (Bastida et al., 2006), which may influence the dynamics of cellulose and lignin.

The first soil was taken in an area of 100 m^2 located in Abanilla in the Province of Murcia, SE Spain. This soil is classified as Calcaric regosol (Soil Survey Staff, 1998) and represents a highly degraded soil with no vegetation growing on it (Bastida et al., 2006). It was selected as an adequate model for studying processes of carbon transformation under pre-desertic conditions. The soil particle distribution was 34.6% clay; 7.7% silt, and 57.7% sand. Abanilla soil has a pH of 7.8, an electrical conductivity of 2.6 dS m^{-1}, a total N content of 1.3 g kg^{-1}, a total C content of 40 g kg^{-1} and total organic C was 5.0 g kg^{-1}.

The second soil was taken in an area of 100 m^2 located in Santomera in the Province of Murcia, SE Spain. The soil was classified as Haplic calcisol (Soil Survey Staff, 1998) and represents a low-degraded soil (Bastida et al., 2006). Plant cover was around 20% and was dominated by xerophytic shrubs. The soil particle distribution was 18.8% clay, 9.5% silt, and 71.7% sand. Santomera soil has a pH of 7.7, an electrical conductivity of 0.3 dS m^{-1}, a total N content of 1.0 g kg^{-1}, a total C content of 71.2 g kg^{-1} and total organic C was 12.0 g kg^{-1}. A detailed description of these soils is provided by Bastida et al. (2006).

Within these areas, three plots ($n = 3$) of 20 m^2 each were selected. Six subsamples were taken from the upper 15 cm of each plot and pooled to obtain one composite sample per plot. Soil samples were sieved by < 2 mm and stored at 4 °C until the beginning of the incubation experiments.

2.2. Experimental design and soil incubations

Two independent incubation experiments with each of the two soils were performed. The first incubation was carried out in containers with 100 g of soil. Each treatment was prepared in identical triplicates for each incubation time (1, 4, 20, 60, and 120 days). Incubation was performed in chambers at 28 °C in darkness and controlled moisture. Soil samples were used for the measurement of the content and the isotopic composition of carbon in bulk soil, water-soluble and sodium-pyrophosphate extracts at long-term, as well as for PLFA and PLFA-SIP analysis.

In the second incubation experiment, the mineralization of cellulose and lignin was studied at short-term. This experiment was carried out in 12 ml-capped glass vials (Labco Limited, Lampeter, UK) containing 1 g of soil. Each treatment was incubated in triplicates at 28 °C in darkness. The concentration and carbon isotope ratio of CO_2 were analyzed after 1, 4, 10, 20 and 30 days of incubation.

In both experiments, two solutions were prepared with uniformly ^{13}C-labeled substrates (>97 atom%) from maize, one of C^{13}-cellulose and the other of C^{13}-lignin (Isolife, Wageningen, The Netherlands). 75 μg C g^{-1} soil of cellulose or lignin was supplied. For non-labeled control experiments, the same concentrations of these substrates were prepared, but using cellulose and lignin with natural isotopic abundance. The amount of substrates is in the same range as carbon applied in field restoration experiments and in previous isotope labeling experiments (Bastida et al., 2013). Furthermore, a control experiment consisting of soil without cellulose or lignin was set up, using the same conditions as the other two treatments. The water-holding capacity of the soil was gravimetrically adjusted to 60% during incubation for all treatments. Before the substrates were added, soils were pre-incubated with distilled water during two weeks in order to avoid drastic effects of moisture on the measured parameters when adding the substrates. All treatments were performed in replicates ($n = 3$).

2.3. Analysis of carbon fractions

The total organic carbon (TOC) of soil samples was determined after acidification with 2N HCl to pH 2 in a Leco Truspec CN elemental analyzer (St. Joseph, MI) (Bastida et al., 2013). Hot water-soluble carbon was extracted with distilled water (1:5, w:v) by shaking for 2 h at 50 °C. Sodium-pyrophosphate extractable carbon was extracted with 0.1 M sodium-pyrophosphate pH 9.8 (1:5, w:v) by shaking for 4 h (Stevenson, 1982; Lucas-Borja et al., 2012). Subsequently, carbon content in both extracts was determined using a Shimadzu TOC5050A Total Organic Carbon Analyzer. Hot water and sodium pyrophosphate extractions were performed in parallel samples, not sequentially. Hot water-soluble carbon represents the easily available carbon for microorganisms, while carbon in sodium pyrophosphate extracts represents both a labile and more stable carbon presented for example in humic substances (Stevenson, 1982).

2.4. Carbon isotope analysis of carbon fractions and CO_2

Analysis of carbon isotope ratios of water-soluble and sodium-pyrophosphate fractions were performed as described by Bastida et al. (2013). Carbon isotope composition of bulk soil and carbon fractions were reported in the δ notation in per mil relative to the Vienna PeeDee Belemnite standard (V-PDB) (Coplen, 2011):

$$\delta^{13}C[‰] = \frac{R_S}{R_{Std}} - 1 \quad (1)$$

where R_S and R_{Std} are the ratios of the heavy isotope to the light isotope ($^{13}C/^{12}C$) in the sample and in the international standard, respectively. Delta values were converted to ^{13}C fraction expressed as % (Coplen, 2011) (Equation (2)):

$$^{13}C\ [\%] = \frac{100}{\frac{1}{\left(\frac{\delta^{13}C}{1000}+1\right)Rstd}+1} \quad (2)$$

where $\delta^{13}C$ is the measured isotope value of the sample and R_{Std} is the carbon isotope ratio of the V-PDB standard with 0.0112372 (Slater et al., 2001).

Four milliliters of headspace gas from each vial of the mineralization experiment was transferred to pre-evacuated glass vials (Labco Limited, UK) for analysis of the amount and isotopic composition of CO_2. Carbon isotope analysis of CO_2 was performed using a Thermo Scientific GasBench-PreCon trace gas system interfaced to a Delta V Plus IRMS (ThermoScientific, Bremen, DE). CO_2 was sampled by a six-port rotary valve (Valco, Houston TX) with a 100 μL loop programmed to switch at the maximum CO_2 concentration in the helium carrier gas. The CO_2 was then separated from N_2O and other residual gases isothermically at 45 °C using a Poraplot Q column (25 m × 0.32 mm ID) and helium as carrier gas at a flow rate of 2.5 mL min^{-1}). Two laboratory standards were analyzed with every 10 samples. The laboratory standards were calibrated directly against NIST 8545.

The $\delta^{13}C$-values of CO_2 were used for calculating the percentage of CO_2–C derived from the added cellulose or lignin, as described previously (Waldrop and Firestone, 2004; Bastida et al., 2013).

2.5. Phospholipid fatty acids analysis (PLFA)

Phospholipids were extracted from 6 g of soil using chloroform–methanol–water extraction as described by Bligh and Dyer (1959). After lipid fractionation on silicic acid columns (Sep-Pak Silica, Waters), the phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al., 1985) and designated as described by Frostegård et al. (1993). The complete dried FAME fraction was dissolved in *n*-hexane containing 0.23 mg ml^{-1} of 21:0 FAME as internal standard. Gas chromatographic-mass spectrometric analysis of FAMEs was carried out as described previously (Bastida et al., 2013). The absolute and relative amounts of FAMEs in the samples were determined according to the concentration of the internal standard added.

The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t are characteristic bacterial fatty acids and were chosen as bacterial biomarkers (Frostegård et al., 1993; Dungait et al., 2011). The fatty acids i15:0, a15:0, i16:0 were taken as representatives for Gram-positive bacteria; and i17:0 cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t were taken as representatives for Gram-negative bacteria (Frostegård et al., 1993; Dungait et al., 2011). The 18:2ω6 was taken as indicator of fungal biomass (Brant et al., 2006; Rinnan and Baath, 2009).

2.6. Carbon isotope analysis of fatty acids

The carbon isotope composition of FAMEs extracted from control, ^{12}C-substrate and ^{13}C-substrate incubations was analyzed using gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS) system as described previously (Bastida et al., 2011). The $\delta^{13}C$ values of the fatty acids reported were corrected for the carbon introduced during derivatization (Abraham et al., 1998) and converted to ^{13}C-fraction (%) following Equation (2). Samples were measured at least in triplicate with an analytical error of less than ±0.5‰ standard deviation.

The amount of ^{13}C incorporated into fatty acids was calculated as described by Boschker (2004). The percentage of ^{13}C incorporated into fatty acids taken as representatives for Gram-positive bacteria (i15:0, i16:0, i17:0, 10Me16:0, 10Me17:0 and 10Me18:0), Gram-negative bacteria (cy17:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t), and fungi (18:2ω6,9) relative to the total amount of ^{13}C in those fatty acids was used as an indication of the flow of ^{13}C in the different compartments of the microbial community.

2.7. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. The data were submitted to ANOVA of repeated measures (Table S1). In the case of PLFA analysis, the intra-subject factor (time) was set up with 5 different levels, and in the case of CO_2 analysis, the intra-subject factor (time) was set up with 6 different levels. The inter-subjects were defined as the treatment (substrate added) and soil (Table S1). ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference, at the 95% confidence interval in the lineal model). In order to determine significant differences among treatments and soils at the same time, the data were also subjected to one-way ANOVA.

3. Results

3.1. Carbon in bulk soil, water extracts and sodium-pyrophosphate extracts

In general, the amount of carbon extracted by hot water was greater in the Santomera soil than in the Abanilla soil and decreased in all microcosms during the incubation (Fig. S1.). The Santomera soil also exhibited about 10 times higher carbon content of sodium-pyrophosphate extracts than the Abanilla soil. Incubation with ^{13}C-lignin resulted in a higher ^{13}C-enrichment within the bulk soil carbon than incubation with ^{13}C-cellulose (Fig. 1). Up to 78% and 25% of the added ^{13}C was found in bulk soil after 4 months of incubation with labeled lignin and cellulose, respectively. In both soils, the ^{13}C-fraction in bulk soil carbon decreased during incubation (Fig. 1).

