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Tesis Doctoral

BENEFICIOS Y RIESGOS DE LA APLICACIÓN DE ENMIENDAS ORGÁNICAS SOBRE LA SALUD DE SUELOS AGRÍCOLAS

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RESUMEN

La creciente intensificación en la explotación de los suelos a escala mundial, derivada de las necesidades y demandas de una población creciente, se traduce en una presión y un deterioro progresivos del ecosistema edáfico que compromete su sostenibilidad funcional y, por ende, la seguridad alimentaria, el bienestar y la supervivencia de nuestra sociedad. En consecuencia, se hace necesaria una transición hacia modelos de producción agrícola más sostenibles que protejan la integridad ecológica y funcionalidad del ecosistema edáfico, al tiempo que promueven la productividad y calidad de los cultivos.

El presente trabajo ha evaluado los beneficios y riesgos potenciales de distintas prácticas agrícolas (*i.e.*, incorporación de rastrojo tras la cosecha, aplicación de enmiendas orgánicas al suelo, inóculos microbianos basados en micorrizas) orientadas a la disminución del uso de agroquímicos en agricultura, estudiando tanto su potencial agronómico como sus efectos sobre la salud del suelo, estimada ésta a partir de parámetros microbianos que reflejan la biomasa, actividad y diversidad de las comunidades microbianas edáficas.

Nuestros resultados indican que: (i) la incorporación del rastrojo de maíz al sueloes una práctica beneficiosa para la salud del ecosistema edáfico; (ii) los beneficios potenciales de la aplicación de enmiendas líquidas obtenidas a partir de la fermentación de residuos orgánicos dependen, entre otros factores, del tipo de suelo y la dosis específica de aporte de enmienda, la cual ha de ajustarse a los requerimientos de nitrógeno del cultivo; (iii) la aplicación de estiércol fresco al suelo agrícola aporta energía y carbono lábil para las comunidades microbianas edáficas, con el consiguiente aumento de su biomasa y actividad metabólica, e incrementa el rendimiento productivo del cultivo de lechuga; no obstante, esta aplicación conlleva un riesgo de diseminación de genes de resistencia a antibióticos a los suelos agrícolas y el medio ambiente. Por otra parte, (iv) tanto el compostaje como la fermentación anaerobia del estiércol conllevan una reducción notable en la carga de determinantes de resistencia a antibióticos; (v) la aplicación a largo plazo de lodos de depuradora urbana digeridos anaeróbicamente y deshidratados conlleva una mejora en las propiedades físico-químicas y biológicas de los suelos agrícolas, pero al mismo tiempo aumenta la abundancia de genes de resistencia a antibióticos y elementos genéticos móviles de forma correlacionada con la concentración total de cobre y zinc en el suelo, lo que sugiere la existencia de mecanismos de co-evolución; (vi) algunas prácticas agrícolas propias de la agricultura orgánica (*i.e.*, laboreo mínimo y empleo de

enmiendas orgánicas) ejercen un efecto negativo sobre la abundancia y diversidad de hongos micorrícicos arbusculares. La inoculación de hongos micorrícicos arbusculares mejora el rendimiento productivo del cultivo de lechuga sin ejercer una alteración notoria en las comunidades edáficas de dichos hongos.

A partir de estos estudios, se concluye que la utilización de enmiendas orgánicas tiene un gran potencial agronómico. Sin embargo, su sostenibilidad como práctica agrícola depende de la minimización de los riesgos derivados de su utilización. Finalmente, la utilización de propiedades microbianas con potencial bioindicador de la salud del suelo representa una opción idónea para la evaluación y monitorización del impacto potencial de la aplicación de enmiendas orgánicas sobre los suelos agrícolas.

LABURPENA

Gero eta handiagoa den populazioaren premiak eta eskaerak asetzera bideratuta dagoen mundu mailako lurzoruen ustiapenaren areagotze etengabeak, ekosistema edafikoaren presio eta narriadura progresiboa dakar. Egoera honek arriskuan jartzen du ekosistema edafikoaren jasangarritasun funtzionala eta, beraz, mundu mailako elikadura-segurtasuna, eta gizartearen ongizatea eta biziraupena. Ondorioz, nekazaritza-ekoizpen jasangarriagoko ereduetaranzko trantsizioa egitea beharrezkoa suertatzen da, ekosistema edafikoaren osotasun ekologikoa eta funtzionaltasuna babestuz eta, aldi berean, laboreen produktibitatea eta kalitatea sustatuz.

Lan honek nekazaritzan agrokimikoen erabilpena murriztera bideratuta dauden hainbat nekazaritza-jardueraren onura eta arrisku potentzialak ebaluatu ditu (hala nola, uzta jaso ondoren uztondoa lurzoruan gehitzea, medeapen organikoen aplikazioa, mikorrizetan oinarritutako mikrobio-inokuluak), haien potentzial agronomikoa eta lurzoruaren osasunean duten eragina aztertuz, azken hori, lurzoruko komunitate mikrobianoen biomasa, aktibitatea eta dibertsitatea islatzen duten parametro mikobiologikoen kalkuluan oinarrituta.

Gure emaitzek adierazten dutenez, (i) arto-uztondoa lurzoruan gehitzea nekazal-jarduera onuragarria da ekosistema edafikoaren osasunerako; (ii) hondakin organikoen hartziduraren bidez lortutako medeapen likidoak aplikatzearen onura potentzialak, besteak beste, lurzoru-motaren eta aplikazio dosi espezifikoaren araberakoak dira, azken hau laborearen nitrogeno-eskakizunetara egokitu behar delarik; (iii) simaur freskoa

energia eta karbono iturri labila da lurzoruko komunitate mikrobianoentzat, hortaz, nekazal lurzoruan aplikatzen denean komunitate mikrobianoen hazkuntza eta aktibitate metabolikoa sustatzen du eta, aldi berean, letxuga laborearen ekoizpena handitu egiten du; hala ere, aplikazio horrek antibiotikoekiko erresistentzia-geneak nekazal lurzoruetara eta ingurumenera zabaltzeko arriskua dakar. Bestalde, (iv) bai simaurraren hartzidura anaerobioak bai konpostatzeak antibiotikoekiko erresistentzia-determinatzaileen kargaren murrizpen nabarmena dakarte; (v) anaerobikoki digeritutako eta deshidratatutako hiri-araztegiko lohiak epe luzean aplikatzeak nekazal lurzoruen propietate fisiko-kimikoak eta biologikoak hobetzen ditu, baina, aldi berean, antibiotikoekiko erresistentzia-geneen eta elementu genetiko mugikorren ugaritasuna areagotzen du, kobre eta zink metal astunen kontzentrazio totalen hazkuntzarekin korrelazioan dagoena, koeboluzio mekanismoen presentzia iradokiz; (vi) nekazaritza organikoko berezko jarduera batzuek (hala nola, gutxieneko laborantza eta medeapen organikoen erabilera) eragin negatiboa dute onddo mikorriziko arbuskuskularren aniztasun eta ugaritasunean. Onddo mikorriziko arbuskularren inokulazioak letxuga laborearen ekoizpena hobetzen du, lurzoruko onddo horien komunitatetan aldaketa nabarmenik eragin gabe.

Emaitza hauetatik ondorioztatzen da medeapen organikoen erabilpenak potentzial agronomiko handia duela. Hala ere, erabilera honen jasangarritasuna nekazal jarduera gisa, erabilpenak dakarren arrisku potentzialak efektiboki minimizatzearen menpe dago. Azkenik, lurzoruaren osasunaren bioadierazle izan daitezkeen propietate mikrobiologikoen erabilpenak, medeapen organikoen aplikazioak izan dezakeen eragina ebaluatzeko eta monitorizatzeko aukera egokia suposatzen du.

ABSTRACT

The increasing intensification of the exploitation of our soils at a global scale, arising from the needs and demands of a growing human population, translates into a progressive pressure and degradation of the soil ecosystem, which compromises its functional sustainability and, hence, global food-security, as well as the survival and well-being of our society. Consequently, there is a need for a transition towards more sustainable agricultural production models which protect the ecological integrity and functionality of the soil ecosystem, while promoting crop quality and productivity.

The present work has evaluated the potential benefits and risks of different agricultural practices (*i.e.*, stover incorporation after crop harvest, the application of organic amendments to soil, mycorrhizal microbial inoculants) aiming to reduce the use of agrochemicals in agriculture, by studying both their agronomic potential and effects on soil health estimated through the assessment of microbial parameters related to the biomass, activity and diversity of soil microbial communities.

Our results suggest that: (i) corn stover incorporation after harvest is a beneficial agricultural practice for soil health; (ii) the potential benefits of the application of liquid amendments obtained from the fermentation of organic residues strongly depend, among other factors, upon soil type and application dose, which should always be adjusted to the nitrogen requirements of the crop; (iii) the application of fresh manure to agricultural soil provides energy and labile carbon for the soil microbial communities, leading to higher values of microbial biomass and metabolic activity, as well as enhanced lettuce crop yield; however, such application poses a risk of dissemination of antibiotic resistance genes into the agricultural soil and the environment. On the other hand, (iv) both composting and anaerobic fermentation of manure lead to a significant reduction of the load of antibiotic resistance determinants; (iv) the long-term application of anaerobically digested and dehydrated sewage sludge leads to an improvement in the soil physicochemical and biological properties but, at the same time, results in an increase in the abundance of antibiotic resistance genes and mobile genetic elements that correlates positively with the total concentration of soil copper and zinc, suggesting the existence of co-evolution mechanisms; (vi) some organic farming practices (*i.e.*, minimum tillage and use of organic amendments) have a negative impact on the abundance and diversity of arbuscular mycorrhizal fungi. The inoculation of arbuscular mycorrhizal fungi increases lettuce crop yield without significantly altering the soil communities of such fungi.

On the basis of these studies, it is concluded that the application of organic amendments has great agronomic potential. Nonetheless, the sustainability of this agricultural practice depends on the minimization of the risks arising from its utilization. Finally, the utilization of soil microbial properties with potential as bioindicators of soil health is a suitable option for the assessment and monitoring of the potential impact of the application of organic amendments on agricultural soils.

ÍNDICE

1| ANTECEDENTES E INTRODUCCIÓN

1. ANTECEDENTES E INTRODUCCIÓN

NOTA INTRODUCTORIA: este primer Capítulo se divide en tres apartados: (1.1) una breve exposición de los antecedentes del estudio a través de la contextualización del marco en el que se encuadra este trabajo dentro de la problemática global de la degradación del suelo derivada de la intensificación agrícola, así como la enfatización dela criticidad de los cambios actuales en el paradigma de los sistemas productivos agrícolas y, análogamente, de la potencialidad y relevancia de la evaluación y monitorización del estado de los suelos agrícolas mediante el empleo de (bio)indicadores; (1.2) una explicación conceptual del desarrollo secuencial del presente trabajo; y (1.3) una revisión bibliográfica (en inglés), a modo de introducción, sobre los principales impactos positivos y negativos del uso de enmiendas orgánicas en agricultura.

1.1. Antecedentes

El suelo, la capa superior de la corteza terrestre, es un sistema dinámico, heterogéneo y extremadamente complejo en el que los componentes sólidos, líquidos y gaseosos interactúan en múltiples procesos físicos, químicos y biológicos. Estas interacciones sustentan la multifuncionalidad del suelo como ecosistema capaz de suministrar un variado elenco de servicios ecosistémicos cruciales para el desarrollo y mantenimiento de la calidad de vida y el bienestar de la sociedad (Figura 1.1). Lamentablemente, desde la Revolución Industrial, los suelos han sufrido un proceso acelerado de degradación derivado, en gran parte, de la acción antrópica, comprometiendo así su capacidad para desempeñar y mantener sus funciones y, por ende, suministrar los citados servicios ecosistémicos. Esta rápida degradación del suelo contrasta con la lentitud de los procesos implicados en su formación (edafogénesis), resultado de la meteorización del material parental por la acción combinada de numerosos factores ambientales y organismos vivos. En este sentido, es importante enfatizar que el suelo se considera un recurso no renovable a escala humana (Hakeem *et al*., 2014).

La degradación del suelo proviene principalmente de los impactos antrópicos derivados de la necesidad de satisfacer la enorme demanda de recursos (alimento, agua, materias primas, superficie física, etc.) de una población en continuo crecimiento. Según análisis recientes de las Naciones Unidas acerca de las perspectivas de evolución y

crecimiento de la población mundial, se estima que en los próximos 30 años la población mundial aumentará en 2.000 millones de personas, llegando a los 9.700 millones en 2050 (UN, 2019). Este rápido y hasta ahora incesante crecimiento de la población mundial implica la necesidad imperiosa y acuciante de aumentar la producción de alimento, entre otras alternativas, mediante el incremento de la productividad agrícola. Por supuesto, la búsqueda de incrementos en la productividad agrícola no es un fenómeno nuevo sino que lleva abordándose desde los albores de la agricultura y, en concreto, con especial énfasis desde la segunda mitad del siglo XX cuando, de la mano de la Revolución Verde, se introdujeron nuevas variedades de cultivos, nuevos desarrollos para la mecanización progresiva de las labores agrarias, mejoras en los sistemas de irrigación, el uso extensivo de agroquímicos sintéticos como fertilizantes y plaguicidas, etc. (Tilman *et al*., 2002). De hecho, la mejora en el rendimiento productivo de los cultivos ha estado históricamente ligada a la intensificación de los sistemas agrícolas (Pretty y Barucha, 2014). Por desgracia, la intensificación agrícola ha causado un fuerte impacto adverso en el medio ambiente y, especialmente, sobre el ecosistema edáfico al que ha sometido a una degradación progresiva y constante, lo que se ha traducido en el preocupante deterioro a escala global de su sostenibilidad funcional, con la concomitante pérdida de fertilidad de los suelos agrícolas (Trivedi *et al*., 2016).

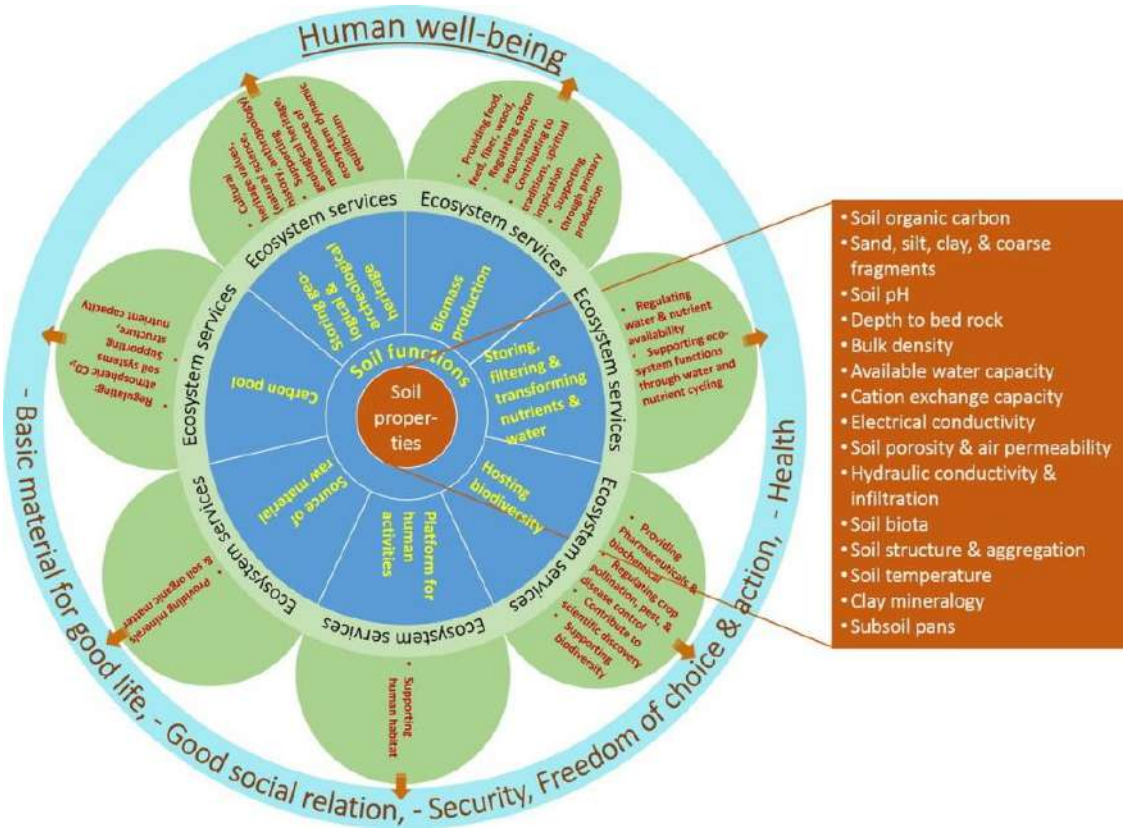

Figura 1.1. Vínculos entre las propiedades del suelo y la realización de funciones que permiten la provisión de servicios ecosistémicos clave para el bienestar humano. *Fuente: Adhikari y Hartemink, 2016.*

Por ello, y dado que el suelo constituye la base de la productividad agrícola, la agricultura actual se enfrenta al reto de garantizar la seguridad alimentaria mundial al tiempo que minimiza los costes ambientales. Este reto parte de la superación de los principios de la agricultura convencional más intensiva, al objeto de promover modelos y tecnologías que (i) integren la sostenibilidad y los procesos ecológicos en los sistemas agrícolas, (ii) concilien aspectos ambientales y altas producciones, y (iii) reduzcan, o incluso sustituyan, los insumos externos. Estos modelos incorporan avances tecnológicos para el uso selectivo de insumos agrícolas, como la agricultura de precisión y la robótica aplicada; tecnologías y pautas para la reducción y reutilización de los residuos agrícolas; y prácticas sostenibles como las recomendadas por la agricultura de conservación y la agroecología (Bommarco *et al*., 2013).

A este respecto, la reutilización de residuos orgánicos como enmiendas agrícolas es una alternativa viable y potencialmente más sostenible, ambiental y económicamente hablando, frente al empleo de los tradicionales insumos procedentes de la síntesis química (*e.g.*, fertilizantes y plaguicidas). Dicha reutilización permite convertir un residuo en un

recurso con potencial agronómico, evitando así su deposición en vertedero (Chojnacka *et al*., 2019). Sin embargo, el efecto de estas enmiendas orgánicas en el ecosistema edáfico y su eficacia en términos de promoción de la productividad agrícola depende, entre otros aspectos, de su naturaleza y origen, así como del tratamiento al que hayan o no sido sometidas.

El efecto potencial que las enmiendas orgánicas más comúnmente utilizadas en agricultura ejercen sobre la salud del suelo se discute de manera exhaustiva en el apartado 1.3 de este capítulo (ver abajo). No obstante, existe un conjunto de "enmiendas orgánicas" constituido por los denominados biofertilizantes, bioestimulantes o inóculos microbianos con potencial promotor del crecimiento vegetal que no han sido incluidos en el siguiente apartado debido a que en la literatura existe cierta controversia acerca de si realmente pueden calificarse como "enmiendas orgánicas" (Abbott *et al*., 2018; Hueso-González *et al*., 2018). En este sentido, el reciente Reglamento de la Unión Europea (UE) 1009/2019 sobre fertilizantes, el cual establece distintas "Categorías Funcionales de Productos" para los fertilizantes, distingue entre "enmiendas orgánicas" y "bioestimulantes de plantas microbianos" (European Parliament and European Council, 2019). Estos bioestimulantes incluyen microorganismos como hongos micorrícicos, bacterias rizosféricas promotoras del crecimiento vegetal y bacterias endosimbiontes que hayan sido seleccionados a raíz de sus demostradas virtudes en la mejora de la eficiencia nutricional de los cultivos y/o en la tolerancia a distintos tipos de estrés (du Jardin, 2015). La utilización de hongos micorrícicos es particularmente interesante para la mejora de la productividad agrícola ya que, entre otros beneficios, tienen la capacidad de incrementar la superficie radicular de las plantas, lo que permite una mayor exploración de la rizosfera y, por tanto, una mayor absorción de agua y nutrientes (Verbruggen *et al*., 2013). Estos microorganismos son capaces de asociarse con la mayoría de los cultivos agrícolas de forma natural, intercambiando carbono por nutrientes. Además de la mejora en la eficiencia de absorción de nutrientes, los hongos micorrícicos mejoran la tolerancia de las plantas frente a condiciones de estrés biótico y abiótico (Gianinazzi *et al*., 2010), y promueven la estructura del suelo mediante la formación y estabilización de agregados (Leifheit *et al*., 2014). Sin embargo, diversas prácticas agrícolas habituales, como el arado y la fertilización química, pueden afectar negativamente la abundancia y diversidad de estos hongos. En consecuencia, y dados los beneficios mencionados, uno de los capítulos de este trabajo se centra en el efecto de la inoculación de hongos micorrícicos sobre la

productividad agrícola y la salud del suelo (en concreto, sobre el crecimiento de la lechuga y la diversidad fúngica del suelo como bioindicador de su salud).

Además de los distintos modelos y tecnologías para la promoción sostenible de la productividad agrícola, la concienciación creciente frente a la progresiva degradación del ecosistema edáfico ha traído conceptos como la "calidad" y la "salud" del suelo. Estos conceptos surgen en la década de los 90 como herramientas para la evaluación de la capacidad de un determinado suelo para realizar sus funciones de forma adecuada y sostenible, y se basan en propiedades inherentes y dinámicas de los procesos edáficos (Karlen *et al*., 1997). En 1994, Doran y Parkin definieron la "calidad del suelo" como "la capacidad de un suelo para funcionar dentro de los límites del ecosistema, sostener la productividad biológica, mantener la calidad del medio ambiente, y promover la salud de las plantas y los animales" (Doran y Parkin, 1994). Posteriormente, basándose en esta definición, el Comité sobre la Calidad del Suelo de la Sociedad Americana de Ciencias del Suelo definió el término calidad del suelo como "la aptitud de un tipo específico de suelo para funcionar, dentro de los límites tanto naturales como gestionados de los ecosistemas, mantener la productividad vegetal y animal, mantener o mejorar la calidad del agua y del aire, y promover la salud humana y la habitabilidad" (Karlen *et al*., 1997).

Por su parte, la "salud del suelo" se define como "la capacidad continua del suelo para funcionar como un sistema vivo, dentro de los límites naturales del ecosistema y de su uso, mantener la productividad biológica, promover la calidad del aire y del agua, y mantener la salud animal, vegetal y humana" (Doran y Safley, 1997). Basándonos en esta definición, puede deducirse que las diferencias entre la salud y la calidad del suelo derivan del componente temporal, que refleja la importancia del funcionamiento adecuado del suelo a lo largo del tiempo, junto con el reconocimiento del suelo como un sistema vivo, haciendo hincapié en la importancia de la biota del suelo para el funcionamiento del ecosistema edáfico, lo que constituye una analogía con respecto a la salud de un organismo o una comunidad. De forma similar, a la hora de señalar las diferencias entre ambos conceptos, Pankhurst *et al*. (1997) enfatizaron que "el concepto salud del suelo incluye los atributos ecológicos del suelo cuyas implicaciones van más allá de su calidad o capacidad para producir un determinado cultivo. Estos atributos son aquellos estrechamente relacionados con la biota edáfica, como la biodiversidad, la estructura de la red trófica, la actividad biológica y la serie de funciones que desempeña" (Pankhurst *et al*., 1997). De esta forma, los autores consideran que, a diferencia de la calidad del suelo, la salud abarca la naturaleza viva y dinámica del ecosistema edáfico.

A pesar de estas diferencias, varios autores consideran que ambos conceptos se solapan en gran medida, utilizándose a menudo como sinónimos. Por ejemplo, en una reciente revisión bibliográfica, Bünemann *et al*. (2018) concluyeron que la distinción entre los conceptos de calidad y salud del suelo ha pasado de ser una cuestión de base a una cuestión de mera preferencia entre los distintos organismos y autores, por lo que consideraron ambos conceptos como equivalentes. Sin embargo, generalmente, el término calidad del suelo se asocia a la aptitud de un suelo para un uso específico, siguiendo la premisa básica del concepto "calidad" desarrollado por Carter *et al*. (1997), mientras que el término salud del suelo hace referencia a la capacidad continua del suelo para funcionar como un sistema vivo (Doran y Zeiss, 2000).