The ^{13}C enrichment was analyzed in water-soluble and sodium-pyrophosphate extractable fractions. Overall, the percentage of ^{13}C recovered with sodium pyrophosphate was much higher than the amount recovered with water in both soils (Fig. 1).

The percentage of ^{13}C recovered with hot water was higher in Santomera soil (up to 1.6%) than in Abanilla soil (up to 1.1%). Noticeably, the recovery of ^{13}C in hot water was higher in soils amended with lignin than in those amended with cellulose in both soils ($P < 0.05$). The percentage of ^{13}C recovered with sodium pyrophosphate was also higher in Santomera than in Abanilla soil. The percentage of lignin-derived ^{13}C in sodium-pyrophosphate extracts reached up to 11% and 13% in Abanilla and Santomera soils, respectively (Fig. 1). However, the maximum recovery of cellulose-

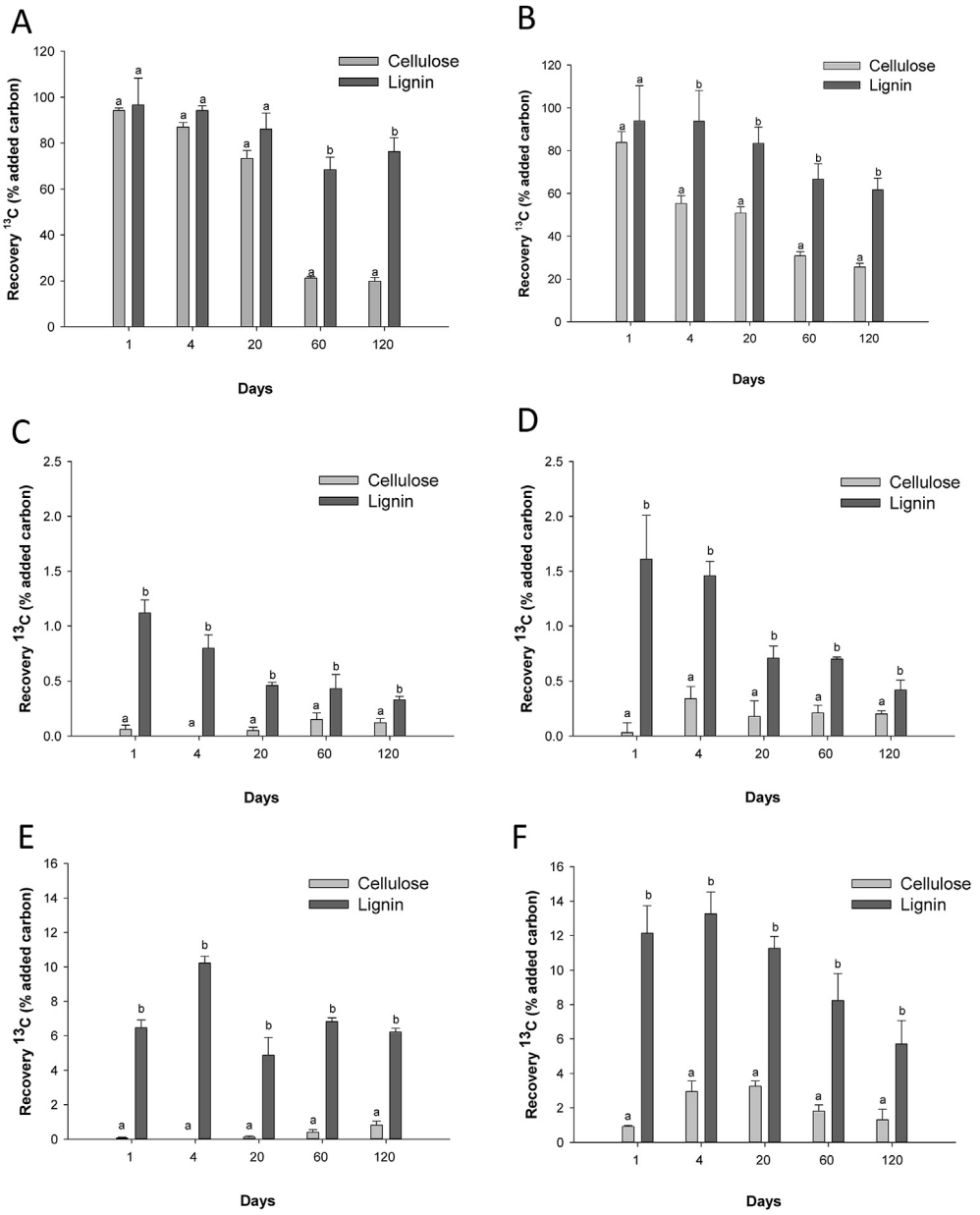

Fig. 1. Recovery of ^{13}C in bulk soil in Abanilla and Santomera, respectively (A and B), water soluble extract (C and D) and sodium pyrophosphate extract (E and F) after addition of ^{13}C-cellulose and lignin. For each incubation time, data followed by the same letter are not significantly different ($P < 0.05$).

derived ^{13}C in sodium-pyrophosphate extracts was 3% in Santomera soil.

3.2. Substrate mineralization

Mineralization of ^{13}C-lignin or ^{13}C-cellulose was already detectable after 0.5 days of incubation but the highest percentage of substrate mineralization occurred after 4 days of incubation. After 30 days of incubation in Santomera and Abanilla soils, 39.7% and 9.3% of the added ^{13}C-cellulose and 34.9% and 8.1% of the ^{13}C-lignin were mineralized, respectively (Fig. 2).

3.3. PLFA concentration and ^{13}C incorporation into PLFA (PLFA-SIP)

Microbial biomass, as estimated by PLFA concentration, was higher in the Santomera soil than in Abanilla soil (Fig. S2 and S3, Supporting information). In both soils, the PLFA concentration of all microbial groups tended to decrease during incubation. The PLFA content of both soils did not respond immediately to substrate addition. Indeed, an increase in the PLFA content of soils with substrates was only observed after 20 days of incubation (Fig. S2 and S3).

Fatty acids extracted from controls as well as from ^{12}C-cellulose and ^{12}C-lignin amended soil exhibited a ^{13}C-fraction of 1.08% ± 0.002. Incubation of soils with ^{13}C-cellulose or ^{13}C-lignin resulted in a fast ^{13}C-labeling of PLFAs already after 1 day of incubation (Fig. 3). For instance, the enrichment in the fungal 18:2w6,9 fatty acid ranged from ^{13}C-fraction of 10%–6% during the incubation of Abanilla soil with lignin, and this enrichment was significantly higher ($P < 0.05$) than in Gram-positive and Gram-negative bacterial representative fatty acids. However, the enrichment of fungal fatty acid did not significantly differ from those of bacterial

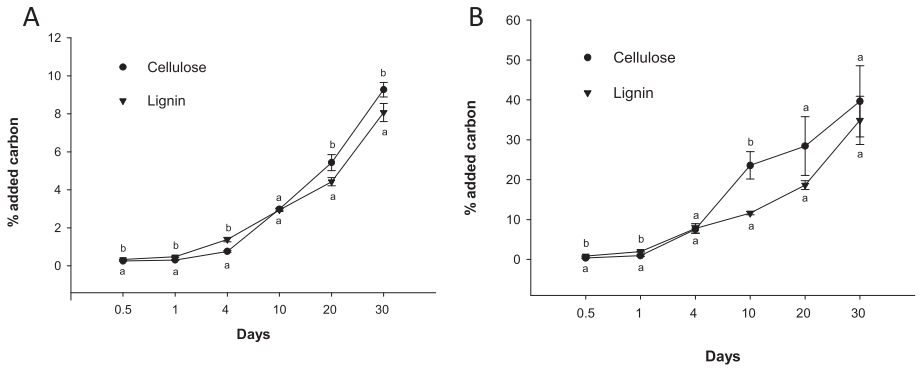

Fig. 2. Cellulose and lignin mineralization in Abanilla (A) and Santomera (B) soil. For each incubation time, data followed by the same letter are not significantly different ($P < 0.05$).

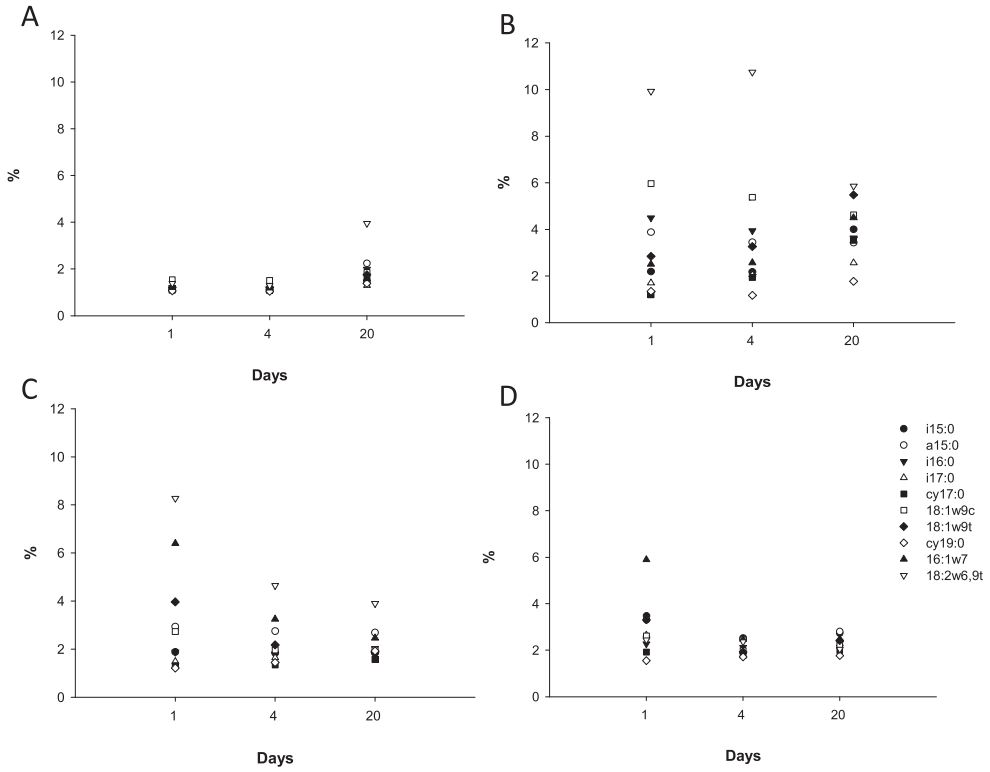

Fig. 3. ^{13}C-fractions in fatty acids given in %. (A) Abanilla soil amended with ^{13}C-cellulose, (B) Abanilla soil amended with ^{13}C-lignin, (C) Santomera soil amended with ^{13}C-cellulose and (D) Santomera soil amended with ^{13}C-lignin. Standard deviation was always below 10% of the mean. i15:0, a15:0, i16:0, i17:0 were taken as indicator for Gram-positive bacteria, cy17:0, 18:1w9c, 18:1w9t, cy19:0, 16:1w7 as measure of Gram-negative bacteria and 18:2w6,9 corresponded to Fungi.

Table 1
Nanograms of ^{13}C content in microbial biomass in Abanilla and Santomera soil during incubation.

		1 day		4 days		20 days	
		Cellulose	Lignin	Cellulose	Lignin	Cellulose	Lignin
Abanilla soil	Bacteria	121.9a	326.0b	82.4a	356.8b	211.1a	448.8b
	Gram-negative	83.6a	261.7a	59.9a	258.6b	141.7a	330.2b
	Gram-positive	38.3a	64.3b	22.4a	98.2b	69.3a	118.6b
	Fungi	9.4a	85.9b	7.1a	70.2a	30.5a	45.8b
Santomera soil	Bacteria	563.6a	669.8a	519.4a	528.2a	445.8a	700.5a
	Gram-negative	364.9a	339.3a	346.2a	321.3a	277.6a	283.7a
	Gram-positive	198.7a	330.5b	173.2a	206.9a	168.2a	416.8b
	Fungi	111.8a	42.6a	103.5b	65.6a	131.7a	49.4a

For each incubation time and microbial group, data followed by the same letter are not significantly different ($P < 0.05$).

fatty acids in Santomera soil (Fig. 3). In soils amended with cellulose, the enrichment in the fatty acid 18:2w6,9 reached up to a ^{13}C-fraction of 4% both in cellulose and lignin treatments after 20 days of incubation.

The total amount of ^{13}C found in fatty acids was much lower than the amount of ^{12}C (please, note the differences units in Tables 1and 2). Overall, the absolute amount of ^{13}C incorporated into PLFAs was higher in Santomera soil than in Abanilla soil. In Abanilla soil, the amount of lignin-derived ^{13}C in the microbial community was significantly higher (with values up to 448 ng ^{13}C allocated in bacterial PLFAs) than the amount of cellulose-derived ^{13}C (maximally up to 210 ng ^{13}C in bacterial PLFAs) ($P < 0.05$). Both bacteria and fungi assimilated a higher amount of lignin-derived ^{13}C than cellulose-derived ^{13}C in Abanilla soil (Table 1). In contrast, fungi assimilated more ^{13}C from cellulose than lignin in Santomera soil during incubation. Regarding bacterial groups, both Gram-positive and Gram-negative bacteria hosted a higher amount of lignin-derived ^{13}C than cellulose-derived ^{13}C in Abanilla soil. However, Gram-negative bacteria in Santomera soil did not differentially assimilate ^{13}C derived from cellulose or lignin during incubation (Table 1).

Out of the total ^{13}C in PLFAs, the percentage of ^{13}C was higher in Gram-negative than in Gram-positive biomarkers, both in cellulose- (up to 67%) and lignin- (up to 68%) amended Abanilla soil (Fig. 4). Out of the total ^{13}C in fatty acids, the percentage of ^{13}C in Gram-positive biomarkers reached its highest value at the first day (25–29%) in Abanilla soil treated with cellulose. In the case of lignin, the percentage of ^{13}C in Gram-positive bacteria increased over time and reached up to 24% at 20 days (Fig. 4).

In Santomera soil, the percentage of ^{13}C was higher in Gram-negative than in Gram-positive bacteria for the cellulose treatment. However, in lignin-treated soils, the percentage of ^{13}C in Gram-positive fatty acids was equal to or higher than the percentage in Gram-negative fatty acids (Fig. 4).

4. Discussion

4.1. Long-term fate of added carbon in semiarid soils

Considering the results of this study together with previous research in the same soil types but amended with ^{13}C-glucose (Bastida et al., 2013), we noticed that lignin-carbon lasted longer than cellulose or glucose carbon in semiarid soils. Hence, our result points to a major importance of the molecular structure of fresh organic matter in its stability in soil, at least in short-term. In contrast, other studies have highlighted that molecular structure alone does not control the stability of soil organic matter at long-term (Schmidt et al., 2011).

The recovery of ^{13}C in sodium-pyrophosphate extracts was always higher than in water extracts (Fig 1.). Water-soluble extracts include the most labile and available fractions (Cook and Allan, 1992), while sodium-pyrophosphate extracts include additionally more stable carbon pools (Stevenson, 1982; Bastida et al., 2013). Our results indicate that a minor portion of cellulose and lignin was quickly subjected to humification. Nevertheless, the contribution of lignin-derived C to the pool of humic substances was higher than that of cellulose in both soils.

The process of humification may involve the degradation of macromolecular compounds into small compounds that are stabilized in soil (Eusterhues et al., 2003). Additionally, compounds derived from the microbial biomass that has been enriched in ^{13}C may be incorporated into the humic substance fraction after cell death.

The recovery of ^{13}C-derived in water-extracts from lignin or cellulose was very low in both cases and followed a different time response. The amount of carbon extracted was higher at the beginning and then progressively declines particularly in the case of lignin. This phenomenon could be attributed to several factors: physical entrapment (Thevenot et al., 2010), chemical transformation and partial mineralization during incubation. However, cellulose behaved differently and the amount of ^{13}C-derived from this substrate extracted with water increased with time. Despite the initially higher level of un-extractability of cellulose-derived carbon, the generation of secondary metabolites by microbial metabolism could explain the higher recovery of cellulose-derived carbon at long-term (Jenkinson et al., 1987).

4.2. Short-term mineralization of lignin and cellulose

A low ^{13}C-enrichment of CO_2 was observed in soils with ^{13}C-cellulose and ^{13}C-lignin at the initial incubation times. These results indicated that, even if the substrate mineralization was low at the beginning, a minor part of the microbial community kept the metabolic capacity for processing these vegetal-like compounds in soils that are highly degraded. In any case, the initial mineralization of these substrates was slow, particularly if we compared with the fast mineralization of cellulose found by other authors (Fontaine et al., 2004). The slow mineralization of substrates at the beginning may be due to the low amount of microbial biomass present in degraded soils (which ranged between 16 and 49 mg C kg^{-1} soil in Abanilla and 200–450 mg C kg^{-1} soil in Santomera) (Bastida et al., 2006). Moreover, a correlation between biomass and substrate respiration has been observed by other authors (Waldrop and Firestone, 2004; Brant et al., 2006; Guenet et al., 2011). Furthermore, the initial un-adaptation of the major part of the microbial community for the mineralization of plant-derived substrates would explain the low mineralization capacity at the initial incubation times. In contrast, the slope of substrate mineralization increased from the 4th day of incubation until 30 days, which could

Table 2
Micrograms of ^{12}C content in microbial biomass in Abanilla and Santomera soil during incubation.

		1 day			4 days			20 days		
		Control	Cellulose	Lignin	Control	Cellulose	Lignin	Control	Cellulose	Lignin
Abanilla soil	Bacteria	13.9b	8.9a	9.6a	18.2b	5.9a	10.1a	10.8a	11.2a	9.8a
	Gram-positive	4.6b	3.2a	2.7a	6.1b	1.9a	2.8a	3.3a	3.8a	3.4a
	Gram-negative	9.3c	5.7a	6.9b	12.1b	4.1a	7.3a	7.5a	7.4a	6.4a
	Fungi	1.3a	0.6a	0.7a	1.2b	0.5a	0.7a	0.6a	0.8a	0.7a
Santomera soil	Bacteria	38.9c	19.3a	24.2b	37.0b	23.1a	23.4a	23.9a	21.1a	30.8b
	Gram-positive	11.8c	8.6a	10.1b	13.9b	8.2a	7.9a	9.0a	7.6a	15.7b
	Gram-negative	27.1c	10.7a	14.2b	23.1b	14.9a	15.6a	14.9a	13.5a	15.0a
	Fungi	2.6c	1.3a	1.9b	2.0a	2.2a	2.5a	1.2a	2.1b	2.2b

For each incubation time and microbial group, data followed by the same letter are not significantly different ($P < 0.05$).