Independientemente del término utilizado, la evaluación y monitorización de la calidad/salud del suelo es de suma importancia a fin de garantizar la funcionalidad del ecosistema edáfico, con todo lo que ello representa. Sin embargo, la evaluación de la calidad y/o la salud del suelo es una actividad sumamente complicada ya que requiere de la integración de propiedades físicas, químicas y biológicas de un ecosistema inherentemente complejo y dinámico. A este respecto, para poder evaluar el estado de un suelo o su respuesta frente a determinadas perturbaciones o estreses es indispensable disponer de una serie de parámetros indicadores de la calidad/salud del suelo. A efectos prácticos, la determinación de los indicadores debe ser sencilla, reproducible y de bajo coste e, idealmente, los parámetros indicadores deben formar parte de una base de datos ya existente (Doran y Parkin, 1996; Bünemann *et al*., 2018). La identificación efectiva de un conjunto reducido de indicadores apropiados para la evaluación y monitorización de la calidad/salud del suelo resulta imperativa, debido a las habituales limitaciones económicas y restricciones temporales. Esta identificación ha dado lugar a varias herramientas para la evaluación de la salud del suelo como el "Soil Management Assessment Framework" (Karlen *et al*., 2003), el "Soil Conditioning Index" (Soil Quality Institute, 2003), el "Agroecosystem Performance Assessment Tool" (Wienhold *et al*., 2006) y el "Comprehensive Assessment of Soil Health" (Moebius-Clune *et al*., 2016), etc. En este sentido, la visión del suelo como un sistema vivo ha promovido que el estudio de las propiedades biológicas del suelo con potencial bioindicador, históricamente ignoradas a favor de las propiedades físico-químicas, haya cobrado relevancia en los programas de evaluación y monitorización de la salud del suelo a nivel global (Garbisu *et al*., 2011). La biota edáfica y, en particular, la microbiota, dado que constituye la fracción más relevante de la biomasa del ecosistema edáfico, tiene una importancia vital

en el funcionamiento del suelo y en el suministro de servicios ecosistémicos (Burges *et al*., 2015). Así, la actividad biológica del suelo se asocia a procesos reguladores del reciclaje de nutrientes (mineralización, desnitrificación, fijación de N_2, etc.) y a la descomposición de la materia orgánica, mientras que la biodiversidad edáfica es responsable, en gran medida, de la estabilidad (procesos de resistencia y resiliencia) del ecosistema edáfico (Isbell *et al*., 2015). Frente a los indicadores físico-químicos, los bioindicadores o indicadores biológicos presentan diversas ventajas entre las que destaca su mayor sensibilidad, rapidez de respuesta, relevancia ecológica y carácter integrador. Y, como se ha mencionado anteriormente, dado su papel clave en el funcionamiento del ecosistema edáfico y su contacto íntimo con la matriz edáfica, los parámetros que reflejan la biomasa, actividad y diversidad de las comunidades microbianas del suelo tienen un enorme potencial como indicadores causales del efecto de perturbaciones y fuentes de estrés ambiental sobre la funcionalidad del suelo.

1.2. Explicación del desarrollo secuencial del presente trabajo

La agricultura debe ser capaz de satisfacer la progresiva demanda de alimento que genera el continuo crecimiento de la población humana y debe hacerlo mediante la adopción de tecnologías y métodos de producción que no pongan en riesgo la sostenibilidad ambiental ni comprometan la salud humana. En concreto, la mejora productiva ligada a la intensificación en el uso de fertilizantes de síntesis química ha causado un importante deterioro del medio ambiente y de la biodiversidad, como se deriva, entre otras razones, del elevado consumo energético asociado a su producción (la demanda de energía para la producción de fertilizantes nitrogenados ha sido satisfecha mediante la quema de combustibles fósiles con el consiguiente impacto en términos de contaminación y cambio climático). Por ello, la incorporación de modelos alternativos que desliguen la producción agrícola del consumo de combustibles fósiles es acuciantemente necesaria. En este sentido, la intensificación ecológica se presenta como la opción más prometedora para la mejora del rendimiento productivo, a la vez que favorece la provisión de servicios ecosistémicos mediante la integración de los procesos ecológicos en las prácticas agrícolas. En este contexto, el presente trabajo profundiza en el potencial de distintas opciones orientadas a la disminución (o sustitución) del uso de agroquímicos en agricultura, incluyendo prácticas propias de la agricultura de

conservación, la agricultura orgánica y el uso de inóculos microbianos. En este sentido, se ha prestado especial interés a la capacidad de estas opciones para mejorar la salud del suelo estimada a partir de parámetros microbianos que reflejan la biomasa, actividad y diversidad de las comunidades microbianas edáficas.

Para ello, en primer lugar, se realizó un estudio acerca del potencial agronómico de la incorporación del rastrojo de maíz al suelo agrícola. Posteriormente, se realizaron varios ensayos con el propósito de evaluar el efecto de distintas enmiendas orgánicas sobre la salud del suelo agrícola. Así, inicialmente se realizaron dos ensayos para evaluar el efecto de la aplicación de enmiendas orgánicas líquidas (localmente conocidas como "bioles"), obtenidas a partir de la fermentación de residuos agrícolas, sobre la productividad agrícola y la salud del suelo. Análogamente, se estudió el efecto del grado de madurez del estiércol equino y la gallinaza sobre el crecimiento del cultivo de lechuga y el riesgo de diseminación de genes de resistencia a antibióticos. A continuación, se estudió el efecto a largo plazo de la aplicación de lodos de depuradora urbana sobre la salud del suelo agrícola y la abundancia y el riesgo de diseminación de genes de resistencia a antibióticos. Por último, se llevó a cabo un ensayo en cámara de crecimiento controlado para el aislamiento y posterior inoculación de hongos micorrícicos como promotores del crecimiento del cultivo de lechuga.

1.3. Introducción

Potential benefits and risks for soil health derived from the use of organic amendments in agriculture

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1.3.1. Ecological intensification

In order to feed the constantly growing human population, it was estimated that food production will have to be doubled within the next few decades (Foley *et al*., 2011). The Green Revolution, which introduced new crop varieties and livestock breeds along with the extensive use of irrigation, machinery, and synthetic agrochemicals (fertilizers, pesticides), led to sharp increases in food production from agricultural systems since the beginning of the 1960s. This global increase in food production was underpinned by intensification rather than spread of agricultural land (Pretty and Barucha, 2014). Seeking

for enhanced crop productivity, agricultural intensification was sustained by indiscriminate inputs of synthetic agrochemicals, an overuse of water, and the alteration of the soil ecosystem, at great expense to the environment. Indeed, replacing soil internal processes with external inputs resulted in the progressive deterioration of the fundamental properties of those soils, including the potential for self-regulation (Bender *et al*., 2016).

Soil is a multi-functional, extremely complex, and highly dynamic three-dimensional system in which solid, liquid, and gaseous components interact in multiple physical, chemical, and biological processes. On the other hand, soil is a non-renewable resource at the human scale (Hakeem *et al*., 2014). Healthy soils support a multitude of functions (Blum, 2005) and the delivery of key ecosystem services. Soil health/soil quality is recurrently defined as the capacity of a given soil to perform its functions. Although both terms are often used interchangeably, *soil quality* is normally associated with a soil's fitness for a specific use, whereas *soil health* is frequently used in a broader sense to indicate "the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality, and maintain plant and animal health" (Doran and Zeiss, 2000). The recovery and conservation of soil health is, thus, of utmost importance for the preservation of life on earth, justifying the concerns of the European Commission in developing a soil legislation framework (CEC, 2006), which was unfortunately withdrawn in 2014.

Developing strategies and tools to promote agricultural sustainability whilst maximizing crop yields will be a major challenge for the next decades, in an attempt to meet the abovementioned goal of food production while protecting the integrity of our environment. In this context, *ecological intensification* was advocated as a suitable approach to integrate ecological processes into agricultural practices, in order to simultaneously enhance the delivery of ecosystem services and reduce, or even replace, the external anthropogenic inputs (Bommarco *et al*., 2013). This innovative approach does not display a consolidated set of guidelines, but rather a suite of alternative models for sustainable intensification, based on a greater reliance on ecological processes and ecosystem services, so as to minimize external anthropogenic inputs without adversely affecting crop productivity. Promising ecological intensification models combine technological advances in agricultural science, such as precision agriculture (Capmourteres *et al*., 2018), the use of sensors (Aranguren *et al*., 2019), and state-of-the-art technologies for agricultural waste reduction and reutilization, with sustainable practices and methodologies aimed at protecting the integrity of the soil ecosystem and,

specifically, its valuable biodiversity. Relying on minimum soil disturbance, a permanent soil organic cover, and crop diversification, *conservation agriculture* was shown to deliver a variety of essential ecosystem services, such as soil carbon (C) storage and sequestration, water regulation, soil erosion control, etc. (Palm *et al*., 2014). However, in certain cases and situations, conservation agriculture was shown to result in a reduction in crop productivity, as compared to conventional agriculture (Pittelkow *et al*., 2014). *Organic farming* relies on natural ecological processes to maintain the integrity of the soil ecosystem and, concomitantly, the provision of ecosystem services, and it is particularly focused on long-term agricultural productivity (Reganold *et al*., 2016). Furthermore, organic farming aims to exclude the use of synthetic fertilizers and claims for their systematic substitution by organic amendments, thus contributing to the valorization of organic waste (Misselbrook *et al*., 2012). Nevertheless, in many cases, organic amendments may harbor traditional and emerging pollutants and, therefore, cause toxicity problems (Kapanen and Itävaara, 2001; Asgharipour and Sirousmehr 2012; Pampuro *et al*., 2017), thus entailing a potential risk to human and ecosystem health.

The aim of this review article (focusing, but not exclusively, on research papers published in the last 10 years) is to highlight the potential benefits and drawbacks associated to the use of organic amendments as agricultural fertilizers, while addressing the existing strategies and technologies to mitigate the potential downsides.

1.3.2. Organic amendments

Our current production systems and transformation processes, designed to create useful goods and services, usually entail the continuous generation and disposal of massive amounts of waste. The required transition toward more ecological and sustainable production systems demands changing the current linear production model, where resources are converted into products and waste, to a circular model which, in a way, attempts to mimic the principles and functioning of natural ecological processes and cycles (Maina *et al*., 2017). The paradigm of circular economy is based on closed-loop models, in which waste and by-products are effectively integrated into the system as valuable assets, thereby reducing natural resource utilization and waste production (Murray *et al*., 2015). This transition was advocated by the European Commission in several documents, such as the *Roadmap to a Resource-Efficient Europe*, which appeals for sustainable production and an efficient use of resources (European Commission,

2011), and the *European Union (EU) Action Plan for the Circular Economy*, which establishes actions covering the whole lifecycle of products and encourages to "close the loop" through greater recycling and re-use (European Commission, 2015).

Within this circular economy paradigm, the reutilization of organic waste and by-products as soil amendments is gaining much interest, since it poses a realistic, cost-effective, and environmentally sound alternative to landfill disposal (the least preferred option for waste management) (Chojnacka *et al*., 2019). Organic amendments, such as composts, animal manures, slurries, crop residues, digestates from the anaerobic treatment of waste, biosolids, etc., are extensively applied to agricultural soil as fertilizers (Abbott *et al*., 2018; Celestina *et al*., 2019) or, alternatively, as amendments in soil remediation and reclamation initiatives (Larney and Angers, 2012; Epelde *et al*., 2014; Galende *et al*., 2014; Gómez-Sagasti *et al*., 2018). Biofertilizers, defined as "mixtures of selected beneficial microorganisms and/or other organic substances (plant growth hormones, vitamins, etc.) for sustainable soil management and plant productivity" (Soil Science Society of America, 2008), are broadly applied worldwide given their promising potential (Schütz *et al*., 2018). Recently, "biofertilization techniques" were included within the group of "organic residues most commonly used as soil amendments" (Hueso-González *et al*., 2018). Nonetheless, other authors (Malusà and Vassilev, 2014; Abbott *et al*., 2018) differentiated "microbial inoculants" from organic waste-derived "organic amendments" or "organic fertilizers". Undeniably, microbial inoculants are of an organic nature and may potentially exert beneficial effects on plant growth and health. However, it is not the purpose of this review article to debate whether or not microbial inoculants should be categorized as "organic amendments", nor to discuss their potential beneficial or adverse effects.

Given that organic amendments may be (i) originated from different sources (agriculture, urban, industry), (ii) subjected or not to treatments (composting, anaerobic digestion, etc.), and (iii) presented in different stages of matter (solid, liquid), it is not surprising that they can have a wide variety of different properties and agronomic potentialities. The most common organic amendments belong to the categories below.

1.3.2.1. Crop residues and green manures

Crop residues are defined as the "non-edible part of the plant that is left in the field after harvest" (Lal, 2005), while the term *green manure* refers to "specific forage or crop varieties that are incorporated into the soil while green or soon after maturing" (Goss *et*

al., 2013). These plant-based amendments are a valuable source of organic matter (OM) and are considered "the greatest source of soil organic matter (SOM)" for agricultural soils (Tisdale *et al*., 1985). Moreover, they can provide protection against soil erosion, suppress weeds (Kruidhof *et al*., 2011), improve soil physicochemical and biological properties, and enhance soil fertility (Turmel *et al*., 2015).

1.3.2.2. Animal manures

Composed of feces, urine, and animal bedding, animal manure was long used as soil organic amendment since it can enhance soil fertility through the supply of essential macro- and micronutrients, as well as OM (Edmeades, 2003; Goss *et al*., 2013). The application of animal manure can improve soil structure by reducing bulk density and increasing soil porosity, water infiltration/percolation rate, and aggregate stability (Edmeades, 2003; Thangarajan *et al*., 2013). Furthermore, manure-based amendments can stimulate soil microbial activity and biomass, as well as alter the composition and diversity of soil microbial communities (Liu *et al*., 2016; Reardon and Wuest, 2016).

1.3.2.3. Biosolids

Biosolids (also referred to as *sewage sludge*) are solid organic residues originated in wastewater treatment plants (Singh and Agrawal, 2008). Given the load of macro- and micronutrients that these organic amendments contain, their application to agricultural soil can be highly beneficial for soil fertility (Haynes *et al*., 2009). Indeed, the application of biosolids to soil was shown to enhance its physicochemical and biological properties (Latare *et al*., 2014; Lloret *et al*., 2016), and was proposed as a suitable practice for C sequestration in agricultural soil (Tian *et al*., 2015a).

1.3.2.4. Compost

The decomposition of OM under controlled aerobic conditions can lead to a stable, humus-like end product known as *compost* (St. Martin and Brathwaite, 2012). Compost can be produced from a wide array of organic materials, including agrarian (crop residues, animal manures) and municipal solid waste and sewage sludge. In fact, compost constitutes the most commonly used organic amendment for agricultural fertilization (Scotti *et al*., 2015). Composted amendments incorporate OM into agricultural soil, thereby improving soil porosity, aeration, water holding capacity, aggregate stability, and nutrient availability (Thangarajan *et al*., 2013), as well as stimulating soil microbial

activity and biomass (Das *et al*., 2017; Hernández *et al*., 2016). Composted amendments contain more recalcitrant organic fractions than the raw components themselves, leading to longer-term positive effects on soil health (Diacono and Montemurro, 2010).

1.3.2.5. Anaerobic digestion

Anaerobic digestion is a biological process via which organic waste is stabilized in the absence of oxygen, resulting in the formation of biogas and an organic by-product known as digestate (Tani *et al*., 2006; Tambone *et al*., 2009). A broad range of organic waste can be subjected to the process of anaerobic digestion, which entails the degradation and mineralization of the labile organic constituents, thereby increasing the stability of the resulting by-product (Tambone *et al*., 2009; Grigatti *et al*., 2011). Given their nutrient-rich composition, digestates, which may then be separated into a liquid and solid fraction, can be used as organic amendments and agricultural fertilizers (Nkoa, 2014). Moreover, the anaerobic digestion of organic waste was reported to effectively reduce the load of potential human pathogens and pollutants within the digested organic material (Li *et al*., 2011; Martín *et al*., 2015).

Regardless of the specific category of amendment, the beneficial or adverse effects that any given organic amendment exert on the (agricultural) soil ecosystem depend on many different factors, ranging from intrinsic characteristics of the amendment (composition, stability, maturity, etc.) to application times and rates, soil type and properties (both physicochemical and biological), cropping system, climatic conditions, etc. (Larney and Angers, 2012; Turmel *et al*., 2015). Hence, in order to properly assess the suitability of a given organic amendment for specific agricultural purposes, an exhaustive characterization of the amendment itself and the agricultural soil and crop needs should be carried out prior to its application.

In any event, it is important to emphasize that, in many rural areas of the world (*e.g.*, regions in southeast Asia, sub-Saharan Africa, etc.), there is a dearth of adequate sources of OM for application to agricultural soil (Cook *et al*., 2016). As an example, agricultural soils in Bangladesh and Nepal are in great need of larger amounts of OM to maintain and improve their fertility, which was for decades driven by chemical fertilizers; however, regrettably, the organic fertilizer subsector in these countries is still at a very early stage of development (Cook *et al*., 2016). Similarly, in sub-Saharan Africa, where agricultural soils often present a high level of degradation and poor fertility, organic inputs are customarily in short supply in smallholder farming systems due to limited affordability

and/or accessibility (Vanlauwe *et al*., 2015). One of the principles of integrated soil fertility management (ISFM)—the combined application of fertilizer and organic resources—can contribute to minimize this limitation and, in general, to the sustainable intensification needed in sub-Saharan Africa to address rural poverty and natural resource degradation (Vanlauwe *et al*., 2015). ISFM is defined as "a set of soil fertility management practices that necessarily include the use of fertilizer, organic inputs, and improved germplasm combined with the knowledge on how to adapt these practices to local conditions, aiming at maximizing agronomic use efficiency of the applied nutrients and improving crop productivity" (Vanlauwe *et al*., 2010). Within the ISFM framework, emphasis is placed on the suitable combination of agronomic practices with mineral and organic inputs and other amendments that are tailored for specific cropping systems and socioeconomic profiles (Vanlauwe *et al*., 2015). Finally, in impoverished rural areas of developing countries, it is imperative to urgently emphasize the critical importance of paying much more attention to SOM, agroecological practices, and the value chains that can provide organic fertilizer in large enough quantities.

Finally, when applying organic amendments to increase the content of SOM, it must always be remembered that SOM is a complex, dynamic, and highly variable soil constituent. In any case, the persistence of OM in soil clearly indicates the existence of protective mechanisms that slow or prevent their decomposition by soil microorganisms. Thus, some OM inputs are accessed easily by soil microorganisms, and mineralized within minutes, hours, or days. In contrast, OM that becomes protected from microbial activity can remain in soils for years, decades, centuries, or even millennia. There are three main mechanisms that stabilize soil organic C (SOC): (i) *physical protection* via aggregation, which decreases the accessibility of organics to microorganisms and enzymes; (ii) *chemical protection*, through the formation of organo-mineral complexes; and (iii) *biochemical protection*, through the chemical recalcitrance of organic molecules (Stewart *et al*., 2008). From these mechanisms, four soil C pools are often considered during the quantification of the soil C sequestration capacity: unprotected (free particulate OM), physically protected (microaggregate-associated C), chemically protected (silt- and clay-associated C), and biochemically protected (nonhydrolyzable C) (Six *et al*., 2002; Stewart *et al*., 2008). This latter pool, the biochemically protected SOC pool, is also known as the passive or recalcitrant pool in SOM models. These SOM stabilization mechanisms are of great relevance in studies on soil C-saturation (Six *et al*., 2002).

1.3.3. Beneficial effects of organic amendments

Aiming to increase crop productivity while protecting agroecosystem health, organic farming was shown, through the proper application of organic amendments, to enhance soil health, as compared to conventional farming which relies on the extensive use of synthetic fertilizers and pesticides (Edmeades, 2003; Bengtsson *et al*., 2005; Chaudhry *et al*., 2012; Gomiero *et al*., 2015; Reganold and Wachter, 2016).

The beneficial or adverse effects of any given agricultural practice on soil health are usually evaluated and monitored through a wide array of indicators, which include physical (*e.g.*, structure, bulk density, porosity, aggregate stability, water holding capacity), chemical (*e.g.*, contents of plant macro- and micronutrients, OM, pH, cation exchange capacity), and biological (*e.g.*, enzyme activities, respiration, potentially mineralizable nitrogen, microbial biomass C and nitrogen, microbial functional and structural diversity, diversity of macro- and mesofauna) soil properties. The latter group (biological properties) and, in particular, soil microbial indicators gained much attention lately, owing to their sensitivity, fast response, integrative characteristic, and ecological relevance (Mijangos *et al*., 2006; Epelde *et al*., 2010; Pardo *et al*., 2014; Garaiyurrebaso *et al*., 2017). Indeed, soil microorganisms, which comprise a major fraction of the soil total living biomass, play a key role in soil functioning and the delivery of crucial ecosystem services (Garbisu *et al*., 2011; de Vries *et al*., 2013; Burges *et al*., 2015). In this sense, belowground soil biodiversity was recognized as the main driver of many critical soil processes (Balvanera *et al*., 2006; Díaz *et al*., 2006; Harrison *et al*., 2014; Bender *et al*., 2016), as well as being responsible, to a great extent, for the stability (resistance and resilience) of the soil ecosystem (Isbell *et al*., 2015). The soil ecosystem is known to harbor an overwhelmingly high biodiversity and is then characterized by a high level of functional redundancy. Accordingly, it was suggested (Strickland *et al*., 2009) that shifts in microbial community composition might not entail relevant changes in soil ecosystem functioning. This assumption can certainly be true for some natural soils, but may nonetheless fall short at low levels of soil biodiversity, as is the case for many agricultural soils (Tsiafouli *et al*., 2015), where increasing species diversity and a higher number of functional groups were reported to improve soil ecosystem functioning (Nielsen *et al*., 2011). Assessing belowground soil biodiversity is, thus, imperative when measuring soil ecosystem functionality and, concomitantly, soil health. Unfortunately, the assessment of structural and functional biodiversity in such a complex ecosystem can

be an extremely difficult and daunting task (O'Donnell *et al*., 2001; Hartmann *et al*., 2015). Aiming to facilitate this somewhat overwhelming task, the use of molecular and, particularly, "omics" methods and techniques is currently being increasingly promoted. Nevertheless, many of these molecular biology tools still have many technical limitations and constraints, which points out the need to be very cautious when drawing conclusions about the responses of soil microbial communities to the application of agricultural practices, including, of course, the application of organic amendments (Hartmann *et al*., 2015; Pershina *et al*., 2015).

One of the main benefits of the application of exogenous OM to agricultural soil is the restoration and maintenance of the SOM content, which greatly contributes to long-term soil fertility and functioning (Lugato *et al*., 2014; Lal, 2016). SOM is, possibly, the most important soil property, as it sustains the physical, chemical, and biological dimensions of soil fertility and health (Hijbeek *et al*., 2017). Moreover, given that SOM simultaneously contributes to both soil fertility and soil C sequestration (and, hence, climate change mitigation), its enhancement was strongly promoted in international food security and climate forums (UNFCCC, 2015).

Regarding the potential benefits of organic amendments for the biological properties of agricultural soils, it is a well-known fact that organic amendments may directly stimulate microbial growth by providing energy and essential nutrients, or indirectly by promoting plant growth and, consequently, the amount of root exudates in the rhizosphere (Bais *et al*., 2006; Abbott *et al*., 2018). Apart from increasing microbial growth and biomass, the presence of diverse substrates susceptible to enzymatic hydrolysis within the amendments themselves leads to the stimulation of soil microbial activities (Singh *et al*., 2011). A higher availability of nutrients and growth substrates may also affect soil microbial diversity and composition, by increasing the number of ecological niches and promoting a variety of ecological interactions such as competition and/or antagonism between organisms (Tian *et al*., 2017; Gómez-Sagasti *et al*., 2018). Biodiversity shifts may then lead to functional changes related, for instance, to plant growth promotion and disease suppression (Larkin, 2015; Das *et al*., 2017). Moreover, increasing structural and functional soil diversity may strengthen the stability of the soil ecosystem, promoting its resistance and resilience against natural and anthropogenic stresses and disturbances (Figure 1.2; not included in the published manuscript) (Kumar *et al*., 2014; Larney *et al*., 2016). These beneficial effects of organic amendments on the biomass, activity, and diversity of soil organisms, in turn, exert a long-term beneficial impact on soil health

(Fließbach *et al*., 2007) and also contribute to the provision of key ecosystem services (C and nutrient cycling, disease suppression, etc.). Yet, it is important to highlight that microbial responses to the application of organic amendments vary greatly depending on the nature and lability of the OM present in the amendments themselves (Dijkstra *et al*., 2009).