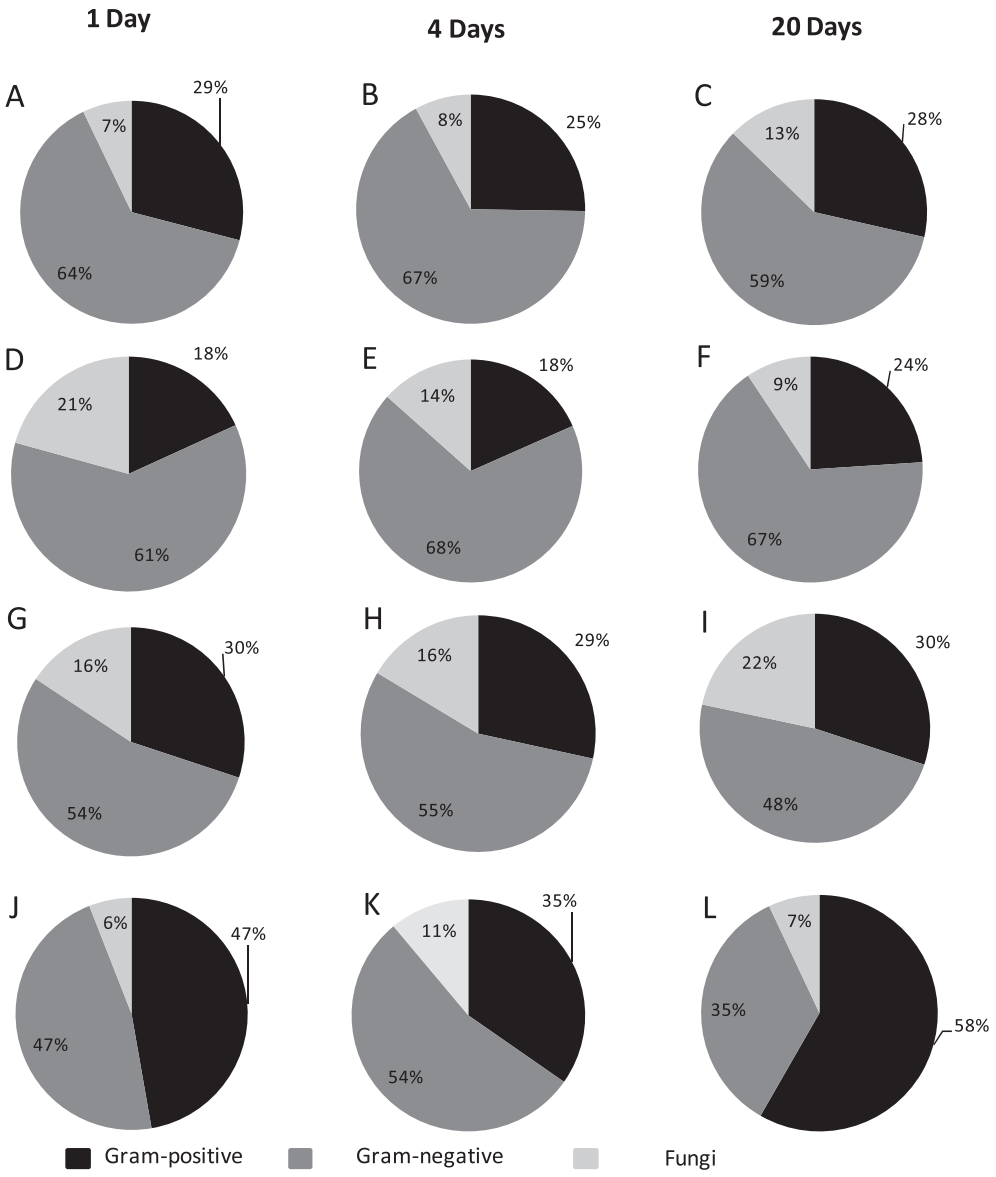

Fig. 4. Percentage distribution of ^{13}C incorporated into PLFA which is shared by specific groups of the community after 1, 4 and 20 days of incubation. (A, B and C) Abanilla soil with added cellulose, (D, E and F) Abanilla soil with added lignin, (G, H and I) Santomera soil with added cellulose, (J, K and L) Santomera soil with added lignin.

suggest an adaptation of microbial community for the mineralization of added substrates.

It is worth of mentioning that the amount of ^{13}C-derived from substrates in microbial biomass at the first day was remarkable in comparison to the scarce amount of substrates that was respired. Products of cellulose and lignin degradation might be stored (and not mineralized) in cell and serve as precursors for fatty acid biosynthesis (Hershberger et al., 1956). Indeed, some authors have noticed that carbon substrates are preferentially stored rather than used for growth (Nguyen and Guckert, 2001) or that respiration is delayed in comparison to storage within microbial cell (Hill et al., 2008).

4.3. Microbial dynamics in relation to cellulose and lignin mineralization

The microbial biomass decreased during incubation in control and amended soils. This decrease could be due, among other factors, to low availability of substrates, as illustrated by the decrease in water-soluble carbon in both soils, and particularly in Abanilla. Water-soluble carbon contains easily available carbon that can be used as an energy source by microorganisms (Cook and Allan, 1992).

It is important to note that the addition of substrates did not generally support the development of microbial biomass, which goes against logic, since microbial growth in semiarid degraded soils is usually hampered by the scarcity of organic matter (Bastida et al., 2006). Indeed, an increase in the PLFA content has been observed after the addition of a similar dose of carbon in the form of glucose in previous studies (Bastida et al., 2013). Noteworthy, carbon quality is particularly important because it restricts the supply of energy for microbial growth in soil (Fontaine et al., 2003). Some authors have even observed a decrease in microbial biomass after the addition of cellulose and lignin (Schutter and Dick, 2001). For instance, DeAngelis et al. (2011) affirmed that lignin can create a non-favorable chemical environment for the development of

microbial biomass, with the exception of certain populations that are able to use lignin. In our study, the only positive response to cellulose or lignin amendment was found at 20 days and was dependent on the soil properties, substrate and microbial group. Besides the fact that an important fraction of added carbon could be immobilized in soil mineral particles (Derrien et al., 2006; Ekschmitt et al., 2008), we also found that only a small fraction of the microbial community assimilated carbon from the added substrates. This is logical, since the enzymatic degradation of macromolecular organic carbon compounds occurs in a limited number of microbial populations (Hammel, 1997; Hu and van Bruggen, 1997; Waldrop et al., 2000). It makes even more sense in our desert-like ecosystems, where the microbial community is not adapted to abundant vegetal inputs. However, it is important to note that the ecological value of such microbial biomass able to assimilate vegetal-like compounds is fundamental, since they are the starters of carbon cycling.

Despite the lower fungal biomass in comparison to bacteria, Fontaine et al. (2011) noted that cellulose decomposition was mainly processed by soil fungi due to stronger competitive advantages over bacteria. We agree with the important role of fungi in cellulose degradation in Santomera soil from the first day of incubation with respect to the high ^{13}C-enrichment of the 18:2w6,9 biomarker in comparison to Gram-negative and Gram-positive bacteria (Figs. 3 and 4). However, in Abanilla soil, fungi were the main drivers of lignin assimilation. In both cases, the assimilation of substrate-derived C into fungal biomass occurred rapidly and decayed during incubation, but always maintained a higher level of enrichment than the bacterial fatty acids. In the case of Abanilla soil, the final decay of fungi enrichment in lignin-amended soil occurred in parallel to a peak of ^{13}C-assimilation in Gram-positive bacteria. This pattern also been found after amendment with ^{13}C-phenol (Brant et al., 2006), and can be explained by an ecological succession related to the degradation of lignin or by a carbon flux from fungi to such bacterial groups as a consequence of grazing phenomena. Rinnan and Baath (2009) explored the assimilation of vanillin in fatty acids of tundra soil. The carbon derived from vanillin, a common product of lignin depolimerization, was quickly assimilated by fungi in tundra soil, but there was a lower level of enrichment in the fungal biomarker (18:2w6,9) than that found in some Gram-negative biomarkers. However, phenol and vanillin are simple structural units constitutive or derived parts of lignin. Nonetheless, the absence of studies based on the stable isotope probing of labeled lignin limits the extension of our discussion.

If we consider the total amount of ^{13}C found in the different microbial groups (Fig 4), Gram-negative bacteria followed by Gram-positive bacteria and fungi are important for the assimilation of lignin and cellulose. These results are in agreement with those found by Haichar et al. (2007) using a DNA-SIP approach with labeled-cellulose. These authors found a higher number of gene sequences related to Gram-negative bacteria in the heavy fractions (i.e. *Sphingomonadaceae*, *Mesorhizobium* sp., *Flavobacterium* sp, *Myxobacterium* sp., etc.) than to Gram-positive bacteria (*Streptomyces* sp.).

5. Conclusions

A major proportion of cellulose- and lignin-derived carbon remained in soil, and only a small fraction of the microbial community was able to assimilate the carbon derived from those macromolecular substrates. However, this community was fundamental for starting the biogeochemical cycling of carbon in desert-like ecosystems. According to the proposed objectives and hypothesis we conclude that:

i) At long-term, the stability of carbon was highly dependent on the chemical nature of the substrate, lignin being more stable than cellulose in both types of soils at long-term. Lignin contributed more than cellulose to the pool of humic substances.
ii) At short-term, substrate mineralization was more related to the soil properties (i.e. microbial biomass) and soil degradation level than on the chemical structure of the substrate.
iii) Microbial assimilation of cellulose- and lignin-derived carbon was higher in the soil with the highest level of biomass (Santomera). The relative contribution of each microbial group to the assimilation of cellulose-derived C was common in both soils. Cellulose assimilation was highly mediated by fungi in both soils, while Gram-negative rather than Gram-positive bacteria exerted an important role on the assimilation of this substrate. However, contrary to the suggested hypothesis, the relative contribution of each microbial group to the assimilation of lignin-derived carbon was different in each soil.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.04.007.

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The effects of fresh and stabilized pruning wastes on the biomass, structure and activity of the soil microbial community in a semiarid climate

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ABSTRACT

The incorporation of organic amendments from pruning waste into soil may help to mitigate soil degradation and to improve soil fertility in semiarid ecosystems. However, the effects of pruning wastes on the biomass, structure and activity of the soil microbial community are not fully known. In this study, we evaluate the response of the microbial community of a semiarid soil to fresh and composted vegetal wastes that were added as organic amendments at different doses (150 and 300 t ha^{-1}) five years ago. The effects on the soil microbial community were evaluated through a suite of different chemical, microbiological and biochemical indicators, including enzyme activities, community-level physiological profiles (CLPPs) and phospholipid fatty acid analysis (PLFA). Our results evidenced a long-term legacy of the added materials in terms of soil microbial biomass and enzyme activity. For instance, cellulase activity reached 633 μg and 283 μg glucose g^{-1} h^{-1} in the soils amended with fresh and composted waste, respectively. Similarly, bacterial biomass reached 116 nmol g^{-1} in the soil treated with a high dose of fresh waste, while it reached just 66 nmol g^{-1} in the soil amended with a high dose of composted waste. Organic amendments produced a long-term increase in microbiological activity and a change in the structure of the microbial community, which was largely dependent on the stabilization level of the pruning waste but not on the applied dose. Ultimately, the addition of fresh pruning waste was more effective than the application of composted waste for improving the microbiological soil quality in semiarid soils.

1. Introduction

Soil degradation is one of the main threats to arid and semiarid ecosystems and is characterized by loss of organic matter (OM) as a consequence of scarce vegetal growth (García et al., 1996). Yet organic matter plays a central role in maintaining key soil functions and global biochemical cycles. Furthermore, it is an essential determinant of soil fertility and resistance to erosion (Ros et al., 2006; Fonte et al., 2009). In scenarios of soil degradation, the application of organic wastes to the soil constitutes an environmental and agricultural means to maintain soil organic matter, reclaim degraded soils and supply plant nutrients. The fresh organic matter present in organic wastes stimulates the development and activity of the soil microbial community (Yang et al., 2003; Bonilla et al., 2012) and can incorporate also exogenous microbes to the soil environment. Moreover, organic matter improves the physical structure of the soil and contributes to carbon sequestration (Foley and Cooperband, 2002; Ros et al., 2006). Nevertheless, the biostimulant capacity of organic amendments depends on their chemical composition (Ajwa and Tabatabai, 1994).

Agricultural and gardening activities produce large quantities of vegetal residues and by-products. These residues cause serious environmental and visual pollution such as formation of pests that can move to new crops, gas emissions, toxic particle accumulation, and so on (Blázquez et al., 2011); particularly in agricultural areas like southeast Spain. A sustainable valorization of this vegetal waste is therefore need. It has been observed that the incorporation of green wastes derived from pruning into soil may improve long term soil fertility and quality (Doran et al., 1988). Moreover, composting these vegetal wastes can also help to reduce waste production. Composting is one of the best known processes for the biological stabilization of solid organic wastes. It involves the accelerated degradation of organic matter by microorganisms under controlled conditions, in which the organic material

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undergoes a characteristic thermophilic stage that allows for sanitization of the waste through the elimination of pathogenic microorganisms (Lung et al., 2001). For this reason, compost is considered to be an environmentally safe and agronomically advantageous organic amendment that stimulates soil microbial activity and crop growth (García et al., 1994; Pascual et al., 1997; Van-Camp et al., 2004). Changes in the properties of organic amendments after composting may directly affect the composition and activity of the soil microbial community (Bastida et al., 2008; Pérez-Piqueres et al., 2006; Cross and Sohi, 2011). However, despite the fact that the restoration of arid and semiarid lands with organic wastes has been a hot topic in recent years, the long-term impact of pruning wastes on the microbial community remains poorly understood.

Several microbial and biochemical indicators are commonly used for evaluating the effects of organic amendments on the soil microbial community. For instance, phospholipid-fatty acid analysis (PLFA) is often used as an indicator of microbial biomass and community structure in soil (Frostegard et al., 1993; Bastida et al., 2008; Williams and Hedlund, 2013). Moreover, community-level physiological profiles (CLPP) are useful for detecting the functional responses of soil microbial communities to a variety of organic amendments (Bastida et al., 2013; Ng et al., 2014).

Soil enzymes play key roles in the biochemical functions of organic matter decomposition and nutrient cycling (Nannipieri et al., 1990; Waldrop and Firestone, 2004). Hence, it has been suggested that soil enzyme activity can be used as an indicator of soil fertility and microbial activity (Badiane et al., 2001) and for evaluating the influence of organic amendments on soil properties (Ng et al., 2014). A better understanding of the enzymes involved in carbon cycling is necessary to improve our knowledge concerning the processes leading soil restoration after application of organic amendments. Given the chemical complexity of soil organic matter contained in plant wastes, including polymeric carbon sources (i.e., cellulose, lignin, etc.), a suite of soil enzymes are involved in organic matter transformation and should be considered in restoration studies. For instance, polyphenol oxidase is involved in lignin degradation and plays an important role in soil C stabilization by favoring humic substance formation through the catalysis of polyphenol oxidation reactions (Weand et al., 2010). Cellulase breaks down cellulose into cellobiose, a sugar composed of two glucose units; and β-glucosidase, which hydrolyzes polymers of plant residues such as cellobiose.

In this experiment, vegetal pruning wastes and compost made from this material were applied as organic amendments in different doses (150 and 300 t ha^{-1}) to a semiarid soil five years ago. We aim to evaluate the long-term effects of these vegetal wastes at different doses and with distinct levels of stabilization (fresh or composted) on the chemical and microbiological properties of a semiarid soil. For this purpose, several indicators of microbial biomass and activity are used in this study. We hypothesized that compost would improve microbial activity and biomass more than fresh waste in the long-term because compost contains a more-stabilized organic matter. Moreover, we expected greater microbial biomass and activity in the plots amended with the highest dose of organic amendments. High doses would incorporate a higher amount of organic carbon and nutrients in soil and promote a most-intense development of the microbial community.

2. Methods and materials

2.1. Study area, experimental design and soil sampling

This study was developed in an experimental field located in Santomera, in the Province of Murcia (southeast Spain) (38° 10′ 91.5″ N; 1° 03′ 79.8″ W), in an area that is greatly affected by soil degradation processes. The area is subjected to a semiarid climate with a mean annual rainfall of less than 300 mm and a mean annual temperature of 18 °C. The soil is classified as Haplic Calcisol (FAO, 2006) and its texture is clay-silt-loam. The pH of the soil is 8.5, its electric conductivity is 250 μS cm^{-1}, its water holding capacity is 40.2 g 100 g^{-1} and the bulk density is 2.57 g cm^{-3}. There was no vegetation growing on the soil at the beginning of this study. The soil was abandoned from agriculture use 20 years ago. This factor, together with inadequate climate conditions of the area and water scarcity, leads soil degradation processes in SE-Spain (López Bermúdez and Albaladejo, 1990). These processes are strongly related to a loss of organic matter (García et al., 1994).

In November of 2008, 15 experimental plots (16 m^2 each) were established in the experimental area. The following treatments were established in replicate plots ($n = 3$): 1) soil with fresh vegetal waste at 150 t ha^{-1} (VW150); 2) soil with fresh vegetal waste at 300 t ha^{-1} (VW300); 3) soil with composted vegetal waste at 150 t ha^{-1} (CVW150); 4) soil with composted vegetal waste at 300 t ha^{-1} (CVW300); and 5) soil without the addition of organic wastes (Control). The chemical properties of the organic amendments are presented in Table 1. Organic materials were incorporated into the first 15–20 cm of soil with a rotovator. The green waste originated mainly from pruning from urban parks. Compost was produced with this type of pruning waste mixed with pig slurry at 3:1 (w:v), for decreasing C/N ratio. For each soil sampling, six subsamples per plot were randomly collected with hand-driven probes to a depth of 15 cm in March of 2014. These subsamples were then mixed to constitute a single sample per plot. The samples were sieved to <2 mm and stored at 4 °C until analysis. Samples were analyzed within one month after sampling.

2.2. Chemical parameters, basal respiration and enzyme activities

Total organic carbon (TOC) was determined using a C analyzer (Thermo Finnigan Flash EA 1112). Water-soluble carbon (WSC) was determined through soil extraction (2 h shaking with a soil:distilled water ratio of 1:5), followed by centrifugation, filtration, and analysis of the extract solution on a C analyzer for liquid samples (Shimazdu 5050 A). An aqueous solution 1:5 (w:v) was used to measure pH in a pH meter (Crison mod.2001, Barcelona, Spain).

Humic substances were extracted with a 0.1 M, pH 9.8 sodium pyrophosphate solution (w/v ratio = 1:5) by mechanical shaking for

Table 1
Characteristics of organic amendments added (VW: fresh vegetal waste; CVW: composted vegetal waste).

	VW	CVW
pH	7.90	8.88
Electrical conductivity (mS)	718.33	787.67
Carbohydrates (ppm)	1671.82	309.13
Water soluble C (ppm)	9601.19	2659.29
Total organic C (%)	34.22	19.32
Humic acids (mg kg^{-1})	1617.01	5241.57
Fulvic acids (mg kg^{-1})	2188.67	1527.34
Polyphenols (μg coumaric ac g^{-1} soil h^{-1})	1300.80	258.75
NH_4 (mg kg^{-1})	<2.5	<2.5
Available P (mg kg^{-1})	135.9	141.40
Total P (g 100 g^{-1})	0.06	0.13
NO_3 (mg kg^{-1})	<5.0	<5.0
N (g 100 g^{-1})	0.87	1.03
Available K (meq 100 g^{-1})	4.65	14.13
Total K (g 100 g^{-1})	0.38	0.75
Cd (mg kg^{-1})	<0.5	0.50
Cu (mg kg^{-1})	14.0	59.60
Cr (mg kg^{-1})	2.7	17.40
Ni (mg kg^{-1})	1.1	7.20
Pb (mg kg^{-1})	1.9	30.50
Zn (mg kg^{-1})	70.6	149.80
(Cellulose + hemicellulose + lignin) (g 100 g^{-1})	35.15	6.76

4 h. Then, the organic carbon concentration in the filtrated extract was estimated by reaction with $K_2Cr_2O_7$ and further measurement of absorbance at 590 nm as indicated by the method of Sims and Haby (1971).

Microbial respiration (CO_2 emission) was measured in 10 ml capped tubes containing 1 g of soil. Soil samples were humified with distilled water at 60% of their water-holding capacity. Vials were hermetically closed and incubated in the dark at 28 °C for 11 days. The concentration of CO_2 was periodically analyzed with a gas chromatograph [Trace Ultra Thermo Scientific, Milan (Italy)] using a packed column [Trace PLOT TG-BOND Q GC, Trace Ultra Thermo Scientific, Milan (Italy)]. The CO_2 emitted per unit of organic carbon was then calculated (qCO_2).

Soil dehydrogenase activity was determined using 1 g of soil, and the reduction of *p*- iodonitrotetrazolium chloride (INT) to *p*-iodonitrotetrazolium formazan (INTF) was measured by the method of Trevors et al. (1982). Soil dehydrogenase activity was expressed as μg INTF g^{-1} soil h^{-1}. The urease activity in the soil was determined by the buffered method of Kandeler and Gerber (1988). In this procedure, 0.5 mL of a solution of urea (0.48%) and 4 mL of borate buffer (pH 10) were added to 1 g of soil and then incubated for 2 h at 37 °C. The ammonium concentration of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate to determine the amount of ammonium produced in the absence of added urea.