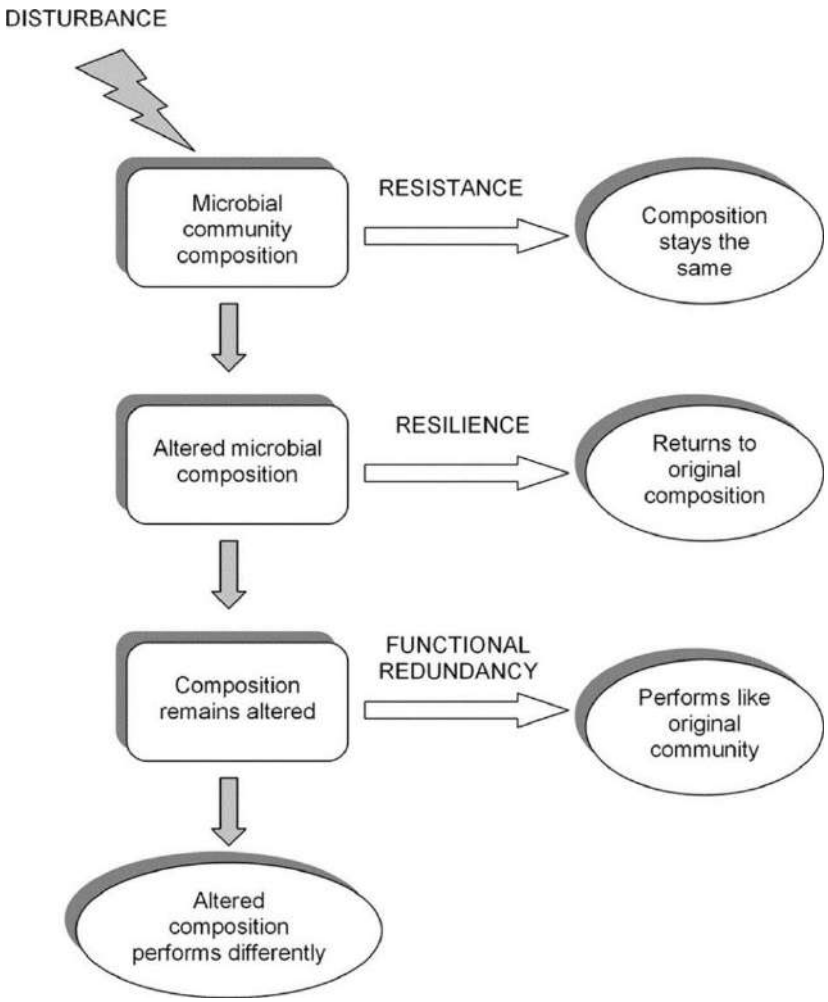


Figure 1.2. Schematic of how disturbance can change microbial composition and thereby affect ecosystem processes versus when disturbance would not have this effect due to ecosystem stability (when the microbial community is resistant, resilient, or functionally redundant). *Source: Allison and Martiny, 2008.*

Positive effects on soil biological properties following the application of organic amendments were substantially evidenced in many studies. A great deal of investigations on the use of organic amendments (Marschner *et al*., 2003; Antolín *et al*., 2005; Kizilkaya and Bayrakli, 2005; Carbonell *et al*., 2009; Dinesh *et al*., 2010; Moeskops *et al*., 2010; Roig *et al*., 2012; Xue and Huang, 2013; Mattana *et al*., 2014; Insam *et al*., 2015; Pershina *et al*., 2015; Hernández *et al*., 2016; Reardon *et al*., 2016; Das *et al*., 2017; Siebielec *et*

al., 2018) reported increases in soil microbial activity and biomass, as well as changes in microbial community composition (with potential concomitant effects on soil functioning) and, to a lesser extent, in microbial diversity. In this sense, a 10-year field experiment was conducted (Ji *et al*., 2018) to study the effects of replacing a mineral nitrogen fertilizer by an organic amendment (fermented pig manure), at different substitution ratios (0, 25, 50, 75, and 100%), on agricultural soil properties. Interestingly, the authors (Ji *et al*., 2018) reported an increase in soil bacterial diversity at increasing ratios of chemical fertilizer substitution. In another study (Ge *et al*., 2008), the long-term effects of organic versus conventional fertilization on soil microbial communities were investigated, finding out that the former modulated microbial community composition while increasing microbial richness and diversity. Similarly, other authors (Liao *et al*., 2018) reported that organic farming promotes soil microbial diversity and the abundance of beneficial soil microorganisms, with concomitant beneficial effects on the stability of the soil ecosystem. An improvement in soil microbial structural and functional diversity, as well as an increase in bacterial richness and evenness, was reported (Aparna *et al*., 2014) after the application of organic amendments to agricultural soil. These authors (Aparna *et al*., 2014) concluded that organic manures can "engineer" the soil ecosystem by selectively modifying the environment, thus enhancing ecosystem sustainability. The beneficial impact of increasing microbial diversity and activity, through the continuous application of organic amendments, on the restoration of saline soils was recently highlighted (Shi *et al*., 2019). The effect of 10 and 20 years of continuous organic farming versus conventional farming practices on agricultural soil health was studied (Bonanomi *et al*., 2016), reporting a stimulation of soil ecosystem functioning under organic management which was driven by the alteration of the soil microbial composition rather than by changes in species richness. A lack of long-term impact of organic amendments on soil microbial alpha-diversity, in the presence of significant shifts in soil microbial community structure, was observed by other authors (Pershina *et al*., 2015; Daquiado *et al*., 2016).

Notably, changes in soil microbial structural and functional diversity were reported after the incorporation of a wide variety of organic amendments, including crop residues (Chen *et al*., 2017; Wang *et al*., 2017), manures (Zhong *et al*., 2009; Shi *et al*., 2019), biosolids (Mattana *et al*., 2014; Mossa *et al*., 2017), composted waste (Orr *et al*., 2015a; Daquiado *et al*., 2016), and digestates (Sapp *et al*., 2015; Ji *et al*., 2018). These changes were identified through the utilization of different techniques: community-level

physiological profiling (Zhong *et al*., 2009; Chaudhry *et al*., 2012; Cesarano *et al*., 2017), phospholipid fatty-acid analysis (Zhong *et al*., 2009; Moeskops *et al*., 2010; Tian *et al*., 2017), Sanger sequencing (Aparna *et al*., 2014), and next-generation sequencing (Chaudhry *et al*., 2012; Li *et al*., 2012; Orr *et al*., 2015a; Bonanomi *et al*., 2016).

In addition to improving soil biological properties, organic amendments are also known to positively influence soil chemical properties. In fact, the abovementioned positive effects of organic amendments on soil microbial communities are often linked to changes in soil chemical characteristics driven by the application of amendments (Li *et al*., 2012; Lloret *et al*., 2016; Liao *et al*., 2018). Several authors (Lauber *et al*., 2009; Rousk *et al*., 2010) evidenced the key role of soil pH for both microbial community structure and function. Indeed, as described above, organic amendments can have a direct effect on soil fertility by supplying a wide variety of macro- and micronutrients, which support plant and microbial growth (Edmeades, 2003). In addition, organic amendments may affect soil pH and alter cation exchange capacity, thus indirectly influencing nutrient availability, microbial activity, and, hence, soil fertility (Abbot *et al*., 2018). Variations in the composition and maturity of the organic amendments may alter their impact on soil pH. Some amendments contain high quantities of calcium and/or magnesium, which may then cause a kind of "liming effect" on acidic soils, increasing their pH (Whalen *et al*., 2000). Liming was shown to significantly increase soil microbial activity (as reflected by the values of soil dehydrogenase activity) in acid soils (Mijangos *et al*., 2010). On the other hand, the application of organic amendments can also result in a decrease in soil pH, owing to the release of humic acids derived from the degradation of the organic C pool provided by the amendment (Singh *et al*., 2011), and/or due to the nitrification of the ammonium present in the amendment (Antolín *et al*., 2005). The addition of organic amendments may enhance the soil cation exchange capacity, mainly through the increase of the soil C pool, leading to enhanced nutrient availability and reduced nutrient leaching (Quilty and Cattle, 2011). Nutrient availability can be affected by the biochemical composition of the amendment. In particular, its carbon-to-nitrogen (C/N) ratio can limit soil microbial growth and activity and, thus, influence the rate of OM decomposition and the patterns of nutrient release (Manzoni *et al*., 2008).

Another beneficial aspect of organic amendments is their ability to immobilize heavy metals through the formation of chemically stable metallo-humic complexes and aggregates (Clemente and Bernal, 2006), or by increasing soil pH (metal bioavailability in soil is commonly reduced at higher pH values) (Soler-Rovira *et al*., 2010). This

beneficial effect was evidenced in many studies (Antolín *et al*., 2005; Singh and Agrawal, 2008; Mohapatra *et al*., 2016). Also, organic amendments were shown to stimulate the degradation and/or mineralization of organic pollutants by providing nutrients and energy to soil degrading microbial populations (Bastida *et al*., 2016).

The soil physical characteristics can also be positively influenced by the application of organic amendments. In this sense, the incorporation of exogenous OM to soil was shown to improve soil structure (better porosity and aggregate stability) (Leroy *et al*., 2008; Liu *et al*., 2014) and water retention capacity, with concomitant positive effects for soil functioning and crop productivity (Young and Ritz, 2000). Likewise, the stimulation of soil microbial communities through the application of organic amendments may indirectly improve soil structure, since microbial activity (through, for instance, the secretion of exopolysaccharides) and, particularly, hyphal growth can markedly influence soil aggregation and aggregate stability (Rillig and Mummey, 2006; Six and Paustian, 2014). On the other hand, an increase in soil porosity often reduces soil crusting and bulk density, which could restrict the movement of water and air through the soil matrix (Zebarth *et al*., 1999; Zhao *et al*., 2009). In turn, this facilitates the development of the rooting matrix and improves the quality of the habitable space for soil biological communities. Furthermore, organic amendments can affect particle size distribution and the total surface area within the soil, increasing the number and types of available niches for biological colonization.

1.3.4. Adverse effects of organic amendments

1.3.4.1. Traditional risks

In spite of all the aforementioned benefits, the application of organic amendments to agricultural soil may also exert some detrimental effects on soil ecosystem health. For instance, organic amendments can harbor potentially harmful constituents such as human pathogens, heavy metal(loid)s, organic pollutants, emerging contaminants (antibiotic-resistance genes, endocrine disruptors, microplastics), etc. (Park *et al*., 2011; Mattana *et al*., 2014; Petrie *et al*., 2014; Mohapatra *et al*., 2016). Moreover, the inappropriate application and/or overuse of organic amendments may result in other undesired environmental risks, including an excess of nutrients (eutrophication), immobilization of essential nutrients, contamination of underground water, emission of greenhouse gases, and soil acidification or salinization (Larney and Angers, 2012; Thangarajan *et al*., 2013;

Alvarenga *et al*., 2015). Altogether, these adverse side-effects threaten the safe usage of organic amendments for agricultural purposes and pose a potential risk to environmental and human health (Goss *et al*., 2013).

Aiming to prevent these potential drawbacks, several legislative tools arose in Europe, including, among others, the Waste Directive (EU) 2018/851, the Directive on the Landfill of Waste (1999/31/EC), the Animal Waste Directive (90/667/EEC), and the Sewage Sludge Directive (86/278/EEC) (Gómez-Sagasti *et al*., 2018). These regulations provide guidelines on waste disposal and, interestingly, set threshold values for the contaminants present in organic waste. Nevertheless, there still exist concerns about the quality of these legislations, specifically regarding the lack of data and regulation for most emerging contaminants. On the other hand, it is widely accepted that bioavailable contaminant concentrations are more significant for environmental risk assessment than total contaminant concentrations. The potential negative effects exerted by, for instance, toxic heavy metals on soil health are known to depend upon their bioavailable concentrations (Kumpiene *et al*., 2009), which, in many cases, are not correlated with total concentration values (Burges *et al*., 2015). In spite of this well-known fact, in most countries, the existing legislation on soil contamination still relies on the values of total contaminant concentration.

Owing to their lack of biodegradability, heavy metals have an extremely long persistence in the soil environment (Zhou *et al*., 2017). Therefore, the regular application of organic amendments may lead to metal accumulation in soil, with concomitant risks of metal bioaccumulation and biomagnification along the trophic chain (Mann *et al*., 2011). As previously addressed, the application of organic amendments can enhance the formation of soil aggregates and metallo-humic complexes, which can then reduce the bioavailability of heavy metals. In contrast, the decomposition and mineralization of OM may increase metal bioavailability due to the disintegration of soil aggregates and the formation of soluble organic metal carriers (McBride, 1995; Parat *et al*., 2007). In this regard, a disruption of nutrient cycling processes derived from the metal toxicity caused by the repeated application of biosolids was reported (Singh *et al*., 2011). Reductions in soil microbial biomass were also observed by several authors (Fließbach *et al*., 1994; Fernández *et al*., 2009), following the application of organic amendments.

In addition to inorganic contaminants, organic amendments can incorporate organic pollutants into the soil ecosystem which, in some cases, may also show a high level of persistence and recalcitrance (Semblante *et al*., 2015; Mohapatra *et al*., 2016; Fijalkowski

et al., 2017). Moreover, the breakdown products and secondary metabolites produced during the degradation of these organic pollutants may happen to be even more toxic and persistent than the parent compounds themselves (Semblante *et al*., 2015). Furthermore, little is known regarding the breakdown rates of many of these organic pollutants and their transformation products in the soil ecosystem, as well as concerning their potential toxic effects on the soil biota. Therefore, there is an urgent need to determine the potential ecotoxicity of those organic pollutants present in organic amendments, in order to ensure the long-term sustainability and safety of this agronomic practice (Clarke and Smith, 2011).

Some organic amendments, particularly those derived from raw, unstable animal by-products or biosolids, can contain potentially pathogenic organisms (Chen *et al*., 2016; García *et al*., 2017), including enteric bacteria, parasites, viruses, and fungi (Fijalkowski *et al*., 2017). In this regard, it was suggested that *Bacillus anthracis* and *Bordetella pertussis* may be dominant human pathogens in animal manure (Fang *et al*., 2015), and *Escherichia coli* and *Klebsiella pneumoniae* in biosolids (Ye and Zhang, 2011). The possibility of pathogen incorporation to agricultural soil through the application of organic amendments is a risk that must be thoroughly prevented given its serious implications for human health. An exhaustive biological characterization of the organic amendments is, thus, imperative in order to minimize, or better avoid, this potential biohazard. As an example, Bibby and Peccia (2013) investigated the viral pathogen load of different biosolids, identifying >40 different human viruses.

An excessive and inappropriate application of organic amendments may also result in an excess of nutrients (*e.g.*, phosphorus, nitrogen), which can eventually cause negative environmental consequences such as contamination of watercourses and eutrophication (Aronsson *et al*., 2007; Stenberg *et al*., 2012; Alvarenga *et al*., 2015). On the other hand, organic amendments with a high C/N ratio can entail the immobilization of mineral nitrogen within the soil microbial biomass, since microorganisms are generally more effective than plants at competing for nutrients (Hodge *et al*., 2000). In addition, the application of organic amendments to soil may trigger the release of gases to the atmosphere, including ammonia and greenhouse gases, most relevantly methane and nitrous oxides (Alvarenga *et al*., 2015; Thangarajan *et al*., 2013; Aslam *et al*., 2014; Bass *et al*., 2016). The emission of these gases depends upon (i) the type of organic waste, (ii) the applied treatments (composting, anaerobic digestion), (iii) the timing, dose, and method of application, etc.

Finally, soil acidification and salinization may occur following the application of organic amendments to agricultural soil which can, in turn, affect soil structure, as well as nutrient availability, and, importantly, the mobility and bioavailability of pollutants, thus threatening agricultural productivity and ecosystem health. Some organic amendments can indeed increase the soil's electrical conductivity (higher salinity and sodicity), with concomitant detrimental effects for crop yield and soil biological activity (Bonanomi *et al*., 2014). Conversely, the use of acid organic amendments or the generation of humic acids (or the activity of some biological processes such as nitrification) may result in soil acidification, often resulting in increased solubility, mobility and bioavailability of soil contaminants (Antolín *et al*., 2005; Singh *et al*., 2011).

1.3.4.2. Emerging contaminants

Microplastics (<5 mm in size) arise from the weathering and fragmentation of plastics into smaller particles (Barnes *et al*., 2009). Microplastics are extremely or completely resistant to biodegradation, and may cause potential detrimental effects on soil ecosystem functioning and, in particular, on soil organisms via their ingestion and accumulation (Horton *et al*., 2017). Furthermore, microplastics can interact with soil contaminants, altering their ecotoxicity and mobility/bioavailability (many contaminants can become adsorbed onto microplastics) (Rochman *et al*., 2013; Wang *et al*., 2016). Domestic and industrial wastewaters can carry substantial loads of potentially harmful microplastics (Horton *et al*., 2017), which eventually end up in the corresponding wastewater treatment plant. Wastewater treatment plants are very effective at removing microplastics from the treated water (Sun *et al*., 2019), resulting in the accumulation of microplastics in the biosolids themselves (Li *et al*., 2018). The application of different rates of biosolids, as drivers of microplastic contamination, into agricultural soil was studied (Corradini *et al*., 2019), finding detectable levels of these potentially harmful emerging contaminants in the amended soils. Nevertheless, existing data on the impact of microplastics on the soil ecosystem are still very scarce (Ng *et al*., 2018).

On the other hand, in the last few decades, the amount of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in the environment increased substantially due to anthropogenic activities, resulting in their current identification as emerging environmental contaminants (Pruden *et al*., 2006). Indeed, the overuse and misuse of antibiotics for human and veterinary applications resulted in a proliferation of clinically relevant ARB and ARGs in the environment (Figure 1.3; not included in the already

published manuscript). Actually, antibiotic resistance is increasingly being recognized as one of the greatest threats for global health, as evidenced by the high-level policy initiatives that recently arose, *e.g.*, the *Transatlantic Taskforce on Antimicrobial Resistance*, the *Global Antibiotic Resistance Partnership*, the *Joint Programming Initiative on Antimicrobial Resistance* (Nahrgang *et al*., 2018), endorsed by the World Health Organization (WHO, 2015), and the *Political Declaration on AMR of the United Nations* (UN, 2016). In Europe, the European Commission published the *Action Plan Against the Rising Threats from Antimicrobial Resistance* (European Commission, 2011b), which contains 12 actions seeking to palliate the detrimental effects of antimicrobial resistance. This Action Plan was later updated by the publication of the *EU One Health Action Plan against Antimicrobial Resistance* (European Commission, 2017). Guidelines, actions, restrictions, and objectives are urgently needed, since it was estimated that antibiotic-resistant infections could cause 10 million deaths per year by 2050 (O'Neill, 2016).

Antibiotics are known to be poorly metabolized in the human and animal body. Hence, a considerable amount of these emerging contaminants are excreted unchanged or as active metabolites of the parent species (Kumar *et al*., 2005), resulting in the presence of a high amount of antibiotics in many wastewaters (Michael *et al*., 2013). Not surprisingly, both livestock manure and wastewater treatment plants are acknowledged as important reservoirs for ARB and ARGs (Rizzo *et al*., 2013; Zhu *et al*., 2013). In this sense, the long-term application of animal manure and biosolids to agricultural soil may lead to the introduction, proliferation, and dissemination of these emerging contaminants in the environment (Marti *et al*., 2013; Mao *et al*., 2015; Wang *et al*., 2015; Peng *et al*., 2017; Urra *et al*., 2019). It was reported that the repeated exposure of the soil environment to amendment-borne ARGs correlates with the emergence and proliferation of ARGs in indigenous soil bacteria (Udikovic-Kolic *et al*., 2014; Xie *et al*., 2018).

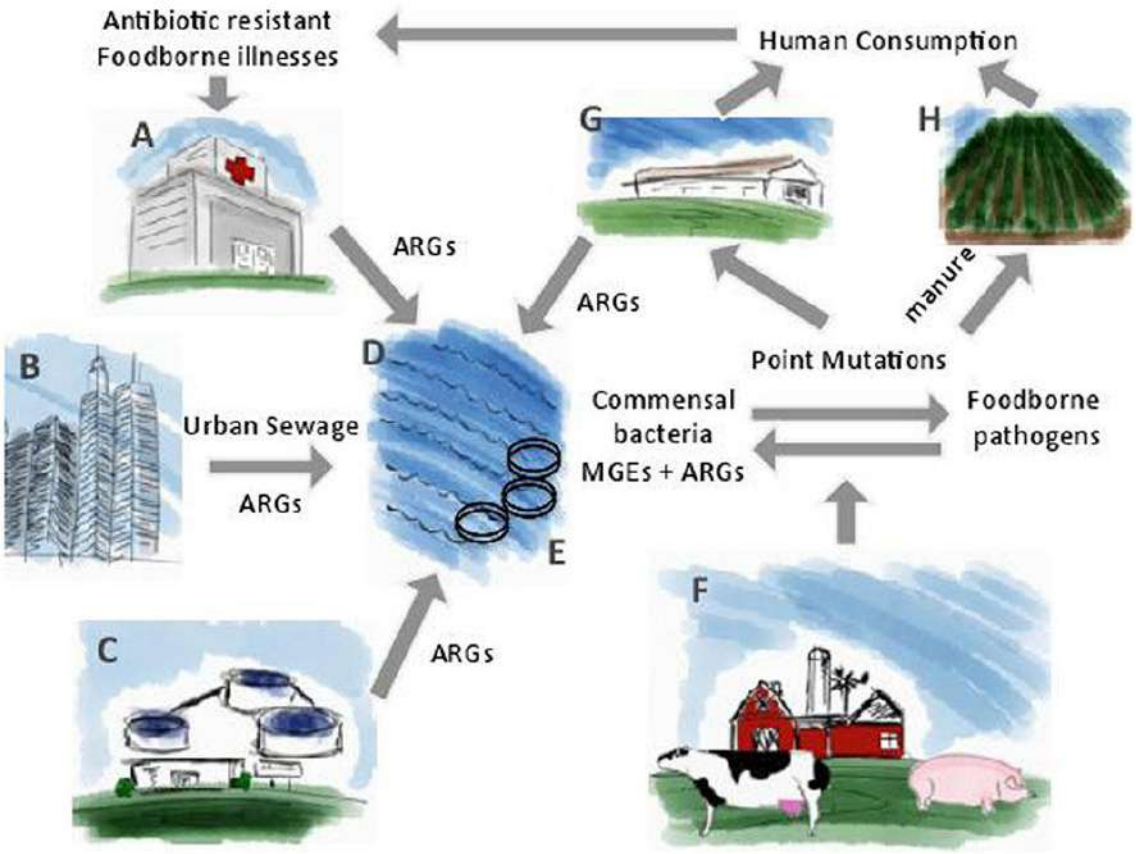


Figure 1.3. The cycle for the dissemination of antibiotic resistance genes from the environment to human consumption. (A) Hospital effluents; (B) urban sewage; (C) wastewater treatment plant effluents; (D) bodies of water such as lakes and oceans; (E) aquaculture; (F) food-producing animals; (G) slaughterhouse; (H) manure application. *Source: Colavecchio et al., 2017.*

The dissemination of ARGs among bacteria is mainly driven by horizontal gene transfer (HGT). Indeed, HGT is the main mechanism for genetic variation in prokaryotic organisms, allowing their adaptation to changing environmental conditions and disturbances. HGT facilitates the colonization of ecological niches (Norman *et al*., 2009; Vos *et al*., 2015) through the acquisition of genes via mobile genetic elements (MGEs), such as plasmids, integrons, and transposons. Although there are three main mechanisms of intercellular DNA movement (transformation, conjugation, transduction) (Frost *et al*., 2005), conjugative plasmid-mediated HGT is considered the most relevant mechanism for the dissemination of ARGs among bacteria (Garbisu *et al*., 2018). MGEs often carry integrons, which act as natural cloning systems and expression vectors of gene cassettes encoding functions of potential adaptive significance, *e.g.*, antibiotic resistance (Gillings, 2014). Relevantly, integrons have a key role in the dissemination of ARGs in manure- and biosolid-amended soils (Burch *et al*., 2014; Sandberg and LaPara, 2016).

In this regard, the rhizosphere was addressed as a major hotspot for HGT (Van Elsas and Bailey, 2002). Interestingly, the phyllosphere was also shown to be conducive to conjugative plasmid transfer (Björklöf *et al*., 1995). Consequently, crops harvested from manure- or biosolid-amended soils can potentially carry ARGs, representing a potential route of exposure to ARB for animals and humans (Pruden *et al*., 2006; Wang *et al*., 2015). The abundance and diversity of ARGs in organically versus conventionally produced lettuce was investigated by high-throughput quantitative PCR (Zhu *et al*., 2017), detecting 134 ARGs in the phyllosphere and leaf endophytes of lettuce samples, which were significantly enriched in the organically produced lettuces. The same research group conducted an analogous study (Wang *et al*., 2015) with lettuce and endive crops in manure-amended soils, obtaining similar results. Other authors (Marti *et al*., 2013) detected ARGs and MGEs in vegetables grown in both manured and inorganically fertilized soils. Some antibiotic determinants were exclusively detected in the manured soils. These authors (Marti *et al*., 2013) highlighted the importance of pretreating the raw organic waste and/or establishing offset times between amendment incorporation and crop harvest for safe consumption. Dolliver *et al*. (2007) found that corn, lettuce, and potato crops were able to accumulate sulfamethazine from manured soils, pointing out the concerns about the consumption of low levels of antibiotics from crops grown in manured soils.

Interestingly, antibiotic resistance is frequently associated with metal resistance (Baker-Austin *et al*., 2006), as the molecular mechanisms underpinning resistance to both antibiotics and heavy metals are often similar (Baker-Austin *et al*., 2006). This phenomenon is due to the evolutionary mechanism of *co-selection*, which drives the simultaneous resistance to different pollutants (*e.g.*, metals, antibiotics, biocides) through *co-resistance* (when different genes encoding for metal and antibiotic resistance are allocated in the same genetic determinant) or *cross-resistance* (when the same gene provides resistance to both antibiotics and metals) mechanisms (Chapman, 2003). Co-selection is a most relevant mechanism for the abovementioned risk of the appearance and dissemination of ARGs associated with the application of organic amendments to agricultural soil, since the presence of heavy metals in the amendments may enhance antibiotic resistance or select for ARB (Bondarczuk *et al*., 2016).