Phosphomonoesterase and β-glucosidase activities were determined following the methods described by Tabatabai and Bremmer, 1969 and a modification of Tabatabai's method (1982), respectively, by adding 2 mL of MUB (modified universal buffer) pH 11 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl phosphate (for phosphatase activity assay) or 2 mL of MUB pH 6 and 0.5 mL of 0.025 mol L^{-1} *p*-nitrophenyl β-D-glucopiranoside (for β-glucosidase activity assay) to 0.5 g of soil. The mixtures were then incubated at 37 °C for 1 h, after which time the enzymatic reactions were stopped by cooling on ice for 15 min. Then, 0.5 mL of 0.5 mol L^{-1} $CaCl_2$ and 2 mL of 0.5 mol L^{-1} NaOH (for phosphatase) or 2 mL of 0.1 mol L^{-1} tris(hydroxymethyl) aminomethane-sodium hydroxide (THAM-NaOH) of pH 12 (for β-glucosidase) were added. For the control test, the respective substrates were added before the addition of $CaCl_2$ and NaOH.

Polyphenol oxidase was determined by the method of Allison (2006) using 50 mM pyrogallol/50 mM EDTA as the substrate, and the absorbance of the supernatant from the reaction was determined directly at 460 nm after 1 h.

Lipase was measured according to the method of Margesin et al. (2002) by reaction with p-nitrophenyl butyrate 100 mM. Cellulase activity was determined following a modification of the method of Deng and Tabatabai (1994) using carboxymethylcellulose as substrate. Finally, catalase activity was determined by the method of Johnson and Temple (1964), using H_2O_2 as a substrate, which was shaken for 20 min. The filtrate was titrated with 0.1 M $KMnO_4$.

2.3. Phospholipid fatty acid analysis (PLFA)

Phospholipids were extracted from 6 g of fresh soil using chloroform-methanol extraction as described by Bligh and Dyer (1959) and fractionated and quantified using the procedure described by Frostegard et al. (1993). Phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al., 1985) and designated as described by Frostegard et al. (1993). The complete dried FAME fraction was dissolved in isooctane containing 0.23 mg ml^{-1} of 21:0 FAME as internal standard. The analysis was perfomed using a Trace Ultra Thermo Scientific gas chromatograph fitted with a 60 m capillary column (Thermo TR-FAME 60 m × 0.25 mm ID × 0.25 μm film), using helium as carrier gas.

The following fatty acids are characteristic bacterial fatty acids and were chosen as bacterial biomarkers: i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7 t, 18:1ω9c, 18:1ω9 t, 10Me16:0 and 10Me18:0 (Frostegard et al., 1993 Dungait et al., 2011). The fatty acid 18:2ω6 was used as indicator of fungal biomass (Brant et al., 2006; Rinnan and Bååth, 2009). The Gram-positive representative fatty acids used were i15:0, a15:0, i16:0, i17:0, 10Me16:0 and 10Me18:0; the Gram-negative fatty acids used were cy17:0, cy19:0, 16:1ω7c, 16:1ω7 t, 18:1ω9c and 18:1ω9 t (Frostegard et al., 1993 Dungait et al., 2011). 10Me-branched FAMES (10Me16:0 and 10Me18:0) were taken as specific actinobacterial biomarkers within Gram-positive bacteria (Dungait et al., 2011).

2.4. Community-level physiological profiles (CLPPs) of the microbial community

Biolog EcoPlates (Biolog, Inc. Hayward, CA, USA), containing 31 different C sources and water were used to determine the microbial community-level physiological profiling based on carbon source utilization (Hitzl et al., 1997). The carbon sources belonged to the following groups: carbohydrates, polymers, carboxylic acids, aromatic compounds, phosphorylated chemicals, amino acids, esters and amines. Two-gram of wet samples were shaken in 20 ml of sterile water at 150 rpm, for 15 min at 4 °C. After incubation, a liquid extract was obtained by centrifuging at 2000 rpm for 10 min; 100 μl were inoculated on each Biolog plate well, and the plates were incubated at 28 °C for 7 days. Microbial biomass of cell extracts was equalized by the use of PLFA content of each sample. This standardization prevents influences of the microbial biomass level in the oxidation of substrates and hence an adequate comparison of the functional community structures of each treatment. The rate of utilization was indicated by the reduction of tetrazolium, a redox indicator dye which changes from colorless to purple. Data were recorded for one week at 590 nm in an automated plate reader (Multiskan Ascen), until a plateau was reached. Microbial activity was expressed as average well color development (AWCD), as described by Garland (1996). The catabolic diversity (Hbio) for each sample was estimated using the Shannon–Weaver index, as calculated from the normalized AWCD data after 32 h of incubation in Biolog EcoPlates (Insam and Goberna, 2004).

2.5. Statistical analysis

Statistical analysis was performed with the IBM-SPSS Statistics (version 19.0) software. In order to determine significant differences between the means of the treatments, data was submitted to one-way ANOVA. ANOVA was followed by Tukey's significant difference as a post hoc test (HSD, honestly significant difference, at the 95% confidence interval in the lineal model). A factor analysis was carried out for relative quantity of PLFA and other for CLPP to explore possible differences between treatments. Correlation analysis was performed to evaluate the relationships between the analyzed variables.

3. Results

3.1. Chemical, biochemical analysis and plant cover

After 5-years, plant cover was 77, 82, 85, 91 and 93% in control, VW150, VW300, CVW150, CVW300, respectively. Plant cover was slightly higher in amended plots than in control plots, but differences were not significant. Moreover, the structure of the plant community was similar in all treatments. The plant community was dominated in both treatments by *Carduus bourgeanus,Piptatherum miliaceum, Hedypnois cretica, Reichardia tingitana, Malva parviflora* and *Bromus Fasciculatus*.

Table 2
Chemical parameters. Enzyme activities and basal respiration of control soil; soil amended with fresh vegetal waste (VW150; VW300); and soil amended with composted vegetal waste (CVW150; CVW300).

	Control	VW150	VW300	CVW150	CVW300
pH	8.58 b	8.09 a	8.13 a	8.40 ab	8.27 a
	(0.06)	(0.15)	(0.03)	(0.22)	(0.04)
TOC	1.10 a	4.63 b	8.37 c	2.27 a	4.44 b
	(0.06)	(0.11)	(0.63)	(0.51)	(0.22)
N	0.13 a	0.40 b	0.49 b	0.22 a	0.39 b
	(0.01)	(0.06)	(0.01)	(0.06)	(0.05)
C/N	8.41 a	11.57 b	17.19 c	10.15 ab	10.59 ab
	(1.12)	(1.31)	(1.62)	(0.65)	(0.57)
WSC	385.47 b	156.20 a	245.99 a	366.35 b	371.36 b
	(49.38)	(2.58)	(33.49)	(21.23)	(89.01)
Humic substances	4743.56 a	16120.34 c	23469.20 d	12369.64 b	22334.36 d
	(741.52)	(932.98)	(153.69)	(1803.62)	(280.22)
β-glucosidase	5.49 a	14.26 b	12.56 b	6.56 a	6.22 a
	(1.09)	(1.83)	(1.44)	(0.26)	(0.27)
Lipase	0.87 a	2.31 b	2.15 b	1.25 a	0.94 a
	(0.05)	(0.36)	(0.03)	(0.16)	(0.13)
Polyphenoloxidase	126.81 ab	132.03 b	127.42 ab	138.81 b	105.28 a
	(18.47)	(4.24)	(6.83)	(2.92)	(3.32)
Cellulase	101.91 a	457.46 c	632.78 d	189.40 ab	282.76 b
	(17.23)	(68.28)	(64.36)	(7.85)	(15.97)
Dehydrogenase	5.72 a	8.08 d	7.68 c	5.84 a	6.24 b
	(0.02)	(0.12)	(0.19)	(0.08)	(0.19)
Catalase	5.69 a	7.29 b	6.84 b	5.82 a	5.85 a
	(0.54)	(0.41)	(0.42)	(0.28)	(0.38)
Phosphatase	4.07 a	10.96 c	11.63 c	6.49 b	7.33 b
	(0.52)	(0.03)	(0.08)	(0.15)	(2.17)
Urease	1.47 a	2.31 c	1.78 b	1.75 b	1.99 b
	(0.06)	(0.10)	(0.02)	(0.05)	(0.18)
Basal respiration	23.55 a	46.57 c	43.91 c	34.62 b	30.08 b
	(3.27)	(7.18)	(1.69)	(3.34)	(2.97)
qCO_2	21.36 c	10.04 b	5.30 a	13.61 b	6.82 a
	(0.95)	(1.30)	(0.75)	(3.20)	(1.08)
H	3.21 b	2.99 a	3.10 ab	3.18 b	3.03 a
	(0.01)	(0.10)	(0.01)	(0.02)	(0.01)

Standard deviations appear between brackets. TOC: total organic carbon (g $100\,g^{-1}$); N (g $100\,g^{-1}$); WSC: water soluble carbon (ppm); humic substances ($\mu g\,C\,g^{-1}$); β-glucosidase (μmol PNF $g^{-1}\,h^{-1}$); lipase (μmol PNF $g^{-1}\,min^{-1}$); polyphenoloxidase (mM pyrogallol $g^{-1}\,h^{-1}$); cellulase (μg glucose $g^{-1}\,h^{-1}$); dehydrogenase (μg INTF $g^{-1}\,h^{-1}$); catalase (mmol H_2O_2 $g^{-1}\,h^{-1}$); phosphatase (μmol PNP $g^{-1}\,h^{-1}$); urease (μmol NH_4^+ $g^{-1}\,h^{-1}$); respiration (mg CO_2-C kg^{-1} day^{-1}); H: Shanon–Weaver index.

TOC was higher in all treatments in comparison to the control (Table 2), particularly in the treatments consisting of fresh waste in comparison to the soil amended with compost. In contrast, WSC was higher in plots treated with composted vegetal wastes than in plots amended with fresh vegetal wastes. HS was higher in soil amended than in control soil, especially in the high dose. However, pH decreased significantly with organic additions except in soil amended with compost at low dose (Table 2).

Soil with fresh vegetal wastes showed significantly higher CO_2 emission than soil with composted green manure at both doses ($P < 0.05$). The qCO_2 quotient was significantly lower than in the control ($P < 0.05$) in all treatments (except in soil treated with a low dose of compost) (Table 2).

Fresh vegetal waste increased the overall enzyme activities in comparison to the control soil. Dehydrogenase and catalase are mainly intracellular activities. As was the case for extracellular enzymes, the activity of these intracellular enzymes was higher in the plots amended with fresh wastes than in the compost treatments ($P < 0.05$) (Table 2). β-glucosidase and lipase activities were significantly higher in soil amended with fresh vegetal waste than in the control soil and soil amended with compost ($P < 0.05$); there were no significant differences between doses. In contrast, cellulase activity was highly dependent on the applied dose, and high doses showed greater activity than low doses ($P < 0.05$) (Table 2).

Phosphatase activity increased significantly for both amendments in comparison to the control soil ($P < 0.05$). The activity of this enzyme, however, was significantly higher in soil amended with fresh vegetal waste than soil amended with compost. Urease activity was significantly higher in fresh and composted vegetal wastes than in the control soil ($P < 0.05$).

Polyphenol oxidase activity behaved differently than the rest of the enzyme activities. In this case, the activity was higher in treated soils than in control soil but there were not differences between treatments at high dose (Table 2). The polyphenol oxidase activity of CWV300 was the lowest ($P < 0.05$).

3.2. Phospholipid fatty acid analysis (PLFA)

Bacterial PLFA content increased significantly in all treatments in comparison to control soil ($P < 0.05$). Bacterial PLFA content was significantly higher in fresh than in composted vegetal waste ($P < 0.05$), and VW300 presented the highest values (Table 3). A similar pattern was observed for the Gram-positive bacteria fatty acid content. However, the Gram-negative fatty acid content did not differ between doses in soil amended with fresh vegetal waste.

Soil amended with fresh waste showed significantly higher actinobacterial PLFA content than both soil amended with compost and control soil. High doses of all materials induced a higher actinobacterial PLFA content than low doses ($P < 0.05$) (Table 3). As occurred in the case of bacterial PLFAs, the fungal PLFA content also increased to a greater extent in soils amended with fresh vegetal wastes in comparison to soils amended with stabilized wastes. VW300 showed the greatest values.

Both the ratio between the PLFA content of Gram-positive and Gram-negative bacteria and the ratio between fungal and bacterial PLFA were higher in the plots amended with fresh waste than those treated with composted waste. The ratio between the PLFA contents of Gram-positive and Gram-negative bacteria was greater in the high-dose treatments than in the corresponding low-dose applications and the control. In contrast, the ratio of fungal to

Table 3
Microbial biomass measured by PLFA analysis (nmol g soil^{-1}) and ratios of control soil; soil amended with fresh vegetal waste (VW150; VW300); and soil amended with composted vegetal waste (CVW150; CVW300).

	Control	VW150	VW300	CVW150	CVW300
Bacteria	42.32 a (3.39)	97.06 c (2.52)	116.20 d (9.55)	56.06 b (5.98)	66.48 b (5.86)
Fungi	5.68 a (0.24)	12.24 b (0.92)	16.50 c (0.11)	5.71 a (1.46)	4.20 a (0.09)
Gram-positive	25.10 a (2.17)	60.79 c (0.58)	77.78 d (6.38)	33.15 a (3.96)	42.29 b (3.41)
Gram-negative	17.21 a (1.25)	36.26 c (2.12)	38.42 c (4.43)	22.90 ab (2.08)	24.19 b (2.51)
Actinobacteria	0.79 a (0.09)	2.06 c (0.09)	2.86 d (0.17)	1.01 a (0.13)	1.32 b (0.16)
Gram+/gram-	1.46 a (0.04)	1.68 ab (0.09)	2.04 c (0.21)	1.45 a (0.06)	1.75 b (0.06)
Fungi/bacteria	0.13c (0.01)	0.13 c (0.01)	0.14 c (0.01)	0.10 b (0.02)	0.06 a (0.0)

bacterial PLFA did not show higher values in the high dose treatments in comparison to the treatments with the corresponding material at a low dose.

In order to estimate the structure of the microbial community, a principal component analysis of the relative abundance of fatty acids was performed. Factor 1 explained 55.20% of the variance of the results and Factor 2 explained 37.52% (Fig. 1). Differences in the community structure appeared. According to Factor 1, the structure of the microbial community of control soil and CVW150 differed from the rest of the treatments. According to Factor 2, two groups could be established: one made up of all treatments except CVW300 and another made up of this last treatment. *Actinobacteria* and Gram-negative bacteria received a high loading score in Factor 1. In contrast, fungal and Gram-positive biomarkers received high loading scores in Factor 2 (Fig. 1).

3.3. Community-level physiological profiles (CLPPs) of the microbial community

The functional diversity and functional structure of each microbial community were analyzed using Biolog EcoPlates. Based on carbon utilization patterns, the functional catabolic diversity was calculated using the Shannon–Weaver index. The catabolic diversity remained equal to the control in soil treated with composted vegetal waste at a low dose, yet was significantly lower in the rest of treatments ($P < 0.05$) (Table 2).

The functional structure of the microbial communities was evaluated using a factor analysis of the normalized AWCD values for each single substrate. Factor 1 accounted for 45.47% of the total system variance and Factor 2 accounted for 25.30% (Fig. 2). Factor 1 separated the treatments into three groups: control soil and CVW150; another group formed by fresh pruning waste in low dose and composted vegetal waste in high doses; and finally, another group with VW300. With respect to Factor 2, soil samples were separated into three groups: one made up of VW300 and CVW300; another made up of control soil and CVW150; and a final group made up of VW150. Polymers, aromatic compounds and phosphorylated chemicals received a high loading score in Factor 1. In the other hand, amines and amino acids received a high loading score in Factor 2.

3.4. Correlations between variables

A correlation matrix was generated to evaluate the relationships between the variables analyzed (Table S3, Supporting information). The amount of TOC positively correlated with all enzyme activities studied except for polyphenol oxidase and urease activities. Furthermore, significant positive correlation coefficients were observed between TOC and the PLFA content of all microbial groups studied ($P < 0.05$).

Significant positive correlation coefficients were also observed between the C of humic substances and the PLFA content of Gram-positive bacteria, Gram-negative bacteria and *Actinobacteria*, but this was not true for fungal PLFAs.

Negative correlation coefficients were observed between the WSC and all enzymatic activities (except polyphenol) and with the PLFA content of various microbial groups. Basal respiration was positively correlated with both TOC and PLFA content ($P < 0.05$). pH was negatively correlated with all variables except for total N and polyphenol oxidase activity. However, pH was positively correlated

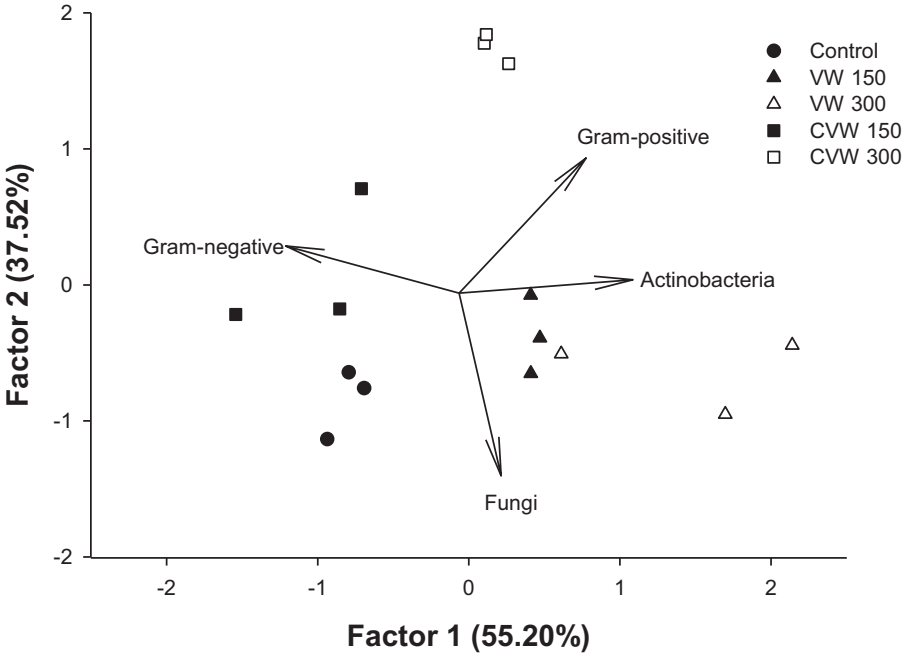

Fig. 1. Principal component analysis of PLFAs in control soil, soil amended with fresh green waste (VW150, VW300) and soil with composted green waste (CVW150, CVW300).

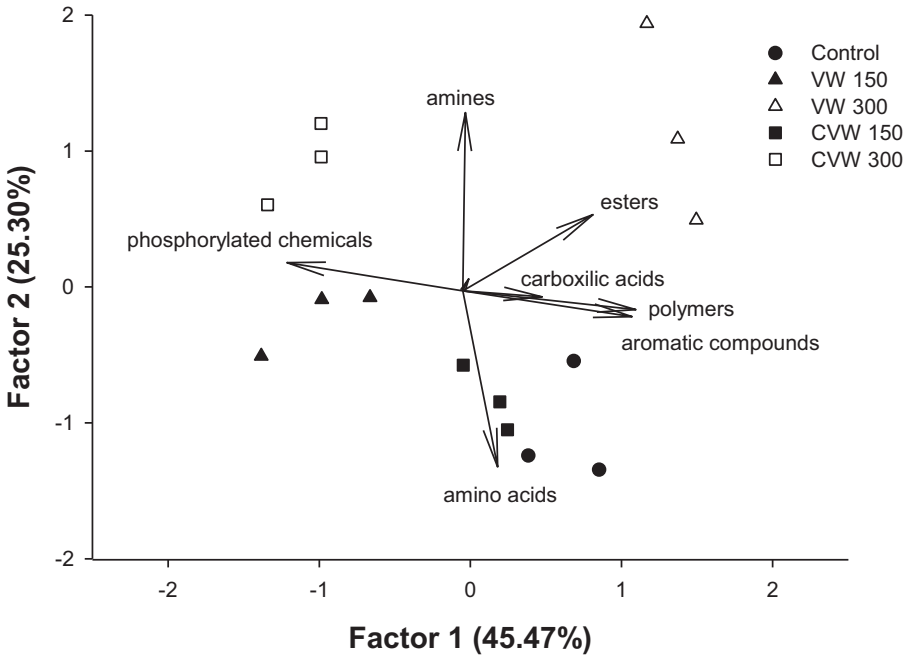

Fig. 2. Principal component analysis of CLPPs in control soil, soil amended with fresh green waste (VW150, VW300) and soil with composted green waste (CVW150, CVW300).

with the functional diversity. Correlations were not observed between plant cover and the rest of variables.