1.3.5. Overcoming the drawbacks

In addition to stabilizing nutrients and OM, which results in a longer-term availability of essential nutrients and a positive effect on soil microbial activity and biomass (Diacono and Montemurro, 2010), composting is a well-known mechanism for minimizing or eliminating many unwanted effects of the application of raw organic waste to agricultural soil (Larney and Angers, 2012). Composting, through the hygienization of organic waste, can significantly mitigate the risk of incorporation of potential human pathogens into the soil ecosystem, although it may not entirely prevent the regrowth of pathogenic strains (Masciandaro *et al*., 2013; Marin *et al*., 2014; García *et al*., 2017). Moreover, composting is acknowledged to be an effective measure to alleviate antimicrobial resistance during the application of organic amendments to agricultural soil (Gou *et al*., 2018; Qian *et al*., 2018). In this sense, total or partial degradation of antibiotic residues through composting processes was widely reported (Dolliver *et al*., 2008; Selvam *et al*., 2012; Wang *et al*., 2012). Moreover, as composting processes entail changes in the physicochemical characteristics of the organic waste, the bioavailability of the antimicrobial compounds may be reduced (Chessa *et al*., 2016). A reduction in the amount of antibiotics or their bioavailability may eventually lead to a decrease in the load of ARGs. The relatively high temperatures reached during many composting processes may also decrease the load of ARB and ARGs (Selvam *et al*., 2012; Qian *et al*., 2016; Qian *et al*., 2018).

The anaerobic digestion of organic waste was also proposed as an effective mechanism to reduce the negative consequences of the application of organic waste to agricultural soil. Indeed, the anaerobic digestion of organic waste was often reported to effectively reduce the levels of organic pollutants and potential human pathogens present in organic amendments (Martín *et al*., 2015; Ghattas *et al*., 2017). Furthermore, many authors (Arikan *et al*., 2006; Mohring *et al*., 2009; Munir *et al*., 2011) reported the potential of the anaerobic digestion for the removal of antibiotic residues and antibiotic determinants in organic waste. As with composting, this process entails the stabilization of the OM and may then influence the bioavailability of organic pollutants by promoting sorption processes (Li *et al*., 2013). Relevantly, physical adsorption was identified as a key mechanism for the removal of antibiotic residues from organic materials (Zheng *et al*., 2019). The anaerobic digestion of organic waste may be carried out under mesophilic or thermophilic conditions, the former being the most widely applied process (Ma *et al*., 2011). However, under thermophilic conditions, better results are obtained regarding the

removal of antibiotic-resistance determinants (Ghosh *et al*., 2009; Miller *et al*., 2016). However, the anaerobic digestion of organic waste was repeatedly reported to inefficiently remove ARGs (Zhang *et al*., 2015). A previous treatment, consisting of applying a thermal hydrolysis prior to the process of anaerobic digestion, was proposed to reduce more efficiently the load of ARB and ARGs (Ma *et al*., 2011), since the high pressure and thermal conditions yielded by this process promote cell lysis and, thus, the release of degradable components (Pei *et al*., 2016).

In any event, both composting and anaerobic digestion recurrently showed their potential for the removal of antibiotic-resistance determinants. Masse *et al*. (2014) concluded that composting was more effective than anaerobic digestion for reducing antibiotic residues from organic waste. Other authors (Chen *et al*., 2010) also found that composted manure contained up to seven orders of magnitude less antibiotic-resistance determinants than the one treated with other aerobic and anaerobic treatments. According to these results, composting appears the best option for reducing the reservoir of antibiotic resistance present in raw organic waste. Nonetheless, some authors showed inconsistent results regarding the positive effect of composting on the reduction of ARGs. For example, Peng *et al*. (2015) compared the abundance and diversity of tetracycline (*tet*) resistance genes in agricultural soils after six years of continuous fresh versus composted manure application. They found nine classes of *tet* genes, and two of them were significantly more abundant in soils amended with composted manure (no reduction in the total abundance of *tet* genes after manure composting was detected).

As described above, owing to the energy and nutrient content of organic waste, many scientists traditionally investigated possible treatment options for such waste, mainly through anaerobic digestion or composting. Currently, within the fields of waste treatment and waste valorization, the utilization of organic waste as substrates for producing insects, mainly as a protein source for the livestock sector or as a source of fats for biodiesel production, appears a most promising alternative (Salomone *et al*., 2017). Processing of (bio)waste with larvae, such as for instance fly larvae, is becoming a promising waste treatment technology. Nonetheless, compared to more conventional waste treatment technologies such as composting or anaerobic digestion, the process performance is variable and the mechanisms driving the decomposition of the organic waste are still poorly understood (Gold *et al*., 2018). The larvae grown on the (bio)waste can then be used for animal feed production, thus providing a protein source to help alleviate the rising global demand for animal feed (Mertenat *et al*., 2019) and,

interestingly, revenues for financially viable waste management systems (Gold *et al*., 2018). In particular, black soldier fly (*Hermetiaillucens* L.; Diptera: Stratiomyidae) biowaste processing is a treatment technology that received much attention over the last few decades (De Smet *et al*., 2018; Zurbrügg *et al*., 2018; Makkar *et al*., 2014). Interestingly, a recent study (Mertenat *et al*., 2019) concluded that black soldier fly biowaste treatment offers an environmentally relevant alternative, with very low direct emissions of greenhouse gases and potentially high reduction in global warming potential.

Finally, the possibility of using CRISPR/Cas (clustered regularly interspaced short palindromic repeats), a prokaryotic immune system which protects bacteria and archaea against phage attack and undesired plasmid replication (Marraffini and Sontheimer, 2010), is being investigated to selectively remove ARGs from bacterial populations. Some studies (Bikard *et al*., 2014; Yosef *et al*., 2015) indeed confirmed the potential of this methodology to remove ARGs and/or the plasmids that encode those genes. Nevertheless, this technology still exhibits several important drawbacks (Pursey *et al*., 2018): (i) finding an appropriate delivery vector, since phages or conjugative plasmids normally show narrow host ranges; (ii) unpredictability of the response of the microbial community following the introduction of a delivery vector, due to the inherent complexity of microbial communities; (iii) evolution of resistance to CRISPR/Cas through mutation of the target hosts and/or by exhibiting anti-CRISPR activity (selection for *arc* genes); and (iv) legislative and social barriers regarding the release of gene-editing systems to the environment, as well as a lack of unanimous acceptance by the scientific community.

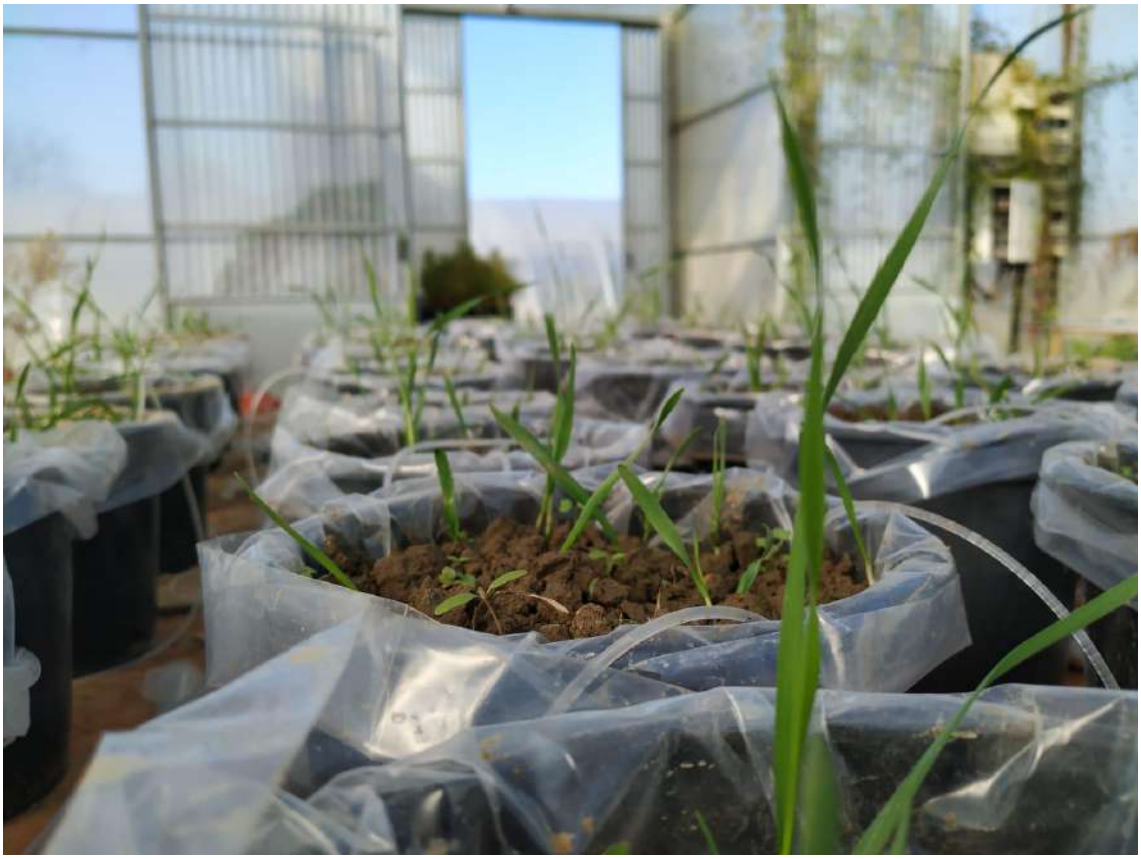
1.3.6. Conclusions

In the search for suitable strategies to optimize agricultural environmental sustainability while maximizing crop productivity and production, the paradigm of ecological intensification recently gained much interest, in an attempt to enhance the provision of ecosystem services through the consideration of natural ecological processes during the design and implementation of agricultural practices and management systems. In this regard, the application of organic waste and by-products as agricultural soil amendments is a common practice, given their potential to increase crop productivity while enhancing the health of the soil ecosystem. Moreover, the integration of organic waste into the value chain as valuable assets meets the current circular economy paradigm.

Organic amendments can be obtained from a wide range of organic materials and origins. Their potential positive effects on soil ecosystem functioning depend upon many factors including their composition, stability, maturity, frequency and rate of utilization, soil type, cropping system, climatic conditions, etc. Therefore, an exhaustive characterization of both the organic amendment and the agroecosystem itself must be performed prior to its application, in order to identify the potentialities and limitations of any given organic amendment for soil and crop health.

Composting and anaerobic digestion are acknowledged to be efficient for overcoming some of the potential adverse impacts that organic amendments may exert on the soil ecosystem and, in general, the environment. Pertaining to the potential adverse effects that organic amendments can exert on the soil ecosystem, some emerging contaminants, such as ARB, ARGs, and MGEs, are currently causing much concern as they pose a serious risk to environmental and human health. Given that biological emerging contaminants such as these (ARGs, MGEs, ARB) can persist in the environment and, worse, make copies of themselves and be transferred by HGT to other biological receptors, there is an urgent need to develop more effective treatments of organic waste, which must go beyond the typical hygienization and bacterial disinfection and effectively destroy DNA.

2| HIPÓTESIS Y OBJETIVOS



2. HIPÓTESIS Y OBJETIVOS

2.1. Hipótesis

La aplicación de enmiendas orgánicas al suelo agrícola es una alternativa sostenible y económicamente viable a la intensificación de la agricultura convencional basada en la aplicación de agroquímicos, al tiempo que promueve la reutilización y revalorización de residuos orgánicos. No obstante, estas enmiendas pueden albergar ciertos riesgos intrínsecos como la presencia de contaminantes tradicionales y emergentes. Los efectos potencialmente positivos y negativos de las enmiendas orgánicas sobre el ecosistema edáfico y su funcionalidad dependen de varios factores como el origen y el tratamiento al que hayan sido sometidas. Por otra parte, las propiedades microbianas edáficas aportan información crucial sobre la funcionalidad del suelo, por lo que tienen un notable valor como indicadores de la salud del ecosistema edáfico. Por consiguiente, el trabajo aquí presentado se sustenta en las siguientes hipótesis:

- *Las propiedades microbianas del suelo son herramientas de gran utilidad de cara a (i) evaluar el impacto de la aplicación de enmiendas orgánicas sobre la salud del suelo e (ii) identificar los riesgos potenciales asociados a su utilización*.
- *Las enmiendas orgánicas ejercen un efecto positivo sobre las comunidades microbianas edáficas y, por consiguiente, sobre la fertilidad y funcionalidad del ecosistema suelo.*

2.2. Objetivo General

El objetivo general de este trabajo fue evaluar el efecto de la aplicación de distintas enmiendas orgánicas sobre la salud del suelo agrícola, estudiando su potencial agronómico y haciendo hincapié en el estudio de la microbiota edáfica como potencial bioindicador de la funcionalidad del ecosistema edáfico.

2.3. Objetivos Específicos

- Estudio de los efectos a medio plazo (6 años) de la incorporación del rastrojo del maíz sobre la reserva de carbono orgánico en el suelo, la producción del cultivo y la salud

del suelo (Capítulo 4). Este objetivo pretende evaluar la idoneidad de una práctica habitual en agricultura de conservación, léase, la incorporación del rastrojo al suelo.

- Estudio del potencial agronómico de enmiendas orgánicas líquidas generadas a partir de la fermentación de residuos vegetales sobre los cultivos de lechuga y maíz. Evaluación del efecto de estas enmiendas sobre las propiedades físico-químicas y microbianas del suelo agrícola (Capítulo 5). Este objetivo pretende evaluar la idoneidad de una práctica creciente en agricultura sostenible, léase, el empleo de enmiendas orgánicas líquidas producidas, en su mayor parte, por los propios agricultores.

- Evaluación del impacto de la aplicación de estiércol equino y gallinaza, en distintos grados de madurez (fresco, compostado y bokashi), sobre la producción y calidad nutricional del cultivo de lechuga, así como sobre la salud del suelo agrícola y el riesgo de diseminación de genes de resistencia a antibióticos en los agroecosistemas (Capítulo 6). Este objetivo pretende evaluar la idoneidad de una práctica habitual en agricultura orgánica, léase, el uso de estiércol en distintos grados de madurez.

- Evaluación del efecto a largo plazo (24 años) de la aplicación de lodos de depuradora urbana digeridos anaeróbicamente sobre la salud del suelo agrícola, con especial énfasis en su impacto sobre la diversidad de las comunidades microbianas edáficas y el riesgo de diseminación de genes de resistencia a antibióticos en los agroecosistemas (Capítulo 7). Este objetivo pretende evaluar la idoneidad de una práctica habitual en agricultura, léase, el uso de lodos de depuradora urbana como enmiendas orgánicas agrícolas.

- Estudio del potencial agronómico de la inoculación de hongos micorrícicos sobre la producción y calidad nutricional del cultivo de lechuga y su impacto sobre las comunidades de hongos del suelo (Capítulo 8). Este objetivo pretende evaluar la idoneidad de una práctica habitual en agricultura, léase, el empleo de hongos micorrícicos para promover el crecimiento vegetal.

3| PROCEDIMIENTOS GENERALES

3. PROCEDIMIENTOS GENERALES

NOTA INTRODUCTORIA: este capítulo expone las características de los distintos ensayos que conforman el presente trabajo a través de la descripción de los emplazamientos, el diseño y las condiciones experimentales, los muestreos y, finalmente, las herramientas de análisis utilizadas para la consecución de los objetivos planteados. En cualquier caso, los materiales y métodos particulares de cada uno de los ensayos se describen con mayor detalle en los capítulos correspondientes. Asimismo, las referencias bibliográficas de los procedimientos aquí descritos se mencionan en los artículos correspondientes. Algunos de los ensayos aquí descritos se realizaron en campo, mientras que otros se llevaron a cabo en una cámara de crecimiento controlado ubicada en las instalaciones de NEIKER en Derio (Bizkaia).

3.1. Incorporación del rastrojo de maíz

Este ensayo, cuyos resultados se discuten en el Capítulo 4, se realizó en colaboración con la Universidad de Lleida. El emplazamiento se encuentra ubicado en el municipio de Almacelles (Lleida, 41°43'56.8"N 0°30'01.7"E) a 324 metros de altitud sobre el nivel del mar (Figura 3.1). Se trata de una zona tradicionalmente agrícola, donde el cultivo mayoritario es el maíz, que se caracteriza por un clima semiárido con bajas precipitaciones (192 mm) y altas temperaturas (19,2ºC) durante la temporada de crecimiento del cultivo. El suelo de estudio es un calcixerept típico, con pH básico y textura limosa. El diseño experimental se basa en dos tratamientos establecidos 6 años atrás que se mantuvieron invariables: (i) la incorporación al suelo del rastrojo del maíz post-cosecha, y (ii) la retirada de dicho rastrojo. Ambos tratamientos se estudiaron por triplicado en parcelas de 18 x 17 m distribuidas de forma aleatoria.

Figura 3.1. Ubicación del ensayo experimental. *Fuentes: Google Earth; NEIKER.*

De forma anual, el maíz (ciclos FAO 600-700) se sembró a principios de primavera con una densidad de siembra de 80.000 plantas por hectárea, dejando una distancia de 71 cm entre filas. Antes de la siembra, se añadieron 150 kg de óxido de fósforo (P_2O_5) y 250 kg de óxido de potasio (K_2O) por hectárea. Por su parte, el fertilizante nitrogenado (300 kg ha^{-1}) se aplicó, en forma de nitrato amónico (NH_4NO_3) en cobertera, en dos fases diferenciadas de la fenología del cultivo: una mitad se aplicó en V3-V4 (desarrollo de la tercera-cuarta hoja) y la otra en V5-V6 (desarrollo de la quinta-sexta hoja). El riego se realizó por aspersión. En toda la superficie cultivada, el control de vegetación arvense se realizó mediante el uso de herbicidas de pre- (Trophy®, 1 L ha^{-1}) y post-siembra (Fluoxypyr 20% y Nicosulfuron, 1,5 L ha^{-1}). El maíz se cosechó a finales de verano y la biomasa aérea se calculó a partir de la retirada de 4 metros de la línea central de cada parcela. Tras la cosecha, la biomasa vegetal que quedaba en la parcela, o rastrojo, se incorporó mediante arado en los tratamientos de incorporación, mientras que en las

parcelas de retirada el rastrojo se recogió de forma mecánica. El muestreo del suelo se realizó mediante la utilización de una sonda manual de 30 cm de profundidad, obteniendo aleatoriamente 6 cilindros de suelo por parcela para la obtención de una muestra representativa de cada parcela. Dado que el procesamiento de las muestras de suelo fue idéntico en todos los ensayos, su descripción se realiza en el apartado 3.6 del presente capítulo.

3.2. Enmiendas líquidas fermentadas

Este estudio se realizó junto con la Fundación HAZI y un grupo de agricultores de la montaña alavesa en un intento de valorizar el potencial agronómico de una enmienda de uso común entre los mismos elaborada a través de la fermentación de residuos agrícolas, cuyos resultados se describen en el Capítulo 5 de este trabajo. Para ello, durante un periodo de dos años, se realizaron distintos ensayos con un cultivo hortícola, la lechuga (3.2.1), y un cereal, el maíz (3.2.2). En el caso de la lechuga, los efectos de las enmiendas sobre el ecosistema suelo se evaluaron a través de un ensayo en microcosmos realizado en una cámara de crecimiento controlado que posteriormente se repetiría en campo. En ambos casos, la duración de los ensayos fue de un solo ciclo de crecimiento del cultivo. Por otro lado, el ensayo con maíz se llevó a cabo exclusivamente en campo, con una duración de dos campañas. En todos los casos se testaron dos enmiendas líquidas diferenciadas en base a su origen: (i) una enmienda comercial, adquirida a través de una empresa local (VITAVERIS SC, Navarra) que además de comercializar el producto, dedica parte de su actividad a la divulgación y asesoramiento acerca de la preparación de estos productos derivados de la descomposición anaerobia de subproductos orgánicos de origen agrícola; (ii) una enmienda fabricada *on site,* siguiendo el procedimiento y directrices proporcionadas por la citada empresa. El procedimiento de elaboración junto con los materiales utilizados, se describen de forma detallada en el Capítulo 5 de este trabajo.

El suelo utilizado como sustrato en el ensayo en microcosmos con lechuga se adquirió de una explotación vinícola en el municipio de Haro (La Rioja, 42°35'47.5"N 2°52'13.3"O; 492 m de altitud el nivel del mar), extrayendo la capa superficial del suelo (0-30 cm) y pasándola por un tamiz de 1,5 cm para su homogenización. Este suelo fue deliberadamente seleccionado para el ensayo en microcosmos debido a la baja cantidad de materia orgánica que presentaba (alrededor de un 1%) y al hecho de no haber sido

tratado con ningún tipo de enmienda orgánica en los últimos 20 años. Se trata del mismo suelo utilizado como sustrato para la realización del ensayo descrito en el punto 3.3 de este capítulo.

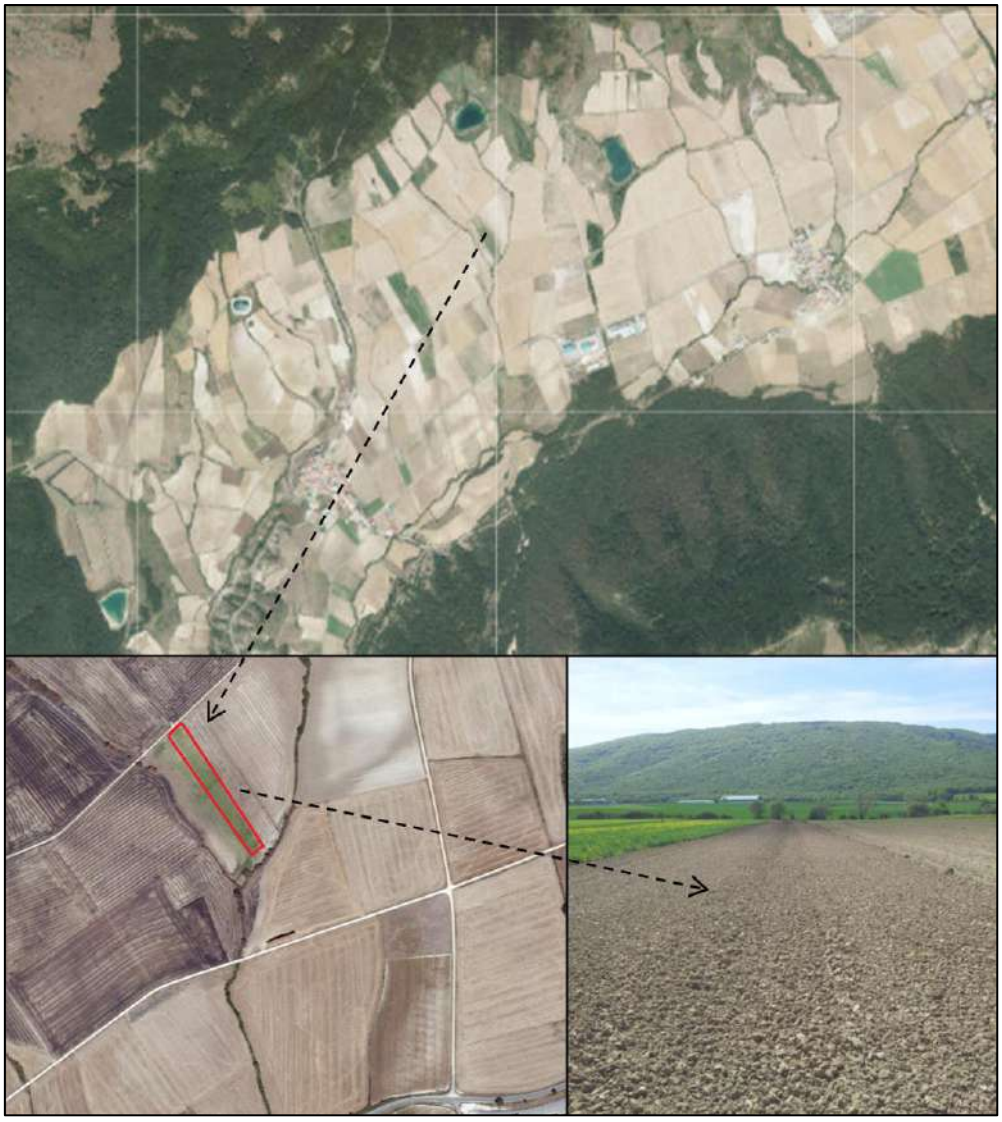


Figura 3.2. Ubicación de los ensayos en campo. *Fuentes: Visor GeoEuskadi; NEIKER.*

Por otro lado, los ensayos en campo con los cultivos de lechuga y maíz se llevaron a cabo en el mismo emplazamiento, una superficie de unos 2.000 m^2 (Figura 3.2) ubicada cerca del municipio de San Vicente de Arana (Araba, 42°45'17.1"N y 2°21'08.2"O; 825 m de altitud sobre el nivel del mar) y propiedad de Ricardo Corres, agricultor de la montaña alavesa que produce en ecológico. La zona se caracteriza por un clima mediterráneo marítimo fresco con una temperatura media anual de 10,6°C y una precipitación media anual de algo menos de 1000 mm. Para la realización de los ensayos,

la superficie se dividió en dos grandes parcelas siendo el suelo en ambos casos básico, con textura fraco-arcillosa y un contenido de materia orgánica del 1,8 y el 1,4% en las parcelas destinadas al cultivo de lechuga y maíz, respectivamente. A continuación, se describen los distintos ensayos llevados a cabo para cada cultivo.

3.2.1. Cultivo de lechuga

Como ya se ha comentado los efectos de las enmiendas líquidas fermentadas en el cultivo de la lechuga se testaron tanto en cámara de crecimiento controlado como en campo. En ambos casos, el diseño experimental se basó en dos factores principales: (i) el ya mencionado origen de la enmienda, evaluando los efectos de la enmienda comercial frente a la enmienda producida en granja; y (ii) la dosis de enmienda, comparando la dosis óptima, o dosis ajustada a la demanda de nitrógeno del cultivo de lechuga (*i.e.* 150 kg N ha^{-1}), frente a la dosis recomendada por el fabricante (400 L de producto diluido al 5% por hectárea). Además de los factores de utilización de enmienda orgánica, se añadió un tratamiento a base de fertilizante mineral NPK con fines comparativos: 150 kg N ha^{-1} en forma de NH_4NO_3 (34,4%); 50 kg P ha^{-1} en forma de P_2O_5 (18%) y 200 kg K ha^{-1}en forma de K_2O (60%). En ambos ensayos, las enmiendas y el fertilizante mineral se aplicaron en fondo, cinco días antes de la plantación (para evitar efectos indeseados sobre las raíces, sobre todo, en las dosis más altas). Para determinar el volumen de enmienda en cada uno de los tratamientos, se determinó la cantidad de nitrógeno total de las enmiendas mediante analizador elemental tras combustión seca (LECO TruSpec CHN-S, LECO Corp. USA) (Figura 3.6A). Además, dado que el agua puede solubilizar el pool de nutrientes presentes en el suelo, a todos los tratamientos se les aplicó el mismo volumen final de abonado, tratándose éste de enmienda orgánica en su totalidad en el caso de los tratamientos de dosis óptima, y una mezcla de enmienda y agua en el caso de los tratamientos de dosis recomendada. En todos los casos, la aplicación se realizó de forma manual, intentando esparcir la enmienda de forma homogénea por toda la superficie de los tiestos/parcelas.

3.2.1.1. Condiciones del ensayo en microcosmos

Para el ensayo en la cámara de crecimiento controlado, se utilizaron macetas de polietileno de 3 L a las que se les añadieron 2,5 kg (peso seco) del suelo de Haro descrito anteriormente. Los tratamientos se añadieron por triplicado y en fondo. Cinco días después de la adición de las enmiendas, las plántulas de lechuga variedad Batavia se plantaron y las macetas se distribuyeron por la cámara de crecimiento controlado de forma

aleatoria (Figura 3.3). Las condiciones de la cámara fueron las siguientes: fotoperiodo 14/10 h, temperatura 24/20°C día/noche, humedad relativa al 70%, intensidad de PAR (radiación fotosintéticamente activa) de 100 μmol m^{-2} s^{-1}. Las plantas se regaban 2 o 3 veces por semana hasta capacidad de campo, vertiendo directamente el agua a los platillos.

Figura 3.3. Detalle del ensayo en microcosmos. *Fuente: NEIKER.*

Tras 8 semanas, las lechugas fueron cosechadas y la biomasa aérea (peso seco) se registró mediante el secado de las plantas en estufa de aire forzado a 70°C. El suelo se muestreó mediante la extracción de la totalidad del volumen de cada maceta, e inmediatamente se procedió a su homogenización para la determinación de parámetros físico-químicos y biológicos indicadores de la salud/calidad del mismo (punto 3.6).

3.2.1.2. Condiciones del ensayo en campo

Los tratamientos se testaron en parcelas de 3 x 5 metros, 6 réplicas por tratamiento distribuidas en un diseño completamente aleatorio (Figura 3.4C). De la misma forma que en el ensayo en microcosmos, la aplicación de las enmiendas y el fertilizante mineral se realizó en fondo 5 días antes de la plantación de las plántulas de lechuga variedad Batavia. La plantación se realizó con la ayuda de una plantadora manual (Figura 3.4A), y se siguió un marco de plantación de 50 x 40 cm, resultando en 6 filas por parcela y 5 plantas por

metro cuadrado. El riego se realizó por aspersión, 2 o 3 veces por semana dependiendo de las condiciones meteorológicas. El control de la vegetación arvense se realizó de forma manual y con ayuda de una biciazada (Figura 3.4B).

Figura 3.4. Ensayo en campo. A, detalle de la plantadora manual; B, detalle de la biciazada; C, detalle del ensayo. *Fuente: NEIKER.*

Al igual que en el ensayo en microcosmos, la cosecha se realizó 8 semanas después de la plantación. La producción de lechuga por planta se determinó mediante la retirada de las plantas de las dos filas centrales de cada parcela y su pesaje tras secado en estufa. De la misma forma que en el punto 3.1, el muestreo del suelo se realizó tras la

cosecha mediante una sonda manual de 30 cm de profundidad, obteniendo de forma aleatoria 6 cilindros de suelo por parcela para la obtención de una muestra compuesta, representativa de cada parcela.

3.2.2. Cultivo de maíz

El ensayo de maíz se realizó durante dos campañas consecutivas en campo. Además de los dos factores experimentales estudiados en los ensayos de lechuga (*i.e.* origen y dosis) en este ensayo se agregó un tercer factor: el tiempo de aplicación de las enmiendas, comparando la aplicación íntegra de las enmiendas en fondo frente a una aplicación espaciada en 4 puntos en el tiempo. En este caso, la cantidad total a aportar de cada enmienda se dividió en 4 dosis idénticas, aplicándose la primera de ellas en fondo y las tres posteriores a los 30, 45 y 60 días tras la siembra. El factor dosis fue el mismo que en el caso del cultivo de lechuga: 150 kg N ha^{-1} frente a 400 litros de enmienda diluidos al 5% por hectárea. Además, como en el caso anterior, se incluyó un un tratamiento a base de fertilizante mineral NPK con fines comparativos: 150 kg N ha^{-1} en forma de NH_4NO_3 (34,4%); 60 kg P ha^{-1} en forma de P_2O_5 (18%) y 100 kg K ha^{-1}en forma de K_2O (60%). Parte de la aplicación del fertilizante mineral se realizó en fondo (50-60-100) y la cantidad restante (100-0-0) se aportó a los 30 días de la siembra, junto con el segundo aporte de enmienda en el factor de aplicación espaciada. En todos los casos, el aporte se realizó de forma manual, aplicándose de forma homogénea por toda la superficie de las parcelas.

Los tratamientos se testaron por triplicado en parcelas de 3 x 5 metros, distribuidas de forma aleatoria al inicio de la primera campaña y mantenidas en la segunda (Figura 3.5C). El maíz grano de ciclo corto (Anjou 456, ciclo FAO 400) se sembró a primeros de junio con una sembradora manual (Figura 3.5A). Se sembraron 4 filas por parcela dejando una distancia de 75 cm entre filas con una densidad de siembra de 66.666 plantas por hectárea. De la misma forma que en el ensayo de campo con lechuga, el riego se realizó por aspersión, 2 o 3 días por semana dependiendo de las condiciones meteorológicas. El control de la vegetación arvense se realizó de forma manual durante el primer mes de crecimiento del cultivo.

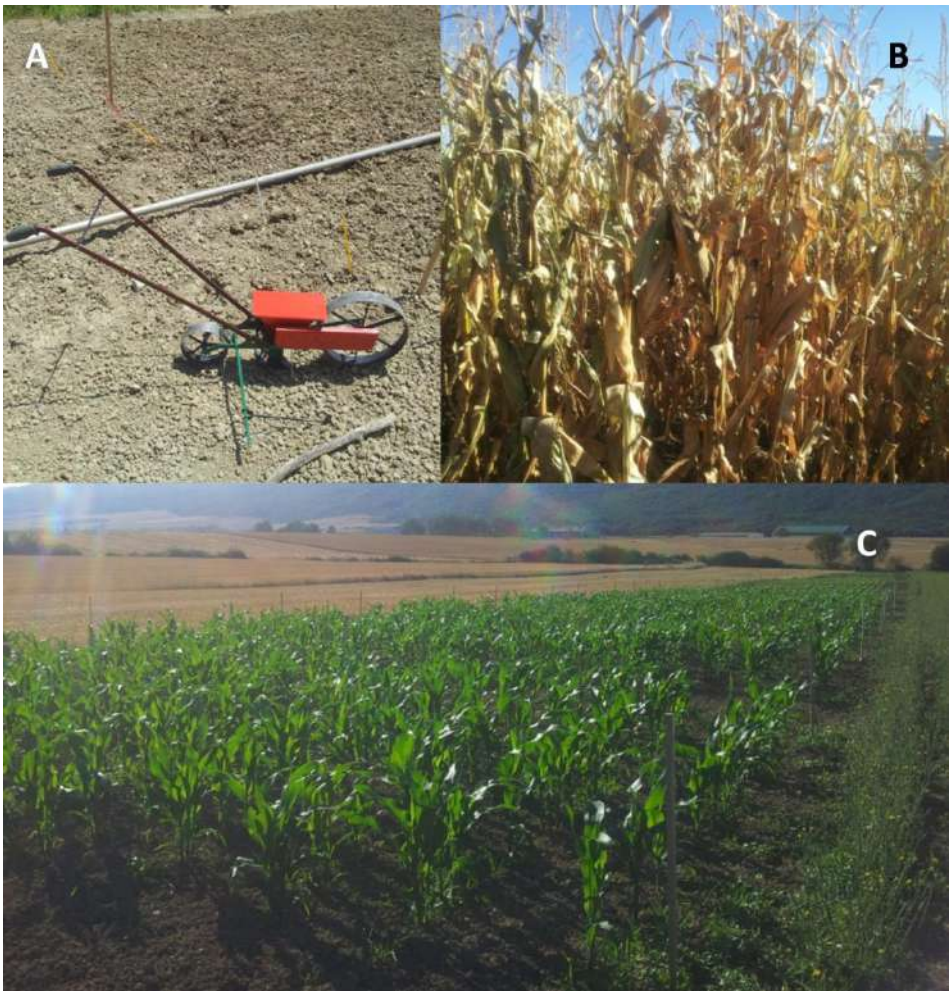


Figura 3.5. Ensayo de maíz. A, detalle de la sembradora manual; B, detalle de las plantas secas para la cosecha de las mazorcas; C, detalle del ensayo. *Fuente: NEIKER.*

El maíz se cosechó a finales de Noviembre (Figura 3.5B) y la producción se determinó a partir de la retirada de 10 plantas de las 2 líneas centrales en cada parcela. Posteriormente, una muestra de grano representativa de cada parcela fue molida para la caracterización de parámetros nutricionales, incluyendo cenizas, proteína bruta, fibra bruta, grasa bruta y almidón, mediante espectroscopia de reflectancia en el infrarrojo cercano (Foss NIRSystem 6500, USA) (Figura 3.6B). El muestreo del suelo se realizó tras la cosecha mediante una sonda manual de 30 cm de profundidad, obteniendo de forma aleatoria 6 cilindros de suelo por parcela para la obtención de una muestra compuesta, representativa de cada parcela.

3.3. Efecto del grado de madurez del estiércol

Para evaluar el efecto del uso de estiércol en distintos grados de madurez sobre la salud del suelo y el riesgo de diseminación de genes de resistencia a antibióticos se realizó un ensayo en cámara de crecimiento controlado a escala microcosmos. El diseño experimental se basó en dos factores principales: (i) el origen de las enmiendas, enfrentando las enmiendas derivadas del estiércol equino y la gallinaza; y (ii) el tipo de enmienda, diferenciada en base al grado de madurez: estiércol fresco, estiércol compostado y bokashi. Esta última es una técnica de origen japonés por la cual la materia orgánica es fermentada por la adición de un inóculo microbiano. Se trata de una enmienda utilizada de forma habitual en la agricultura orgánica cuyo periodo de maduración es significativamente menor al del compost. La descripción del proceso y materiales para la elaboración del bokashi utilizado en este ensayo se describe de forma detallada en el Capítulo 6. Por otra parte, el estiércol equino fresco y compostado, y la gallinaza freca y compostada se obtuvieron de las empresas Bolaleku S.A.T. (Bizkaia) y Productos Flower S.A. (Lleida), respectivamente. Se determinó el nitrógeno total de cada enmienda mediante analizador elemental para ajustar la cantidad de aporte de cada una a 150 kg N ha^{-1}. Las enmiendas se dispusieron por triplicado en macetas de tres litros que contenían, como en el caso del punto 3.2.1.1, 2,5 kg (peso seco) de suelo franco-arenoso de Haro que se había dejado acondicionar durante 10 días a 20°C. La adición de las enmiendas se realizó en su totalidad en fondo, mezclándose con la parte más superficial del suelo. Además de los tratamientos a base de enmiendas se añadió un tratamiento control, utilizando únicamente el suelo agrícola como sustrato para el desarrollo vegetal, como referencia para el estudio de la abundancia de genes de resistencia a antibióticos derivada de la presencia de enmiendas de origen animal. Las plántulas de lechuga variedad Batavia se plantaron y las macetas se distribuyeron a lo largo de la cámara de crecimiento controlado de forma aleatoria. Las condiciones de la cámara fueron las mismas que en el punto 3.2.1.1: fotoperiodo 14/10 h, temperatura 24/20°C día/noche, humedad relativa al 70%, intensidad de PAR de 100 μmol m^{-2} s^{-1}. El riego se realizó manualmente, vertiendo el agua directamente al platillo de cada maceta, 3 veces por semana hasta capacidad de campo.

Tras 8 semanas, las lechugas fueron cosechadas y la biomasa aérea (peso seco) se registró mediante el secado de las plantas en estufa de aire forzado a 70°C. Posteriormente, la biomasa aérea se molió para la determinación del nitrógeno total por

combustión seca y analizador elemental, calculando después la proteína cruda al multiplicar el nitrógeno resultante por el factor empírico 6,25. Los contenidos de fibra bruta y el almidón se calcularon siguiendo sendos procedimientos internos de NEIKER (PEC/EN/A-005 y PEC/EN/A-008, respectivamente), mientras que la concentración de minerales (*i.e.* P, Ca, Mg, Na, K, S, Cu, Zn, Fe y Mn) se determinó mediante espectrometría de emisión óptica de plasma acoplado inductivamente (ICP-OES, Varian VISTA-MPX) tras digestión con ácido nítrico/perclórico (Figura 3.6A). El suelo se muestreó mediante la retirada de todo el volumen de cada maceta, e inmediatamente se procedió a su homogenización para la determinación de parámetros físico-químicos y biológicos indicadores de la salud/calidad del mismo y para la cuantificación de genes de resistencia a antibióticos (punto 3.6).

Figura 3.6. A, espectrofotómetro de emisión óptica por plasma acoplado inductivamente (ICP-OES); B, espectrofotómetro NIR; C, analizador elemental LECO CHN. *Fuente: NEIKER.*

3.4. Lodos de depuradora

Este estudio se realizó en colaboración con la Mancomunidad de la Comarca de Pamplona (MCP) que, junto con el INTIA, viene realizando estudios acerca de los potenciales impactos ambientales y agronómicos derivados del uso de los lodos de depuradora generados en el proceso de depuración de las aguas residuales urbanas de la comarca de Pamplona desde principios de los 90. En concreto, el estudio cuyos resultados se discuten en el Capítulo 7, se encuentra ubicado en una finca experimental contigua a la estación depuradora de aguas residuales (EDAR) de Arazuri (Navarra, 42°48'41.7"N 1°43'29.3"O; 392 metros de altitud sobre el nivel del mar) (Figura 3.7), una zona donde el clima es mediterráneo templado (húmedo) con una temperatura media anual de 12,4°C y precipitaciones anuales en torno a los 760 mm. El suelo experimental es un cambisol calcáreo con pH básico y textura arcillo-limosa.

Figura 3.7. Ubicación del ensayo experimental. *Fuentes: Visor GeoEuskadi; NEIKER.*

El ensayo objeto de estudio se estableció en 1992 al objeto de valorizar el potencial agronómico de los lodos de depuradora urbanos a largo plazo. Para ello, se estableció un diseño de aplicación de lodos basado en dos factores experimentales que actualmente se mantiene: (i) la cantidad de aporte de lodo (40 y 80 t ha^{-1}); y (ii) la frecuencia de aporte (cada 1, 2 y 4 años). Además de los tratamientos resultantes de la combinación de estos dos factores, se estableció un tratamiento control sin ningún tipo de aporte de enmienda. Los tratamientos se dispusieron en parcelas de 35 m^2, 6 réplicas por tratamiento distribuidas aleatoriamente en 3 bloques y mantenidas a lo largo del tiempo. En cuanto a la cubierta vegetal, desde el principio del ensayo se estableció una rotación siguiendo la secuencia cereal – cereal – no cereal. No hubo riego ni control de la vegetación arvense. Antes de su aplicación al suelo agrícola, el lodo se estabilizó y se higienizó mediante un proceso de digestión anaerobia seguida de un proceso de deshidratación por centrifugado. Posteriormente el lodo se aplicó a la superficie del suelo mediante esparcidora y se incorporó mediante arado de disco a unos 30 cm de profundidad. El muestreo del suelo se realizó tras la cosecha mediante una sonda manual de 30 cm de profundidad, obteniendo 6 cilindros de suelo por parcela de forma aleatoria para la obtención de una muestra compuesta y representativa de cada parcela. Las muestras de suelo se llevaron al laboratorio de NEIKER para su procesado y determinación de parámetros físico-químicos y biológicos indicadores de la salud/calidad del mismo (punto 3.6).

Figura 3.8. Lodo antes de ser incorporado al suelo. *Fuente: MCP*.

3.5. Inoculación de micorrizas arbusculares

Para el desarrollo de este estudio el primer paso fue la recolección de suelo. Para ello se seleccionaron tres huertos gestionados bajo las directrices de la agricultura ecológica: dos de ellos ubicados en la provincia de Araba (Salcedo, 42°44'06.8"N 2°57'41.7"O, a 530 metros de altitud sobre el nivel del mar; y Monasterioguren, 42°48'08.7"N 2°38'37.2"O, a 590 metros de altitud sobre el nivel del mar), y el tercero en Bizkaia (Larrabetzu, 43°15'31.5"N 2°47'17.5"O, a 97 metros de altitud sobre el nivel del mar). En todos los casos, los suelos recibían aportes periódicos de estiércol compostado y se les aplicaba laboreo mínimo. En los tres emplazamientos, además del suelo agrícola de dentro de los huertos, también se recogió suelo natural de los pastos adyacentes a los huertos, con fines comparativos. Para la recogida del suelo, la parte más superficial del mismo (0-10 cm) se retiró mediante una sonda manual, realizando 10 pinchazos en cada huerto/suelo adyacente para la obtención de una muestra compuesta representativa de cada punto de estudio. Inmediatamente después de la recogida, los suelos se trasladaron al laboratorio de NEIKER para su caracterización físico-química, así como para el estudio de la estructura y diversidad de las comunidades fúngicas y micorrícicas.

Tras la caracterización de los suelos, se procedió al aislamiento y multiplicación de los hongos micorrícicos. Este aislamiento se realizó mediante cultivos trampa de sorgo y alfalfa en cámara de crecimiento controlado a escala microcosmos, utilizando como sustrato una parte de suelo de cada una de las huertas muestreadas y los correspondientes suelos adyacentes, por tres partes de arena de cuarzo estéril (Figura 3.9). En esta técnica de cultivos trampa, el suelo sirve como inóculo inicial de esporas micorrícicas, mientras que un sustrato inerte como la arena de cuarzo facilita el desarrollo de las mismas tras su germinación, mediante la colonización de las raíces de las plantas hospedadoras, donde se propagan y multiplican produciendo nuevas esporas. Se establecieron 6 cultivos trampa por suelo muestreado. Antes de la siembra, las semillas de sorgo y alfalfa se esterilizaron superficialmente. La fertilización se realizó mediante la adición de un fertilizante comercial encapsulado de lenta liberación, Osmocote Pro 5-6M (17-11-10 + 2MgO + TE), a una dosis de 0,5 g kg de suelo^{-1}. Las condiciones de la cámara para el desarrollo de los cultivos fueron: fotoperiodo 14/10 h, temperatura 20/16°C día/noche, humedad relativa al 70%, intensidad de PAR de 150 μmol m^{-2} s^{-1}. El riego se realizó manualmente, vertiendo el agua a la base de las macetas de forma periódica según las necesidades de los cultivos.

Figura 3.9. Cultivos trampa para la multiplicación de micorrizas arbusculares con sorgo y alfafa en la cámara de creciento controlado. *Fuente: NEIKER.*

Tras 6 meses, el sorgo y la alfalfa se cosecharon y todo el suelo rizosférico así como la biomasa radicular se extrajeron de todas las macetas. El suelo se mantuvo a 4°C para ser utilizado como inóculo de micorrizas en los posteriores ensayos de biofertilización en microcosmos. Antes de dichos ensayos, se determinó la correcta multiplicación de esporas en todos los suelos estudiados. Para ello, una muestra representativa del suelo rizosférico (50 g) de cada maceta se suspendió en agua y se filtró por una serie de tamices de 500, 100 y 50 µm. Las partículas contenidas en el tamiz de mayor poro se desecharon, mientras que la materia que quedó en los tamices de 100 y 50 micras se almacenó por separado en tubos falcon de 50 ml donde la mitad del volumen se había ocupado con una solución de sacarosa al 80%. Los tubos se agitaron para posteriormente centrifugarse a 3500 rpm durante 3 minutos. Con la ayuda de una bomba de vació, el sobrenadante se filtró por una membrana de 0,45 µm. Dado que las esporas superan el tamaño de poro de la membrana, tras el filtrado la membrana se trasladó a una lupa para la cuantificación visual de las esporas retenidas (3.10A). Además de la cuantificación, también se determinó el porcentaje de colonización de raíces de las plantas hospedadoras por parte de las micorrizas arbusculares. Para ello, las raíces se decoloraron sumergiéndose en una solución de KOH al 10% a 70°C durante 45 minutos para posteriormente ser teñidas mediante una solución de azul de tripano al 0,05%. Tras la tinción, las raíces se lavaron con agua del grifo y se mantuvieron en lactoglicerol (1:1:1

– ácido láctico:glicerol:H_2O) hasta la determinación del porcentaje de colonización por observación mediante microscopía óptica (Figura 3.10B) siguiendo las directrices del Centro de Estudios sobre la Monoxénica de Micorrizas Arbusculares (CESAMM) de la Universidad de Lovaina, Bélgica.