4. Discussion

The maintenance of a vegetal cover is fundamental for soil fertility and helps to preserve a basic level of microbial activity and biomass in semiarid ecosystems (García et al., 1994; Bastida et al., 2006). In this study, scarce differences in the plant cover or community composition were observed between control plots and plots that received amendments 5 years ago. It is likely that initial plowing activities together with organic amendments at the time the experiment was established favored the development of opportunistic plant cover.

4.1. Carbon fractions and microbial activity

The addition of organic waste of plant origin (pruning waste), both fresh and stabilized through composting, increased the soil TOC even 5 years after application. This increase was found to depend on the degree of stabilization of the material used and the dose applied, as other authors have described (Elfstrand et al., 2007; Crecchio et al., 2004; Bastida et al., 2008). The increase in organic C and nutrients in the soil resulting from the initial addition of waste caused a greater increase in the basal respiration of soils treated with amendments than in the control soil, as other authors have also observed (Dube et al., 2009; Iqbal et al., 2010). In general, the parallel increase in basal respiration and hydrolase activities, especially those activities related to the C cycle, reflects the relationship between the degradation of organic matter at the extracellular level and gains in energy at the intracellular level through respiration.

However, considering that the water-soluble C fraction contains labile substrates that are capable of sustaining microbial growth (De Luca and Keeney, 1993; García et al., 2002), it would be expected that the microbial activity would be higher in plots receiving compost treatments (in which WSC values were higher even 5 years after amendment) (Bastida et al., 2008). On the contrary, basal respiration was higher in the plots amended with fresh waste than in plots amended with compost. One possible explanation for this finding is the greater amount of microbial biomass in fresh waste-treated soils (Martens et al., 1992; Goyal et al., 1993). This result might be explained by the high values of organic carbon and nitrogen observed in the fresh-waste treated plots in comparison to other treatments (Marschner et al., 2003). Moreover, this explanation is supported by positive correlation between carbon and nitrogen, and PLFA contents. Enzymatic activities 5 years post-treatment were more dependent on the degree of stabilization of the amendment than on the dose applied. As other authors have noted, this may be due to the existence of a threshold beyond which enzyme activity does not increase in conjunction with increases in the dose of exogenous organic material applied (Crecchio et al., 2004; Bastida et al., 2007). In fact, the dose could even cause an inhibitory effect on some enzymes, as several authors have described (Marcote et al., 2001; Xue and Huang, 2013). In our case, polyphenol oxydase activity was lower in amended soils than in the control. Despite the fact that the behavior of polyphenol oxydase is not entirely clear, since it often does not correlate with hydrolase activity (Sinsabaugh, 2010), some authors have demonstrated that this enzyme may either decrease in quantity or be inhibited by the addition of exogenous organic matter or as a result of the N content of the soil (Sinsabaugh et al., 2005; Stursova and Sinsabaugh, 2008). These observations are consistent with our results.

In comparison to the overall increase in enzymatic and microbial activity in soils amended with fresh material, soils treated with compost produced no clear increase in the majority of activities related to the carbon cycle (i.e., cellulase, β-glucosidase and lipase activities) compared to the control soil, although a number of authors have shown an increase in these activities after several years of compost treatments (Guisquiani et al., 1994, 1995; Crecchio et al., 2001). It is likely that the distinct chemical nature of the added materials was primarily responsible for the observed long-term response of enzyme activities. In fact, considering the differences in carbon quality of the added materials, the higher level of stabilization of compost may still influence results and

could under-stimulate the response of enzyme activities involved in C-cycling. The lack of increase in enzyme activity in compost-amended soil could thus be due to the fact that compost components are more difficult to degrade compared to fresh pruning waste because of the stabilization process (Katterer et al., 2014). Furthermore, the compost treatment seems to induce a lower microbial biomass capable of generating such enzymes than the fresh green waste treatment.

Contrary to what was observed for enzyme activities related to the C cycle, the activity of hydrolase enzymes involved in the P cycle (phosphatase) and N cycle (urease) increased in the compost-amended soil compared to the control. The increase in the phosphatase activity, especially in fresh green waste treatments, may be related to the fact that the activity of this enzyme is closely linked to the amount of microbial biomass rather than to the availability of P (Bowles et al., 2014).

4.2. Biomass, microbial community structure and catabolic diversity

The application of different organic amendments caused differences in the microbial biomass and community structure. Such differences in the structure of the microbial community may be related to the chemical nature of the materials used as amendments (i.e., fresh vs composted) (Saetre and Baath, 2000; Ng et al., 2014). This postulation is consistent with previous studies that have shown that composting alters carbon quality and microbial community (Pérez-Piqueres et al., 2006; Bastida et al., 2008; Cross and Sohi, 2011).

Microbial biomass was influenced by the dose of the material applied and the type of material (Table 3). This could be due to a positive correlation between microbial biomass and the TOC and N content in a soil, all of which are dependent on the dose (Marschner et al., 2003). The microbial biomass increased to a greater extent in soil amended with fresh green waste over the long term, particularly at the highest dose. Some studies have found that microbial biomass is positively related to the TOC and WSC content of amended soils (Marschner et al., 2003; Carrasco et al., 2010). In our study, we found positive correlation between the amount of TOC and the C/N ratio, but this was not the case with WSC. Moreover, there were significant and negative correlations between pH and PLFA groups. This result may indicate that pH exerts a control on the microbial community (Fierer and Jackson, 2006; Lauber et al., 2009). At the bacterial level, the Gram-positive/Gram-negative ratio and C/N ratio were higher in VW300 than in the other treatments. This could be due to the fact that Gram-positive bacteria have a greater ability to degrade complex carbon substrates (Paul and Clark, 1989; Halverson et al., 2000), which could result from the high cellulose content present in fresh, non-composted waste.

Although fungi are characterized as being related to the production of polyphenol oxidase and therefore the formation of humic substances (Allison and Jastrow, 2006; Sinsabaugh, 2010; Weand et al., 2010), this microbial group showed no correlation with either of these variables in our study. As has been observed in other studies, polyphenol oxidase activity can be produced by bacteria and by fungi (Bugg et al., 2011; Ng et al., 2014). This is consistent with our data, as the fungi showed an increase in biomass compared to the control soil with the addition of fresh waste pruning waste, although there was a much greater increase in Gram-positive bacteria. Furthermore, bacterial PLFA content positively correlated with humic substance carbon. In fact, within the Gram-positive bacteria group, the fatty acid content representative of *Actinobacteria* was greater in the soil amended with fresh waste than in the composted or control soils. The Gram-positive bacteria group is associated with lignin decomposition since it is able to use lignin-derived compounds (Kirby, 2006); its increase is therefore not surprising subsequent to the addition of fresh pruning waste.

The changes in microbial biomass described above may be related to changes in the structure of the microbial community in the amended soils. For this reason, the microbial community structure was studied through a principal component analysis of the fatty acids. The microbial community structure in soil amended with fresh green waste and in CVW300 varied with respect to that of control soil. Moreover, the structure community in soil amended with CVW300 was different respect to soil amended with fresh green waste (as regards of the Factor 2). Nevertheless, the structure was similar in both control soil and CVW150. These results indicate that the dose of compost (but not fresh material) and the stabilization level impacted the structure of soil microbial community. The addition of structured and stabilized organic matter (compost) at the lowest dose can provide the most similar organic matter to that of the soil itself and therefore induce less changes in the microbial community structure than high dose.

The change in microbial community structure was mainly due to *Actinobacteria* and Gram-negative bacteria. This fact was not surprising because green residues formed by complex polymers of carbon such as cellulose or lignin and some Gram-positive bacteria (including *Actinobacteria*) are capable of degrading complex substrates of carbon (Paul and Clark, 1989; Halverson et al., 2000). Gram-negative bacteria, on the other hand, are characterized by the fact that they mainly utilize simple carbon forms (Holding, 1960).

Variations in the microbial community structure (assessed by PLFAs) were associated with a shift at the catabolic level (CLPPs). As in the case of PLFAs, the catabolic structures of CVW150 and the control soil were similar, but they were different compared to the other treatments. Catabolic structures of the microbial community were different depending on the dose and stabilization level of the added material. This fact reflects a long-term influence of amendment in the catabolic functionality of the soil microbial community.

Similarly, the control soil and CVW150 showed greater catabolic diversity than the other treatments. In comparison to results in short-term studies that have shown an increase in catabolic diversity with the addition of organic amendments (Gómez et al., 2006; Ros et al., 2006; Hu et al., 2011), other authors have observed that, in the long term, catabolic diversity decreases with the application of high doses of municipal waste (Bastida et al., 2013).

5. Conclusions

The degree of stabilization of the added organic material (5 years ago) had a persistent effect on the microbial community of a semiarid soil. The addition of fresh pruning waste promoted an increase in microbial biomass and more notable activity than the addition of composted waste. However, the dose of vegetal waste applied did not have a notable effect on microbial activity but impacted the functional structure of the microbial community. We may conclude that it is not necessary to compost green waste in order to improve the microbiological quality of semiarid soils in the medium- long term.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2014.12.009.

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