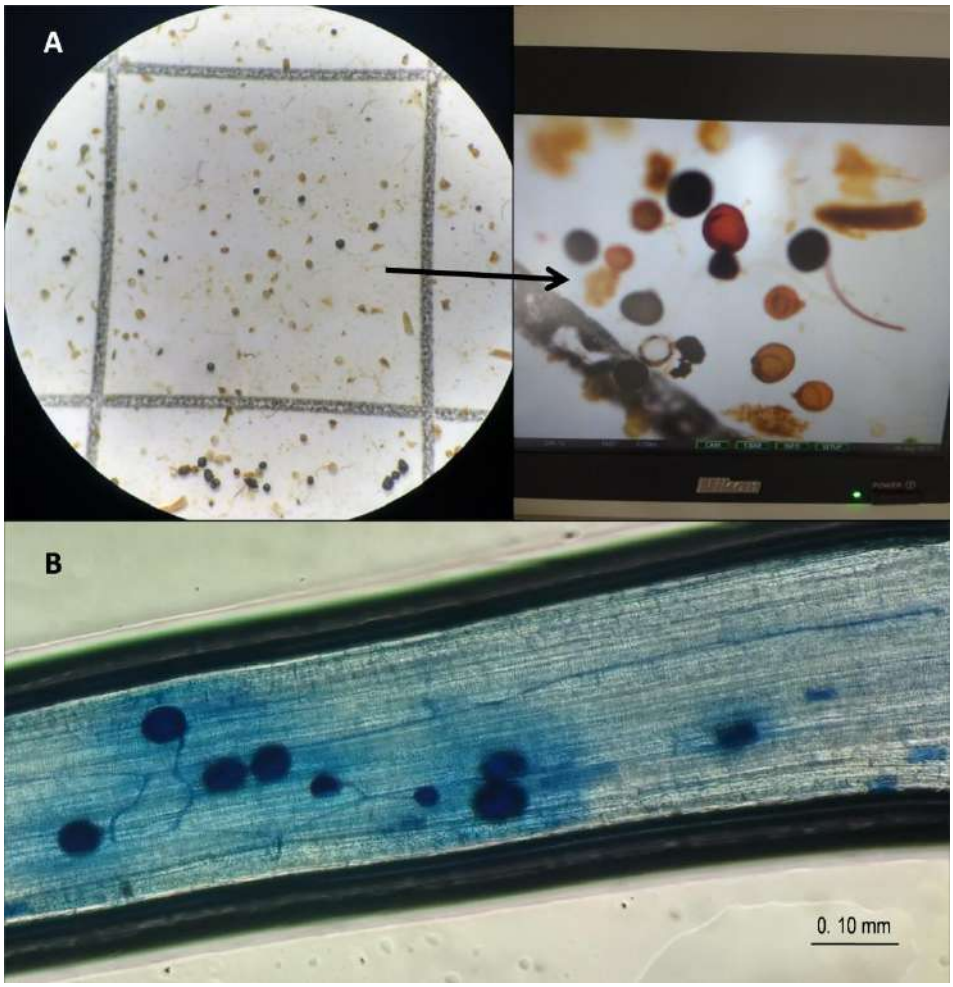


Figura 3.10. A, Cuantificación de esporas de micorrizas arbusculares mediante lupa; B, Detalle de vesículas e hifas en una raíz. *Fuente: NEIKER.*

Después de verificar la correcta multiplicación de esporas micorrícicas arbusculares en los distintos suelos, se llevó a cabo un ensayo con lechuga en cámara de crecimiento controlado a escala microcosmos para evaluar el efecto de la inoculación de micorrizas arbusculares en la producción y calidad nutricional de la lechuga, así como en las comunidades de micorrizas arbusculares del suelo. Para ello, cada uno de los suelos muestreados al principio del experimento y utilizado en los posteriores cultivos trampa se estudiaron por triplicado como tratamientos de inoculación independientes: (1) inoculación con micorrizas multiplicadas del huerto de Salcedo y del (2) suelo natural

adyacente; (3) inoculación con micorrizas multiplicadas del huerto de Monasterioguren y del (4) suelo natural adyacente; (5) inoculación con micorrizas multiplicadas del huerto de Larrabetzu y del (6) suelo natural adyacente. Además de estos 6 tratamientos, se incluyó un control sin ningún tipo de aporte de inóculo. La cantidad de suelo rizosférico a aportar en cada caso se calculó en base al inóculo final, que se igualó en todos los casos a un aporte total de unas 25.000 esporas de hongos micorrícicos arbusculares por tiesto. A cada maceta de 3 litros de volumen se le añadieron 2 kg de suelo control del huerto de Monasterioguren menos la cantidad de inóculo aportado por tratamiento, además de medio kilo de arena de cuarzo. Las semillas de lechuga se sembraron directamente en las macetas que se dispusieron de forma aleatoria a lo largo de la cámara de crecimiento. Las condiciones de la cámara fueron las mismas descritas para los cultivos de sorgo y alfalfa.

Tras 7 semanas, la biomasa aérea se cosechó y se realizó una nueva siembra de lechuga en las mismas macetas y bajo las mismas condiciones para un nuevo ciclo de crecimiento, que duró otras 7 semanas. Al final de cada ciclo de crecimiento, la producción de biomasa aérea se determinó mediante el secado de las plantas en estufa de aire forzado a 70°C durante 48 h y posterior pesaje. Posteriormente, la biomasa aérea se molió y fue digerida con ácido nítrico/perclórico para la determinación de la concentración de minerales (*i.e.* P, Ca, Mg, Na, K, S, Al, Fe, Mn y Mo) mediante espectrometría de emisión óptica de plasma acoplado inductivamente (ICP-OES). El nitrógeno total se determinó mediante analizador elemental tras combustión seca (LECO TruSpec CHN-S). Al final del ensayo en microcosmos tres tratamientos fueron seleccionados para el estudio de la diversidad fúngica y de micorrizas arbusculares en la biomasa radicular: (i) el tratamiento control o tratamiento sin aporte de inóculo; (ii) el tratamiento a base de inóculo de micorrizas del suelo no cultivado adyacente al huerto de Larrabetzu; y (iii) el tratamiento a base de inóculo de micorrizas del suelo no cultivado adyacente al huerto de Monasterioguren. Esta selección se realizó en base a la producción de biomasa aérea tras el segundo ciclo de cultivo, que resultó significativamente superior en los tratamientos arriba mencionados que en el control. Para el estudio de las comunidades fúngicas en la biomasa radicular, las raíces de lavaron exhaustivamente con agua destilada y se molieron en un mortero con nitrógeno líquido. El DNA de las muestras radiculares se extrajo mediante un kit de extracción comercial (NucleoSpin® Plant II, Marcherey Nagel). Partiendo de ese material genético, se prepararon librerías de amplicones del gen ITS del RNA ribosómico para su posterior secuenciación con Illumina MiSeq y análisis de la diversidad y estructura de las comunidades fúngicas. El proceso de

preparación de las librerías de amplicones así como el análisis de los datos de secuenciación, se describen en el punto 3.6.

Por último, en lo que al análisis del suelo se refiere, una muestra representativa de suelo de cada maceta se recogió tras cada uno de ciclos de cultivo de lechuga y se dejó secar a temperatura ambiente. Los suelos se tamizaron (<2 mm) y se procedió a la determinación de la proteína del suelo relacionada con la glomalina fácilmente extraíble. Los resultados de estas determinaciones se discuten en el Capítulo 8.

3.6. Parámetros analíticos de suelo

El procesamiento y análisis de las muestras de material vegetal para cada ensayo se han descrito de forma individual. Sin embargo, los análisis realizados en matriz suelo son, en su mayoría, comunes para todos los ensayos realizados en el transcurso de este trabajo. Por ello, este punto describe el procesamiento de las muestras de suelo tras su llegada al laboratorio, así como las técnicas analíticas utilizadas para la consecución de uno de los objetivos comunes en todos los ensayos: la evaluación del estado de la salud/calidad del ecosistema edáfico mediante la determinación de parámetros físico-químicos y biológicos.

3.6.1. Procesamiento de las muestras

A su llegada al laboratorio, las muestras de suelo se dividieron en dos partes: (i) la primera de ellas, destinada al análisis de los parámetros físico-químicos, se dejó secar a temperatura ambiente para después ser tamizada por un tamiz de 2 mm de diámetro; (ii) la segunda porción, destinada a la determinación de los parámetros biológicos, fue tamizada en fresco por un tamiz de 2 mm de diámetro. Una vez tamizadas, la parte correspondiente a los análisis físico-químicos se almacenó a temperatura ambiente, mientras que la que correspondía a los parámetros biológicos se almacenó a 4°C hasta su posterior análisis.

Para los ensayos de análisis moleculares, el DNA de las muestras de suelo se extrajo con el kit de extracción PowerSoil DNA Isolation Kit (Mo-Bio Laboratories) partiendo de 0,25 g de suelo por muestra y siguiendo las instrucciones del fabricante. Tras la extracción, la cantidad y calidad del material genético obtenido en cada muestra se determinó con un espectrofotómetro Nanodrop (Figura 13C).

3.6.2. Parámetros físico-químicos

A pesar de que una de las hipótesis de este trabajo es la relevancia y utilidad de los parámetros biológicos del suelo como indicadores de la salud del mismo, los parámetros físico-químicos tradicionalmente empleados, siguen siendo herramientas muy útiles para la evaluación del estado del ecosistema edáfico, aportando información complementaria acerca de la salud del mismo. A continuación se exponen los parámetros físico-químicos analizados en el transcurso de este trabajo. La mayoría de los mismos se determinaron siguiendo métodos oficiales de análisis (MAPA, 1994):

- pH, establece los límites para el desarrollo de los cultivos y la microbiota edáfica, y determina el comportamiento y movilidad de los elementos presentes en el suelo.
- Materia orgánica, componente fundamental del suelo ligado a la mayoría de indicadores físico-químicos y biológicos del mismo, determinante de la fertilidad del suelo.
- Carbono y nitrógeno totales, nitrógeno mineral en forma de amonio y nitrato, fósforo y potasio extraíbles, concentraciones totales de minerales y oligoelementos, indican la cantidad de nutrientes y la disponibilidad de los mismos para las plantas y la microbiota edáfica.
- Textura del suelo, relacionada con la retención y transporte de agua, nutrientes y compuestos químicos.
- Concentración total de metales pesados y metaloides, elementos contaminantes que pueden ejercer toxicidad sobre las plantas y microorganismos. Asimismo, para la evaluación de la fracción biodisponible, en el Capítulo 7 se midieron las concentraciones extraíbles de estos elementos con tres extractantes: (i) $CaCl_2$ 0,01M (Houba *et al*., 2000); (ii) $NaNO_3$ 0,1M (Gupta y Aten, 1993); (iii) solución de ácidos orgánicos de bajo peso molecular (Feng *et al*., 2005).

3.6.3. Parámetros biológicos

Si bien las propiedades físico-químicas resultan útiles a la hora de evaluar determinados aspectos del estado de un suelo, por sí solas no son suficientes para determinar el efecto de las enmiendas orgánicas sobre los organismos y sobre las interacciones de éstos con el ecosistema edáfico. En este sentido, como ya se ha mencionado, las propiedades biológicas, y especialmente las microbianas, se presentan como indicadores óptimos de

la salud del suelo por su carácter integrador de las propiedades físicas, químicas y biológicas del suelo, así como su rapidez de respuesta, alta sensibilidad y relevancia ecológica. A continuación se detallan los parámetros microbianos que reflejan la biomasa, actividad y diversidad de las comunidades microbianas edáficas analizados en el presente trabajo:

3.6.3.1. Actividades enzimáticas

Las enzimas del suelo son proteínas que median y catalizan reacciones asociadas a procesos de vital importancia en el suelo como son la descomposición de la materia orgánica o la mineralización y el reciclaje de los nutrientes. Debido a su papel en estos y otros procesos, presentan un gran potencial para suministrar una evaluación integradora de la salud del ecosistema edáfico. Estas proteínas pueden proceder de microorganismos, vivos o muertos, raíces y partes residuales de plantas, o micro- y mesofauna, y estar asociadas tanto a células viables como en forma extracelular complejadas a la matriz edáfica o en la solución del suelo. Así pues, la actividad enzimática en suelo es consecuencia de la suma de la actividad de las comunidades microbianas viables y de la actividad de enzimas estabilizadas a largo plazo, de forma que pueden reflejar los cambios acumulados en el tiempo sobre la salud del ecosistema edáfico. Las actividades enzimáticas estudiadas en este trabajo se basan en la determinación colorimétrica del producto liberado por la actividad cuando el suelo tamponado es incubado a una temperatura óptima, en condiciones de substrato saturantes (Figura 3.11D), y se describen a continuación:

- *Actividad β-glucosidasa* (Capítulos 4, 5, 6 y 7). Se trata de una enzima implicada en la liberación de glucosa. Hidroliza polímeros de los residuos vegetales (*i.e.*, celobiosa y maltosa) aportando los esqueletos de C y energía esenciales para el crecimiento de los organismos heterótrofos del suelo. La determinación de esta actividad se realizó siguiendo el método descrito por Dick (1997) y Taylor *et al*. (2002).
- *Actividad β-glucosaminidasa* (Capítulo 7): Esta enzima cataliza la hidrólisis de quitina, un polisacárido natural presente en la pared celular fúngica, en acetilglucosamina, un amino azúcar. Se determinó siguiendo el procedimiento descrito por Parham y Deng (2000).

- *Actividad fosfatasa alcalina* (Capítulos 4, 5, 6 y 7): Esta enzima está implicada en la mineralización de esteres de fósforo de la materia orgánica del suelo para liberar fosfato disponible para las plantas. La determinación de esta actividad se realizó siguiendo el método descrito por Dick (1997) y Taylor *et al*. (2002).
- *Actividad arilsulfatasa* (Capítulos 4, 5, 6 y 7): Cataliza la mineralización de ésteres orgánicos de azufre presentes en la materia orgánica del suelo para producir sulfato inorgánico. La determinación de esta actividad se realizó siguiendo el método descrito por Dick (1997) y Taylor *et al*. (2002).
- *Actividad ureasa* (Capítulos 4, 5 y 6): Cataliza la hidrólisis del nitrógeno orgánico (urea) en amonio disponible para las plantas. Se determinó según el procedimiento descrito por Kandeler y Gerber (1988).
- *Actividad arginina deaminasa* (Capítulo 7): Cataliza la hidrólisis del aminoácido arginina, liberando amonio. Se determinó siguiendo el procedimiento descrito por Kandeler (1996).

3.6.3.2. Nitrógeno potencialmente mineralizable

La mineralización del nitrógeno es un proceso por el cual el nitrógeno orgánico, contenido en la materia orgánica principalmente en forma de aminoácidos y proteínas, es convertido en distintas formas minerales de nitrógeno inorgánico que pueden ser incorporados por los cultivos. El primer paso de este proceso consiste en la conversión del nitrógeno orgánico en amonio mediante un proceso llevado a cabo exclusivamente por microorganismos heterótrofos, la amonificación. En este sentido, el nitrógeno potencialmente mineralizable se basa en la medición de la cantidad de nitrógeno orgánico que puede transformarse a amonio por acción microbiana, durante un tiempo conocido, para obtener una estimación de la tasa de mineralización del nitrógeno en un suelo determinado. Se trata de un parámetro con gran valor indicador de la fertilidad del suelo. La determinación de este parámetro se realizó en los Capítulos 4, 5, 6 y 7, siguiendo el procedimiento descrito por Powers (1980).

Figura 3.11. A, Determinación colorimétrica de carbono de la biomasa; B, valoración de NaOH en la determinación de la respiración del suelo; C, placas Biolog EcoPlates™; D, determinación colorimétrica de actividades enzimáticas. *Fuente: NEIKER.*

3.6.3.3. Respiración del suelo e inducida por sustrato

La respiración del suelo es un parámetro bien establecido para la monitorización de la descomposición de la materia orgánica del suelo. Se trata de un proceso clave en el sistema edáfico, por el cual al metabolizar una fuente de carbono, los microorganismos liberan CO_2 a la atmósfera. La tasa de respiración de un suelo viene determinada por diversos factores tales como la temperatura, la humedad, la cantidad y composición de la materia orgánica presente, y un largo etcétera, que hacen que este parámetro sea muy variable. Además, es un parámetro muy sensible a los cambios como la presencia de materia orgánica exógena y la presencia de contaminantes. Debido a esta sensibilidad así como a su relevancia ecológica, la respiración del suelo se presenta como un indicador muy útil a la hora de evaluar el impacto producido por la adición de enmiendas orgánicas de distinto origen y composición en el suelo agrícola. La determinación de este parámetro se realizó en los Capítulos 4, 5, 6 y 7, de acuerdo a la norma ISO 16072 (2002).

Por otro lado, la respiración inducida por sustrato se basa en suministrar a la biomasa microbiana un sustrato fácilmente mineralizable (generalmente glucosa) a concentración saturante y la posterior determinación de la tasa de respiración tras un tiempo relativamente breve (alrededor de 5 horas). La tasa de respiración ante una fuente de carbono no limitante debería representar la velocidad de reacción metabólica máxima y, por tanto, ofrecer una estimación de la biomasa microbiana potencialmente activa. Este parámetro se determinó en los Capítulos 4 y 6, siguiendo el procedimiento establecido en la norma ISO 17155 (2002).

3.6.3.4. Carbono de la biomasa microbiana

La biomasa microbiana constituye la fracción viva de la materia orgánica del suelo, y su determinación se basa en la medición de la cantidad de carbono contenida dentro de los organismos que componen la comunidad microbiana edáfica. Se trata de un parámetro que responde rápidamente a los cambios en las propiedades edáficas, así como a la incorporación de materia orgánica exógena y a la presencia de contaminantes, siendo un indicador muy útil para la evaluación de los efectos de prácticas agrícolas tales como la adición de enmiendas orgánicas. En este trabajo, la determinación del carbono de la biomasa microbiana se realizó en los Capítulos 4, 5, 6 y 7, siguiendo la metodología de fumigación/extracción descrita por Vance *et al*. (1987).

3.6.3.5. Abundancia de genes estructurales por qPCR

La abundancia del número de genes estructurales de una comunidad puede determinarse mediante PCR cuantitativa (qPCR) a tiempo real. Para ello, se realiza una amplificación a tiempo real de un gen diana del DNA molde mediante cebadores o primers (secuencias nucleotídicas cortas) específicos. La acumulación de las copias del gen amplificado se cuantifica al final de cada ciclo en base a un patrón de concentración conocida. Es por ello, que a este tipo de cuantificación se le denomina “amplificación absoluta”. En el presente trabajo, se determinó la abundancia de copias de los genes estructurales universalmente conservados 16S y 18S del RNA ribosómico (rRNA) para organismos procariotas y eucariotas, respectivamente, en los Capítulos 4, 5, 6 y 7. Para ello, se emplearon cebadores específicos para ambos genes, que se encuentran descritos, junto con las mezclas de reacción y las condiciones de PCR en el Capítulo 4. En todos los capítulos dicha cuantificación se utilizó como una estimación de la biomasa de las comunidades edáficas bacterianas y fúngicas.

3.6.3.6. Abundancia de genes funcionales por qPCR

La abundancia de genes funcionales como los genes de resistencia a antibióticos (ARGs) y elementos genéticos móviles (MGEs) puede cuantificarse de la misma forma que la de los genes estructurales, mediante cebadores específicos para cada región a amplificar. Los genes funcionales pueden cuantificarse de forma "absoluta" utilizando una recta patrón, o de forma "relativa" mediante la cuantificación simultánea de un gen funcional y uno estructural y relativizando los valores de abundancia al tamaño poblacional. En los Capítulos 6 y 7 de este trabajo se determinó la cantidad de una batería de genes funcionales mediante cuantificación relativa, relativizando la cantidad de ARGs y MGEs a al gen estructural de procariotas 16S rRNA. Sin embargo, las técnicas de amplificación utilizadas en dichos capítulos difieren: tratándose de qPCR estándar en el caso del Capítulo 6; y qPCR de alta resolución (HT-qPCR) en el del Capítulo 7. La HT-qPCR permite la amplificación simultánea de 96 genes en hasta 96 muestras de DNA molde mediante la tecnología de nanofluídica (sistema de qPCR BioMark HD Nanofluidic qPCR System en combinación con chips de 96.96 Dynamic Array IFCs de Fluidigm Corporation). Esta determinación se realizó en colaboración con la unidad de secuenciación y genotipado de Sgiker (UPV/EHU). Los cebadores y condiciones de PCR para la cuantificación de los genes funcionales vienen descritos en los Capítulos 6 y 7.

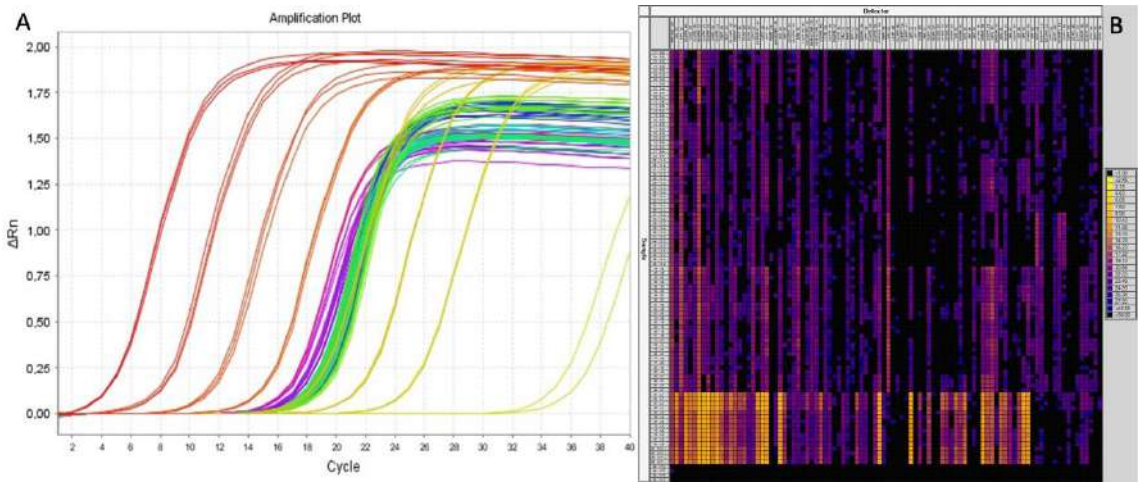


Figura 3.12. A, Gráfica de amplificación obtenida por qPCR en tiempo real; B, heatmap con los datos de amplificación obtenidos mediante HT-qPCR. *Fuente: NEIKER.*

3.6.3.7. Perfiles fisiológicos a nivel de comunidad (Biolog EcoPlates™)

Los perfiles fisiológicos de utilización de fuentes de carbono a nivel de comunidad obtenidos mediante placas Biolog EcoPlates™ (Figura 3.11C), permiten realizar una

estimación de la diversidad metabólico-funcional de las comunidades de microorganismos heterótrofos y cultivables del suelo. Estas placas, constan de 31 pocillos (por triplicado) cada uno de los cuales contiene un sustrato de carbono diferente (salvo uno de ellos, que se utiliza como blanco) además de tetrazolio que, en caso de reacción catabólica, se reduce y desarrolla una coloración morada. La intensidad y el patrón de formación de dicha coloración a lo largo de los 31 pocillos, proporciona un perfil fisiológico para cada muestra, que es representativo de la diversidad funcional de la comunidad microbiana, y que puede utilizarse para comparar el efecto de distintos tratamientos experimentales sobre las comunidades de microorganismos heterótrofos del suelo. La determinación de este parámetro se realizó en los Capítulos 4 y 5, siguiendo el procedimiento descrito por Epelde *et al*. (2008).

3.6.3.8. Diversidad estructural por secuenciación masiva (NGS)

Es sabido que la gran mayoría de microorganismos edáficos no son cultivables *in vitro*. Es por ello que en los últimos años ha habido un rápido y monumental desarrollo de métodos "ómicos" para el análisis de las comunidades microbianas edáficas que parten del material genético de la matriz edáfica, desembarazándose de la necesidad de cultivar. En este sentido, las técnicas de secuenciación masiva de nueva generación (NGS) han permitido el desarrollo del DNA metabarcoding, o caracterización de comunidades de microorganismos presentes en una muestra de suelo mediante la creación de librerías de amplicones de un gen estructural y la posterior secuenciación de las mismas. Partiendo de una muestra de DNA molde de suelo, las librerías de amplicones se preparan mediante la amplificación de los genes estructurales diana mediante PCR en dos estapas: (i) en la primera etapa, o primera PCR, se amplifica el gen diana mediante cebadores específicos; (ii) en la segunda, se incluye una secuencia nucleotídica conocida como "barcode", o código de barras, que es específico para cada muestra, y que permite la identificación de una muestra concreta tras el proceso de secuenciación. Después de cada PCR, se realiza una electroforesis en gel de agarosa al 1% para confirmar la correcta amplificación de las librerías (Figura 3.13A), tras la cual se realiza la purificación de los amplicones mediante microesferas magnéticas AMPure XP de Agencourt (Figura 3.13B). Tras cuantificación de la concentración final de DNA de doble cadena mediante fluorescencia (Qubit), las librerías se secuencian mediante la tecnología Illumina MiSeq V2. Los datos obtenidos con este proceso de secuenciación permiten el estudio de la diversidad, estructura y composición taxonómica de los microorganismos del suelo.

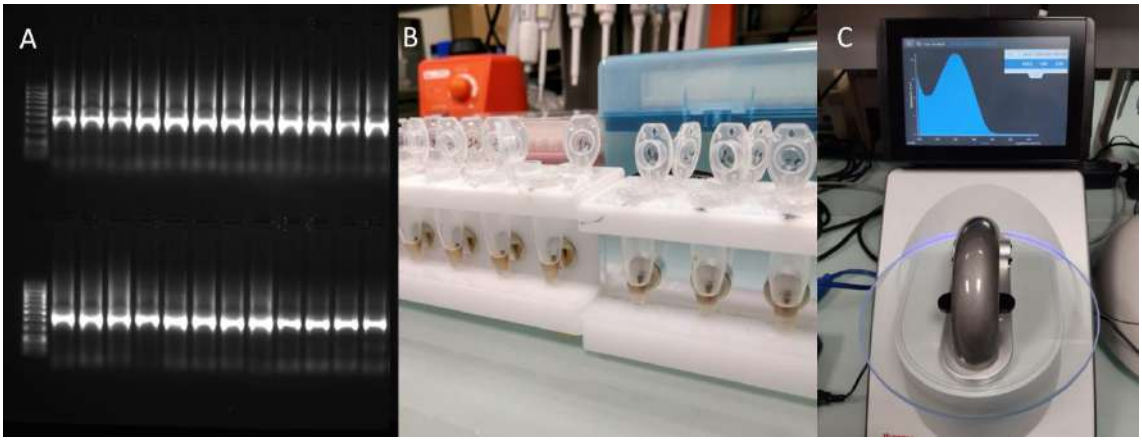


Figura 3.13. A, Gel de agarosa para confirmar la amplificación del gen diana; B, proceso de purificación del DNA; C, cuantificación de DNA por espectrofotometría. *Fuente: NEIKER.*

En este trabajo, este proceso de secuenciación mediante metabarcoding para el estudio de las comunidades microbianas edáficas se realizó en todos los capítulos, con algunas diferencias: la secuenciación del gen 16S rRNA para organismos procariotas se llevó a cabo en los Capítulos 4, 5, 6 y 7; la secuenciación del gen 18S rRNA para organismos eucariotas se estudió en los Capítulos 4 y 7; la secuenciación del gen ITS para hongos se estudió en el Capítulo 8. El proceso de preparación de las librerías de amplicones se describe de forma detallada en el Capítulo 4. Los primers para la preparación de amplicones del gen ITS se describen en el Capítulo 8. En todos los casos, la secuenciación se llevó a cabo en Tecnalia (Araba).

4| EFFECTS OF CORN STOVER MANAGEMENT ON SOIL QUALITY

4. EFFECTS OF CORN STOVER MANAGEMENT ON SOIL QUALITY

Urra, J., Mijangos, I., Lanzén, A., Lloveras, J., Garbisu, C., 2018, published in ***European Journal of Soil Biology****,* 88, 57-64.

Abstract

The incorporation of stover into soil can bring beneficial effects in terms of soil fertility, stabilization of soil structure, maintenance of soil organic carbon, etc. We evaluated the effects, after 6 years of consecutive treatment, of corn stover incorporation *versus* corn stover removal on soil quality, using physicochemical and biological parameters as indicators of soil quality. Throughout the experimental period, soil organic carbon decreased as a result of stover removal (from 20.1 to 14.7 g kg^{-1}). Substrate-induced respiration and bacterial gene abundance decreased by stover removal over the same period (24.0 and 47.6%, respectively). Biolog EcoPlates™ data showed faster rates of D-xylose and D-mannitol utilization by the soil bacterial communities under stover incorporation. 16S and 18S rRNA Illumina sequencing data did not show significant differences in terms of microbial diversity and composition between stover incorporation and stover removal treatments. Finally, the incorporation of stover resulted in higher values (27.2% higher) of soil quality, as reflected by the value of a Soil Quality Index, which integrates the values of a variety of microbial indicators of soil quality. In conclusion, incorporation of stover after corn harvest is a beneficial agronomic practice which enhances soil N and C pools and stimulates microbial communities, leading to an increase in soil quality.

4.1. Introduction

Corn (*Zea mays* L.) stover has been identified as potential feedstock for cellulosic ethanol production because of its high cellulosic content, large volume of biomass production and wide availability around the world (Woiciechowski *et al*., 2016). However, the removal of stover can lead to a decline in soil quality and, hence, agricultural productivity by decreasing the content of soil organic carbon (SOC) and increasing the risk of soil erosion (Mann *et al*., 2002; Blanco-Canqui and Lal, 2009; Johnson *et al*., 2013). Conversely,

incorporation of stover into agricultural soil can improve soil quality through a variety of processes such as stabilization of soil structure, prevention of soil erosion, maintenance of SOC, nutrient recycling, provision of energy for soil microbial communities, etc.

Soil microorganisms play an essential role in soil functioning and the delivery of soil ecosystem services. Thus, soil microbial parameters related to the activity, biomass and diversity of soil microbial communities are frequently used as indicators of soil quality (Epelde *et al*., 2010; Pardo *et al*., 2014), owing to their sensitivity, fast response, integrative character and ecological relevance. Nonetheless, there are limited reports on the effects of stover incorporation *versus* stover removal on soil microbial communities. Lehman *et al*. (2014) reported a reduction in the fungi-to-bacteria ratio as a result of the removal of stover. Johnson *et al*. (2013) found a decrease in soil enzyme activities after three consecutive cycles of stover removal. Moebius-Clune *et al*. (2008) observed a reduction in decomposition activity and glomalin concentration in soil after 32 years of stover removal. In contrast, the long-term incorporation of stover has been found to increase soil microbial biomass (Halpern *et al*., 2010; Sheibani *et al*., 2013).

The aim of this work was to evaluate the effects of six years of corn stover incorporation *versus* corn stover removal on agricultural soil quality, with special emphasis on the changes induced in soil microbial parameters that provide information on the activity, biomass and diversity of soil microorganisms. In particular, there is limited information on the changes induced by stover management on soil microbial diversity and composition. Recent developments in sequencing technologies have facilitated the sequencing of the genomes of soil microbial communities. Therefore, 16S and 18S rRNA gene-based Illumina sequencing were used to study differences between soil microbial communities subject to stover incorporation *versus* stover removal. We hypothesized that, after six years, the incorporation of corn stover from agricultural fields would positively impact soil quality by increasing soil organic carbon and stimulating soil microbial communities.

4.2. Materials and methods

4.2.1. Experimental design

A field experiment was conducted in Almacelles (NE Spain, 41º43’ N, 0º26’ E) at an altitude of 324 metres above sea level. The climate in this region is semiarid with low

precipitation (192 mm) and high temperature (daily average temperature of 19.1ºC) during the corn-growing period. The soil is a Typic Calcixerept with loamy texture, well drained and without salinity problems.

Experimental plots of 18 x 17 m were arranged following a completely randomized design with three replications. Treatments consisted of: (1) corn stover incorporation (SI), carried out annually after crop harvest by disc plowing to a depth of approximately 30 cm, and (2) corn stover removal (SR) carried out mechanically.

Corn hybrids belonging to the 600 to 700 FAO cycle were planted annually (continuous corn cropping sequence) in early spring at a density of 80,000 plants ha^{-1} with a distance of 71 cm between rows. Plots were irrigated by sprinkler 2-3 times per week depending on the weather conditions, resulting in approximately 1,000 mm of water per season. Nitrogen, phosphorus and potassium fertilizers were applied annually at a rate of 300 kg NH_4NO_3 (33.5% N) ha^{-1}, 150 kg P_2O_2 ha^{-1} and 250 kg K_2O ha^{-1}, respectively. Plots were treated with 3.3 L ha^{-1} of the pre-emergence herbicide Trophy (40% Acetochlor + 6% Dichlormid) and 1 L ha^{-1} of the post-emergence herbicide Fluoxypyr 20% plus 1.5 L ha^{-1} of Nicosulfuron in order to control *Abutilon theophrasti* M. and *Sorghum halepense* L., respectively.

Aboveground corn biomass was determined at physiological maturity, by harvesting 4 m of the central row in each plot.

4.2.2. Soil parameters

After crop harvest, composite soil samples (from six cores taken at a 0-30 cm soil depth) were randomly collected from each plot. Immediately after collection, soil was sieved to less than 2 mm and subjected to physicochemical characterization. Soil pH, the content of carbonate, limestone, nitrate, ammonium, total nitrogen and organic carbon were measured according to standard methods (MAPA, 1994). Heavy metals and minerals were quantified by inductively-coupled plasma atomic emission spectrophotometry (ICP-AES).

For the determination of soil microbial parameters, soils were stored fresh at 4ºC for a maximum of one month until analysis. Sub-samples for molecular analyses were stored at -20ºC. Soil enzyme activities were determined at optimal conditions of temperature, pH and substrate concentration, so as to get an assessment of maximum potential enzyme activity in soil. β-glucosidase, arylsulphatase and alkaline phosphatase

were determined according to Dick *et al*. (1996) and Taylor *et al*. (2002). Urease was measured following Kandeler and Gerber (1988).

Potentially mineralizable N, an indicator of biologically active soil N, was measured as described by Powers (1980). Respiration and substrate-induced respiration (SIR) were measured following ISO 16072 Norm (2002) and ISO 17155 Norm (2002), respectively. Microbial biomass carbon (MBC) was determined following Vance *et al*. (1987). Community-level physiological profiles (CLPPs) of soil cultivable heterotrophic bacteria were determined with Biolog EcoPlates™ following Epelde *et al*. (2008).

For the molecular analyses, DNA extraction was performed as soon as samples were processed and carried out from three aliquots (each of them corresponding to 0.25 g dry weight soil) of each sample using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA). Prior to DNA extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) to wash away extracellular DNA.

The amount of DNA in the samples was measured on a ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE). For the estimation of the abundance of 16S rRNA gene fragments for total bacteria and 18S rRNA gene fragments for total fungi, qPCR measurements were carried out using the primers, reaction mixtures and PCR conditions described by Epelde *et al*. (2014) (Table 4.1):

Table 4.1. Primers and PCR conditions for qPCR assays.

Primers	**PCR conditions**	**References**
qPCR for total fungi: Fung5F and FF390R	95ºC for 30 s, 94ºC for 30 s, 52ºC for 30 s, 72ºC for 1 min (40 cycles); 95ºC for 15 s, 60ºC for 1 min, 95ºC for 30 s for the melt curve, final extension of 60 ◦C for 15 s	Lueders *et al*. (2004)
qPCR for total bacteria: Ba519F and Ba907R	95ºC for 30 s, 94ºC for 30 s, 50ºC for 30 s, 72ºC for 1 min (40 cycles); 95ºC for 15 s, 60ºC for 1 min, 95ºC for 30 s for the melt curve, final extension of 60ºC for 15 s	Lueders *et al*. (2004)

4.2.3. Gene amplification, sequencing and data processing

16S rRNA gene amplicon libraries were prepared using primers 519F (CAGCMGCCGCGGTAA) adapted from Øvreås *et al*. (1997) and 806R (GGACTACHVGGGTWTCTAAT) modified from Caporaso *et al*. (2012), targeting the V4 hypervariable region. For the 18S rRNA, primers 1183F (AATTTGACTCAACRCGGG) and 1443R (GRGCATCACAGACCTG) (Ray *et al*., 2016) were used. Amplification was carried out using a dual indexing approach modified from Lanzén *et al*. (2016). Briefly, adapter-linked forward and reverse primers were used in the first amplification step using the following reaction, to a total of 20 µl volume: 1 µl template community DNA; 1 µM each of forward and reverse primers; and 1x KAPA3G Plant PCR mix (Kapa Biosystems, Wilmington MA). The following PCR parameters were used: initial denaturation at 95ºC for 15 min, followed by 25 cycles of 95ºC for 20 s, 55ºC for 30 s, 72ºC for 30 s with a final extension at 72ºC for 7 min. Amplicon libraries were cleaned using AMPure XP (Beckman Coulter Genomics). Barcoded primers were used in the second amplification step (10 cycles) to a total of 50 µl volume as described in Lanzén *et al*. (2016). Sequencing was carried out using an Illumina MiSeq with the V2 kit and pair-ended 2x250 nt at Tecnalia, Spain.

Read-pairs from 16S rRNA amplicons were overlapped using *vsearch* (Rognes *et al*., 2016; options *fastq_maxdiff=5, fastq_allowmergestagger*). Overlapped reads were trimmed from primer sequences from both ends using *cutadapt* (Martin, 2011) and then truncated using *vsearch--fastq_filter* to a length of 252 nt (discarding shorter sequences and those with >0.5 expected errors). 18S rRNA gene reads were treated in the same manner except for truncation due to their length polymorphism in the targeted region. Reads shorter than 200 nt were discarded.

All quality-filtered overlapped sequences from 16S rRNA and 18S rRNA amplicons, respectively, were first clustered into fine-scale OTUs using SWARM v2 Mahé *et al*., (2015), then subjected to reference-based (with the *rdp_gold dataset for 16S and SilvaMod106 for 18S)* and thereafter *de novo* chimera filtering, using UCHIME as implemented in *vsearch* (Rognes *et al*., 2016). Resulting representative (centroid) sequences from chimera-filtered OTUs were thereafter subjected to a second clustering using *vsearch* with 97 and 98% similarity thresholds for 16S and 18S rRNA sequences, respectively (Rognes *et al*., 2015).

Representative OTU sequences were aligned to the SilvaMod v106 (16S and 18S rRNA) reference databases using *blastn* (v.2.2.25 + task megablast) and taxonomically classified with CREST using default parameters (Lanzén *et al*., 2012). Resulting taxon distributions were studied at order rank as determined by CREST.

4.2.4. Statistical analysis

The effects of corn stover incorporation (SI) *versus* corn stover removal (SR) on soil physicochemical and microbial properties were evaluated by Student´s t-test using IBM SPSS Statistics 21.0. Despite the limited number (n=3) of field replicates, all statistical tests were considered significant at $p<0.05$. Multivariate analyses were performed to explore relationships between experimental treatments and response variables using Canoco 5: (i) a principal component analysis (PCA) was performed on microbial activity and biomass parameters; (ii) a redundancy analysis (RDA) and variation partitioning analysis were performed in order to explore the effect of physicochemical parameters on biological parameters.

The Soil Quality Index (SQI) described by Mijangos *et al*. (2010) was calculated as follows:

$$SQI = 10^{logm+\frac{\sum_{i=1}^{n}(logn_i-logm)}{n}}$$

where *m* is the reference value (set here to 100% for mean values obtained in SR-treated soil) and *n* corresponds to the measured values for each parameter as a percentage of the reference value. For this calculation, the following parameters were taken into consideration: enzyme activities (β-glucosidase, arylsulphatase, alkaline phosphatase and urease activity), potentially mineralizable N, respiration, SIR, MBC, bacterial and fungal gene abundance, and Biolog™ data (AWCD, NUS, H').

R package *vegan* was used to perform multivariate statistics, diversity indices and visualization of amplicon sequencing data (Oksanen *et al*., 2015). Rarefied richness estimates interpolating the expected richness at the lowest sample-specific sequencing depth were used to compensate for variation in read numbers across samples. Function *decostand* was used to transform OTU distributions into relative abundances. Subsequent calculation of Bray-Curtis dissimilarity matrices comparing community composition between samples was performed as described by Lanzén *et al*. (2016).

These matrices were used to perform non-metric multidimensional scaling (NMDS) with function *metaMDS*. t-test was used to perform correlation analysis between treatments and taxon abundances or diversity parameters. Bayesian 95% credibility intervals of total 16S and 18S sample richness were estimated using the parametric method described by Quince *et al*. (2008).

4.3. Results

4.3.1. Crop yield

After 6 years of treatment, corn yield was significantly ($p<0.05$) higher in SI-treated (33.8 Mg ha^{-1}) *versus* SR-treated (28.3 Mg ha^{-1}) plots.

4.3.2. Soil physicochemical parameters

Soil physicochemical parameters, after 6 years of stover removal led to significantly ($p<0.01$) lower values of SOC than stover incorporation (Table 4.2). From the first to the sixth year of the experiment, SOC values decreased from 20.1 to 14.7 g SOC kg^{-1} dry weight (DW) soil (corresponding values in plots where corn stover was annually incorporated were 18.7 and 18.9 g kg^{-1} DW soil). No significant differences were detected between SI-treated and SR-treated soil for the other physicochemical parameters.

Table 4.2. Effect of treatments (SI: stover incorporation; SR: stover removal) on soil physicochemical parameters. Mean values (n = 3) ± SD.

	SR	**SI**
pH	8.75 ± 0.02	8.73 ± 0.08
Carbonate (%)	27.22 ± 4.78	27.66 ± 5.82
Limestone (%)	8.80 ± 2.15	9.63 ± 2.29
Nitrate (mg kg^{-1})	16.15 ± 9.00	21.44 ± 13.59
Ammonium (mg kg^{-1})	0.41 ± 0.11	0.48 ± 0.10
Total N (%)	0.12 ± 0.01	0.14 ± 0.01
SOC (g kg^{-1})	14.7 ± 0.1	18.9 ± 1.2 **
Al (mg kg^{-1})	31.85 ± 3.7	29.96 ± 3.4
P (mg kg^{-1})	0.69 ± 0.16	0.68 ± 0.09
Ca (mg kg^{-1})	113.00 ± 12.9	109.45 ± 14.8
Mg (mg kg^{-1})	6.03 ± 0.50	5.43 ± 0.52
Na (mg kg^{-1})	1.02 ± 0.13	0.86 ± 0.08
K (mg kg^{-1})	10.98 ± 0.78	10.33 ± 0.85
S (mg kg^{-1})	0.65 ± 0.39	0.89 ± 0.40
Fe (mg kg^{-1})	21.17 ± 1.61	19.97 ± 1.80
Mn (µg kg^{-1})	356.51 ± 57.6	315.93 ± 37.8
Mo (µg kg^{-1})	0.65 ± 0.01	0.65 ± 0.01
Cu (µg kg^{-1})	17.17 ± 3.7	17.49 ± 2.3
Zn (µg kg^{-1})	68.20 ± 11.8	58.96 ± 6.9
Cd (µg kg^{-1})	0.97 ± 0.07	1.04 ± 0.06
Pb (µg kg^{-1})	16.20 ± 7.8	14.09 ± 1.1
Cr (µg kg^{-1})	15.19 ± 4.7	14.84 ± 1.4
Ni (µg kg^{-1})	24.69 ± 7.9	20.38 ± 1.9
As (µg kg^{-1})	12.25 ± 1.13	12.77 ± 0.17

**Significant ($p<0.01$) differences between the two treatments according to Student's t-test.

4.3.3. Soil microbial parameters

No significant differences were observed between treatments (SI *versus* SR) for any of the measured parameters on soil microbial activity; however, all of them showed higher values in SI-treated *versus* SR-treated soil (Table 4.3). Soil microbial biomass, values of SIR and bacterial gene abundance were significantly ($p<0.05$) higher in SI-treated than in SR-treated soil. The same trend was observed for the other parameters used to determine soil microbial biomass, but, in this case, differences were not statistically significant. None of the parameters for microbial activity or microbial biomass showed lower values in SI-treated than in SR-treated soil, indicating a stimulation of these parameters as a result of the incorporation of corn stover (Figure 4.1). In fact, in relation

to the values of soil microbial activity and biomass, both treatments were separated by the PCA (Figure 4.2A). According to the variation partitioning and RDA analysis (Figure 4.2B), SOC and nitrates correlated positively (pseudo-F= 4.2, $p<0.01$) with values of soil microbial activity and biomass parameters, and accounted for 78% of the explained variation. Individually, soil nitrate content (pseudo-F= 4.7, $p<0.05$) and SOC (pseudo-F= 3.2, $p<0.05$) correlated positively with microbial biomass and activity, and accounted for 72.4 and 42.0% of the explained variation, respectively.

Table 4.3. Effect of treatments (SI: stover incorporation; SR: stover removal) on soil microbial activity and biomass. Mean values (n= 3) ± SD.

	Enzyme	Units	SR	SI
ACTIVITY	Arylsulphatase	mg ρ-nitrophenol kg^{-1} DW h^{-1}	84.7 ± 7.8	104.1 ± 17.8
	Alkaline phosphatase	mg ρ-nitrophenol kg^{-1} DW h^{-1}	136 ± 19	174 ± 39
	β-Glucosidase	mg ρ-nitrophenol kg^{-1} DW h^{-1}	140 ± 13	168 ± 24
	Urease	mg $N\text{-}NH_4^+$ kg^{-1} DW h^{-1}	33.2 ± 1.4	46.3 ± 13.8
	Potentially mineralizable N	mg $N\text{-}NH_4^+$ kg^{-1} DW h^{-1}	1.3 ± 1.0	2.5 ± 1.3
	Basal respiration	µg C g^{-1} DW soil h^{-1}	0.74 ± 0.04	0.83 ± 0.10
BIOMASS	Substrate-induced respiration	µg C g^{-1} DW soil h^{-1}	2.5 ± 0.2	3.1 ± 0.2 *
	Microbial biomass carbon	mg C kg^{-1} DW soil	176 ± 44	238 ± 55
	Bacterial gene abundance	x 10^{10} copies g^{-1} DW soil	1.05 ± 0.25	1.55 ± 0.11*
	Fungal gene abundance	x 10^{8} copies g^{-1} DW soil	1.18 ± 0.6	1.67 ± 0.2

*Significant ($p<0.05$) differences between the two treatments according to Student's t-test.

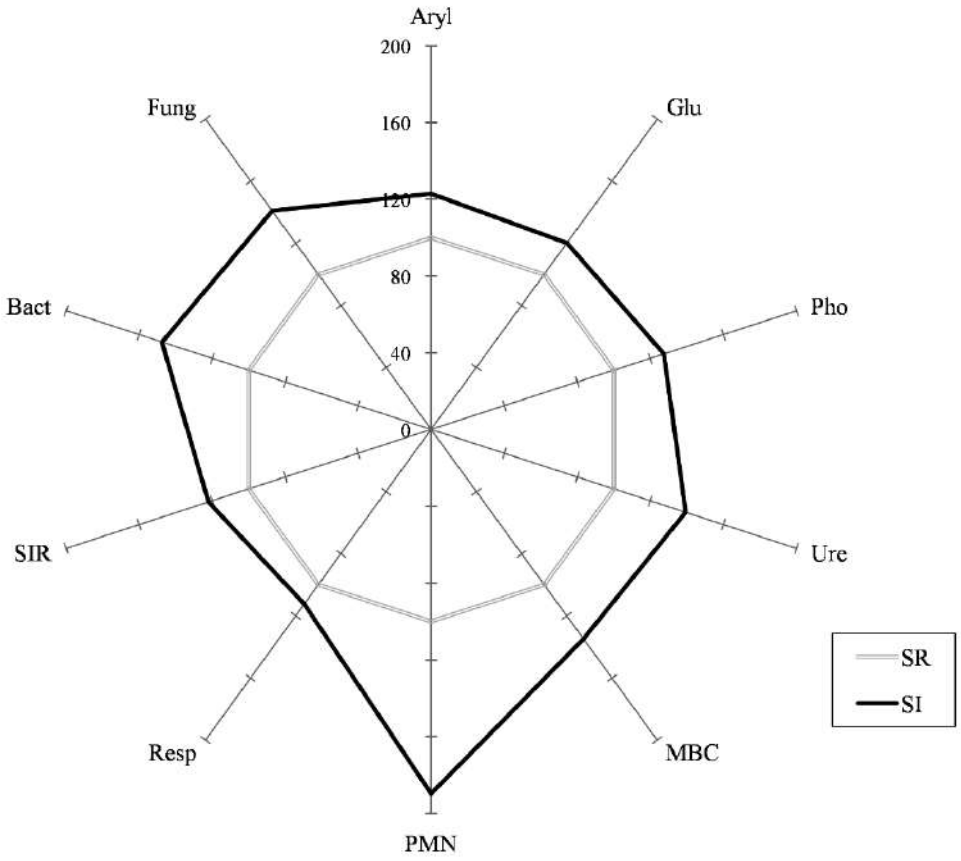


Figure 4.1. Sunray plot of soil microbial activity and biomass parameters. A value of 100 corresponds to the mean value obtained for each parameter in the stover removal treatment. SI: stover incorporation; SR: stover removal; Aryl: arylsulphatase; Pho: alkaline phosphatase; Glu: β-glucosidase (mg ρ-nitrophenol kg^{-1} DW soil h^{-1}); Ure: urease; PMN: potentially mineralizable N (mg N-NH_4^+ kg^{-1} DW soil h^{-1}); Resp: Respiration (µg C g^{-1} DW soil h^{-1}); SIR: substrate-induced respiration (µg C g^{-1} DW soil h^{-1}); MBC: microbial biomass carbon (mg C kg^{-1} DW soil); Bact: total bacterial gene abundance (x 10^{10} copies g^{-1} DW soil); Fung: total fungal gene abundance (x 10^8 copies g^{-1} DW soil).

The effect of treatments on soil microbial functional diversity was estimated from the community-level physiological profiles obtained from Biolog EcoPlates™ data. In particular, the average well colour development (AWCD), the number of utilized substrates (NUS) and the Shannon's diversity index (H') were calculated for both treatments, resulting in no clear differences between SI-treated and SR-treated plots: AWCD, NUS and H' values were 1.08, 24.65 and 4.43, respectively, in SI-treated soil; corresponding values for SR-treated soil were 0.97, 24.83 and 4.49. However, regarding the pattern of use of specific carbon substrates, D-xylose and D-mannitol were used to a significantly ($p<0.05$) greater extent in SI-treated than SR-treated soil (Table 4.S1).

Values of the SQI were significantly ($p<0.05$) higher in SI-treated than in SR-treated plots (SQI = 124±8 and 98±13 in SI-treated and SR-treated soil, respectively).

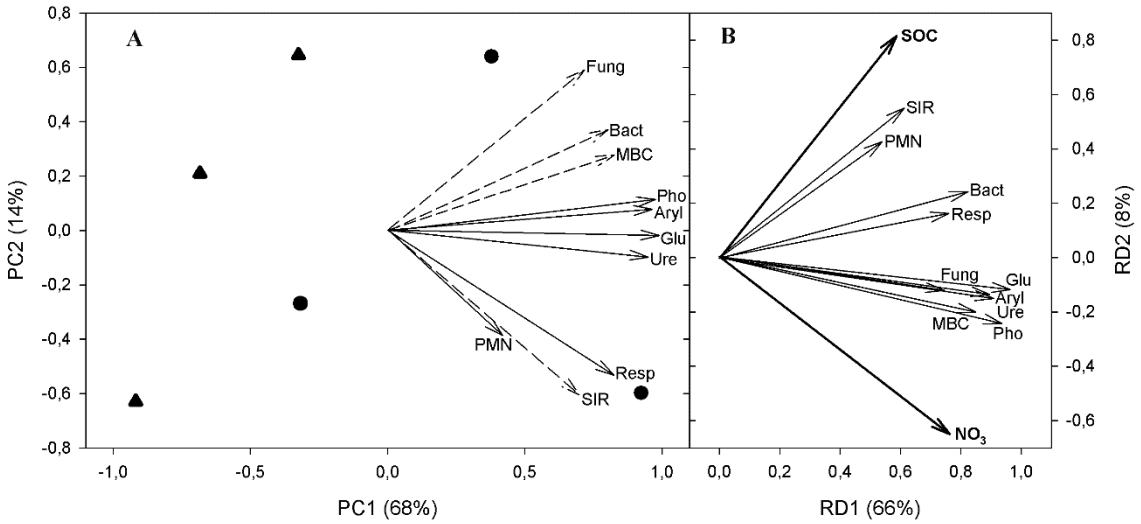


Figure 4.2. Biplots of: **(A)** Principal component analysis (PCA) of soil microbial activity (solid arrows) and biomass (dashed arrows) parameters under stover incorporation (circles) and stover removal (triangles); PC1 and PC2 account for 68 and 14% of the variance, respectively; **(B)** Redundancy analysis (RDA) explaining the variation of soil microbial activity and biomass parameters explained by SOC and nitrate concentration (bold arrows); RDA1 and RDA2 account for 66 and 8% of the variance, respectively. Pho: alkaline phosphatase; Glu: β-glucosidase; Aryl: arylsulphatase; Ure: urease; PMN: potentially mineralizable N; MBC: microbial biomass C; Resp: basal respiration; SIR: substrate-induced respiration; Bact: total bacterial gene abundance (x 10^{10} copies g^{-1} DW soil); Fung: total fungal gene abundance (x 10^{8} copies g^{-1} DW soil); SOC: soil organic carbon; NO_3^-: soil nitrate concentration.

In relation to soil microbial genetic diversity and composition, amplicon sequencing resulted in over 801,183 prokaryotic 16S rRNA sequences clustered into 14,833 OTUs, while 662,595 eukaryotic 18S rRNA reads clustered into 6,357 OTUs, after quality filtering and removal of singletons. The number of reads correlated significantly with total OTU richness for 16S data ($p=0.016$), indicating that the sequencing effort was insufficient to obtain full coverage of soil prokaryotic diversity. Instead, rarefied and Bayesian parametric richness estimates were used. Regarding 18S, there was no significant correlation between number of reads and OTU richness ($p=0.39$), but differences in number of reads between samples were so marked (sample with less reads = 91,180; sample with more reads = 131,600) that rarefied richness estimates were also used. These data, together with other alpha diversity parameters for prokaryotic and eukaryotic communities in SI-treated and SR-treated plots, are shown in Table 4.4 and Figure 4.3. Mean values for rarefied richness, Pielou's evenness and Shannon's index

showed no differences between treatments for none of the evaluated amplicon data. Nonetheless, the Bayesian parametric estimates of diversity showed an increasing trend in eukaryotic OTU richness for the SI-treated plots, with minimum, maximum and median values rated higher in all SI-treatment samples for both prokaryotic (16S; Figure 4.3A) and eukaryotic communities (18S; Figure 4.3B).

Table 4.4. Effect of treatments (SI: stover incorporation; SR: stover removal) on soil microbial diversity. Mean values (n = 3) ± SD.

	16S rRNA			18S rRNA		
	RR	**H'**	**J'**	**RR**	**H'**	**J'**
SR	6896 ± 551	7.2 ± 0.2	0.81 ± 0.01	2393 ± 72	4.9 ± 0.2	0.6 ± 0.03
SI	7236 ± 105	7.2 ± 0.1	0.81 ± 0.01	2453 ± 181	4.8 ± 0.3	0.6 ± 0.04

RR: rarefied richness; H': Shannon´s diversity index; P': Pielou's evenness.

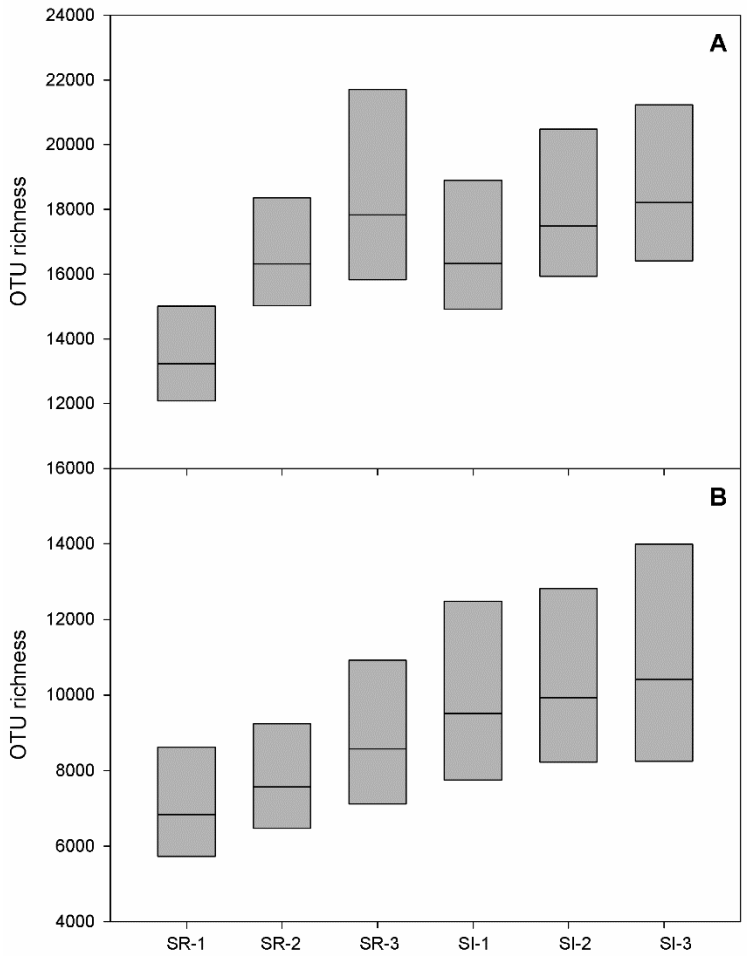


Figure 4.3. Boxplots showing Bayesian parametric estimates for (A) prokaryotic 16S and (B) eukaryotic 18S amplicon diversity data for each sample. SR-1, SR-2, SR-3: stover removal replicates; SI-1, SI-2, SI-3: stover incorporation replicates.

The NMDS performed based on Bray-Curtis dissimilarities of community composition (Figure 4.4) did not separate soil sample communities by treatment into clear groups for any of the amplicons. No big differences at phylum rank could be observed for bacterial, protist and fungal community compositions between treatments (Figure 4.5).

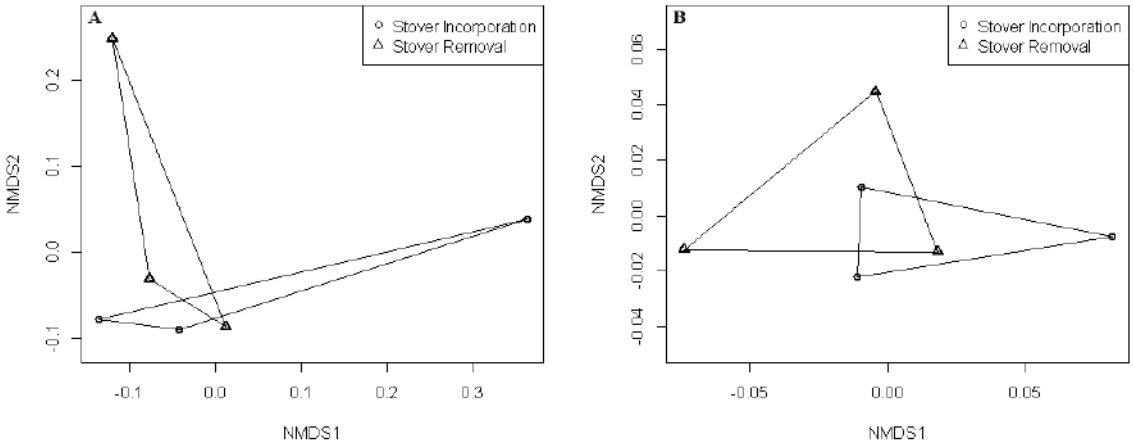
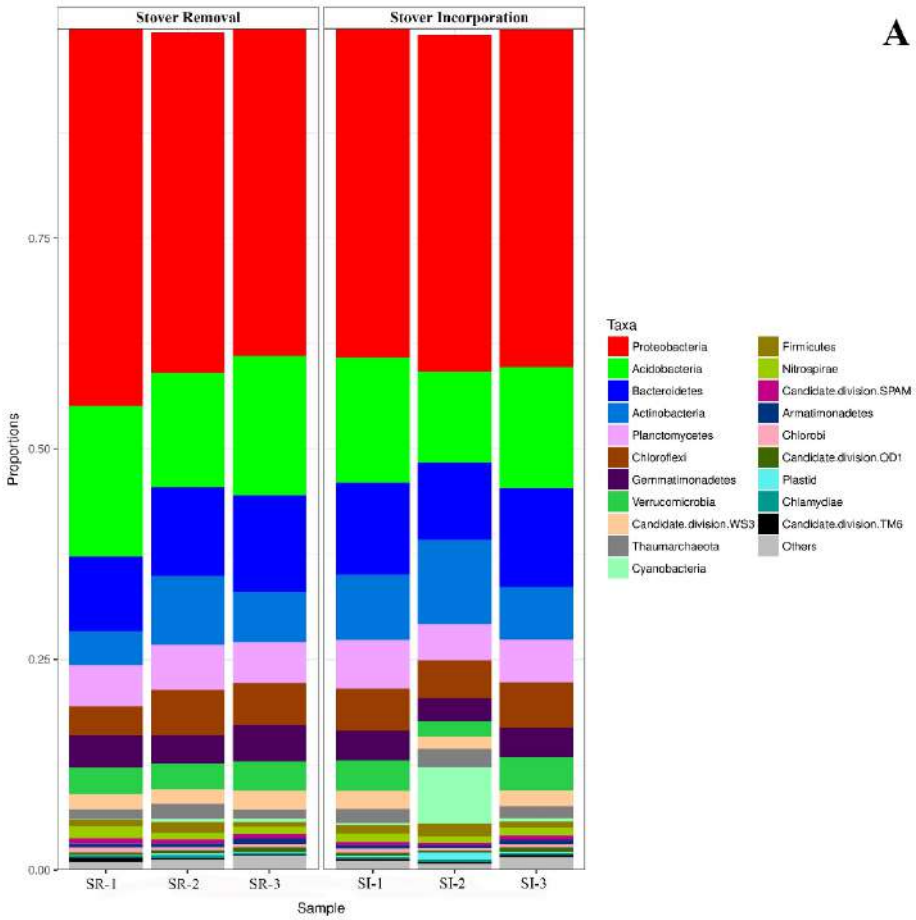


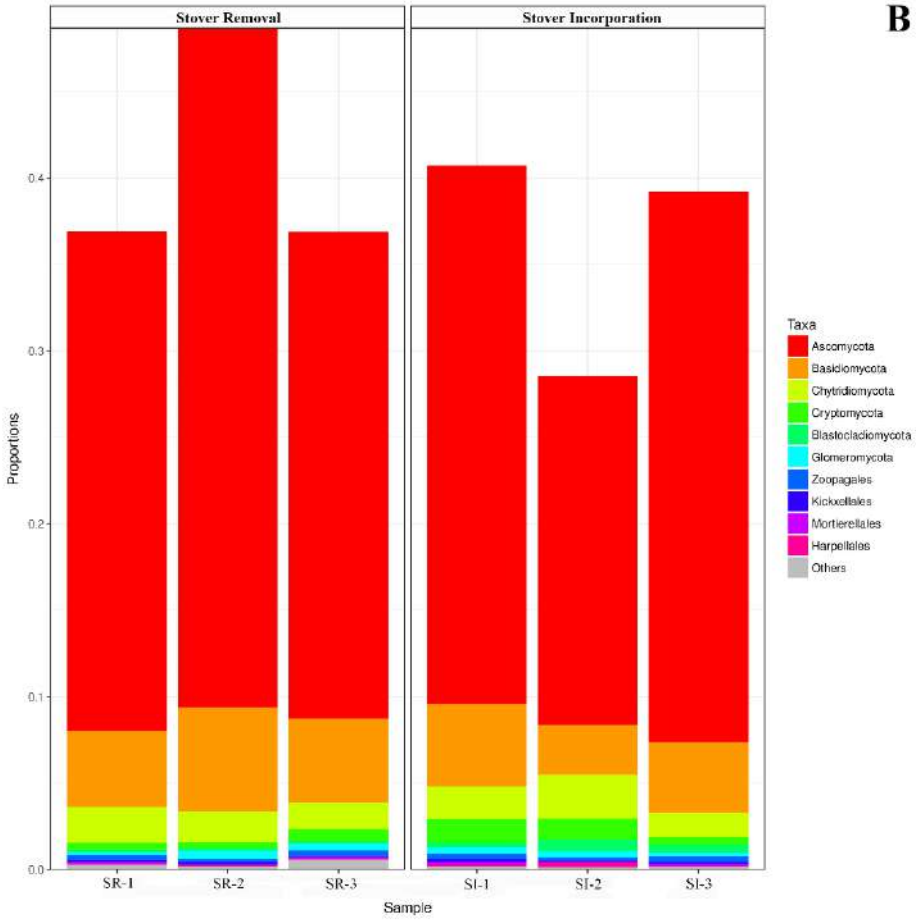
Figure 4.4. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of soil microbial community composition. Composition was based on relative OTU abundances from (A) prokaryotic 16S and (B) eukaryotic 18S amplicon data. Samples are labelled according to legend.

Stover management did not have a significant impact on dominant bacterial community composition, as no differences in relative abundances were found at phylum or class level (Table 4.S2). However, some differences ($p<0.05$) between treatments at order level could be observed on the abundance of rare taxa such as *Desulfurellales* (relative abundance of 0.17 and 0.12% in SI-treated and SR-treated plots, respectively), *Acidobacteria group 15 - JG37-AG-116* (0.06 and 0.08%) and *Acidobacteria group 1* (0.02 and 0.04%).

In relation to fungal community composition, no differences between treatments were observed on dominant taxa. Nevertheless, at phylum level, *Mucorales* showed higher relative abundance ($p<0.05$) in SR-treated soil (0.10 and 0.03% in SR-treated and SI-treated soil, respectively). *Wallemiales*, a fungal taxonomic order containing just one family, *Wallemiaceae*, appeared to be more abundant in plots where corn stover was incorporated (0.002% in SI-treated plots *versus* no abundance in SR-treated plots).

Finally, the protists community did not show any differences among taxa, neither for the dominant nor for the rare taxa (Table 4.S2).


A
Stover Removal
Stover Incorporation
Proportions
0.00
0.25
0.50
0.75
SR-1
SR-2
SR-3
SI-1
SI-2
SI-3
Sample
Taxa
Proteobacteria
Acidobacteria
Bacteroidetes
Actinobacteria
Planctomycetes
Chloroflexi
Gemmatimonadetes
Verrucomicrobia
Candidate.division.WS3
Thaumarchaeota
Cyanobacteria
Firmicutes
Nitrospirae
Candidate.division.SPAM
Armatimonadetes
Chlorobi
Candidate.division.OD1
Plastid
Chlamydiae
Candidate.division.TM6
Others


B
Stover Removal
Stover Incorporation
Proportions
0.0
0.1
0.2
0.3
0.4
SR-1
SR-2
SR-3
SI-1
SI-2
SI-3
Sample
Taxa
Ascomycota
Basidiomycota
Chytridiomycota
Cryptomycota
Blastocladiomycota
Glomeromycota
Zoopagales
Kickxellales
Mortierellales
Harpellales
Others

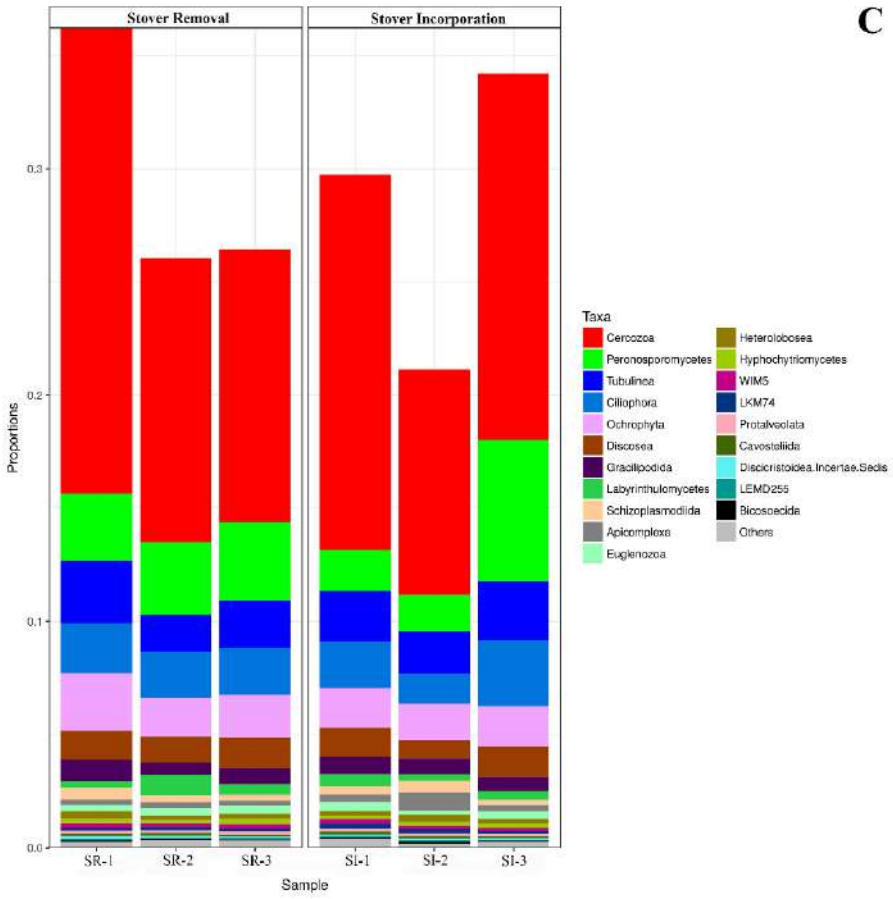


Figure 4.5. Barplots representing the taxonomic composition distribution at phylum level of the 20 most abundant prokaryotic taxa (A), 10 most abundant fungal taxa (B), and 20 most abundant protist taxa (C). S-1, SR-2, SR-3: stover removal samples; SI-1, SI-2, SI-3: stover incorporation samples.

4.4. Discussion

There is increasing interest in the use of crop residues (*e.g.*, corn stover) to improve soil fertility and quality, leading to a reduction in the use of synthetic chemical fertilizers. Here, corn yield was significantly higher in SI-treated *versus* SR-treated plots after 6 years of treatment. Values of mineral and total nitrogen were greater (though differences were not statistically significant) in SI-treated plots, which could most likely explain the higher crop yields under stover incorporation. Our corn stover had a high C/N ratio (42:1), which favours N immobilization over soil N mineralization by soil microbial populations (Taylor *et al*., 2002). Stover incorporation has been reported to have no impact (Karlen *et al*., 1994; Linden *et al*., 2000) or to cause a positive effect (Blanco-Canqui and Lal, 2007; Varvel *et al*., 2008; Karlen *et al*., 2014) on crop yield, depending on, among other factors, the duration of the treatment and the specific soil characteristics, etc.

In any case, values of SOC were significantly different between treatments (SI *versus* SR). The content of SOC decreased progressively in SR-treated plots throughout the 6-year experimental period, as previously reported in many studies (Blanco-Canqui and Lal, 2007; Halpern *et al*., 2010; Stetson *et al*., 2012). Many authors (Mann *et al*., 2002; Zhao *et al*., 2015) have studied the impact of stover management on carbon and other nutrients dynamics. Interestingly, Blanco-Canqui and Lal (2009) concluded that *ca.* 25% of corn stover could be removed from the field without negatively affecting SOC, soil fertility and structural stability. In any case, that conclusion can only be applied to the specific soil-climatic conditions present in their experimental site. An increase in SOC values can be beneficial from a variety of viewpoints, such as mitigation of global warming, crop productivity, soil and water quality, nitrogen dynamics, soil resistance to erosion, etc. (Mann *et al*., 2002).

Johnson *et al*. (2010) found their corn stover (stalk and cob) to contain 19.4 g N kg^{-1}, 2.7 g P kg^{-1}, 25.6 g K^+ kg^{-1}, 1.4 g S kg^{-1} and 6.5 g Ca^{2+} kg^{-1}, with concomitant consequences for the nutrient supply of crops and soil microbial communities. However, in this case significant differences were not observed between treatments regarding the content of soil nutrients. In contrast, when stover was removed from the plots at a high rate, Karlen *et al*. (2014) reported a decrease in N, P and K^+ concentrations in soil. Similarly, Blanco-Canqui and Lal (2009) observed that the soil content of available P and exchangeable K^+, Ca^{2+} and Mg^{2+} decreased as a result of stover removal at a soil depth of 0-10 cm. After 5 years of stover removal, Villamil and Nafziger (2015) also observed a reduction in soil P and K^+ at 0-15 and 15-30 cm depth intervals. Finally, in a long-term experiment, Rogovska *et al*. (2016) reported significant differences on soil Zn concentration between no-removal and 90% stover removal, but no changes were observed for the other nutrients studied (*i.e.*, P, S, K^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}) at a soil depth of 0-15 cm). Our results correspond to a soil depth of 0-30 cm, comprising the whole plow layer. It has been stated that analyzing soil depth covering the entire plow layer is imperative when assessing the effects of different agricultural practices on soil quality (Mijangos and Garbisu, 2010).

A proper assessment of soil quality requires the consideration of physical, chemical and biological indicators (Karlen *et al*., 1994; Moebius-Clune *et al*., 2008). In particular, microbial parameters are increasingly being used as indicators of the impact of disturbances on soil quality, owing to their sensitivity, rapid response, integrative character and ecological relevance (Alkorta *et al*., 2003).

Soil microbial communities have a key role in the decomposition of plant material. Here, although no significant differences were observed in terms of soil microbial activity between SI-treated and SR-treated plots, all of the parameters determined here to quantify microbial activity (*i.e.*, β-glucosidase, arylsulphatase, alkaline phosphatase and urease enzyme activities; potentially mineralizable N; respiration) did show higher values in SI-treated *versus* SR-treated soils, suggesting, in agreement with other studies (Moebius-Clune *et al*., 2008; Johnson *et al*., 2013), that stover removal reduces soil microbial activity, with concomitant consequences for soil functioning. The lack of statistical significance observed here is due to the high variation among experimental replicates, in spite of the fact that composite soil samples following a random pattern were collected. Besides, despite the limited number (n=3) of field plot replicates, differences were considered significant only at $p<0.05$.

On the other hand, soil microbial biomass, as reflected by the values of SIR and bacterial gene abundance, significantly decreased in SR-treated plots, compared to SI-treated plots. Values of fungal gene abundance and MBC increased in SI-treated compared to SR-treated soils, but, in this case, differences were not statistically significant. In their long-term (>10 years) studies, Halpern *et al*. (2010) and Sheibani *et al*. (2013) reported a reduction in soil microbial biomass as a result of stover removal. Furthermore, Kushwaha *et al*. (2000) found a decrease in soil MBC after just one season of stover removal. Values of SOC correlated positively with values of microbial activity and biomass parameters (Figure 4.2B), suggesting that the reduction in SOC content caused by stover removal is responsible for the observed decrease in soil microbial activity and biomass. The availability of organic C is a well-known factor limiting the activity of heterotrophic microorganisms. A decreased microbial activity and biomass can lead to an alteration of soil quality, as microbial communities play a key role in essential soil functions and ecosystem services (Burges *et al*., 2015). In concordance with our results, Kushwaha *et al*. (2000) reported that an increased supply of carbon from residue incorporation increased soil microbial activity and biomass. However, after 16 years of study, Halpern *et al*. (2010) reported that values of soil microbial biomass and activity are not always clearly reflected in the SOC pool.

In contrast, functional microbial diversity, as reflected by the values of AWCD, NUS and H' from Biolog EcoPlates™, did not vary between treatments. Nonetheless, stover incorporation did have an impact on the carbon substrate utilization patterns of cultivable soil bacterial communities, as the rates of D-xylose and D-mannitol utilization

increased in SI-treated *versus* SR-treated plots. The monosaccharide D-xylose has been reported as a constituent of root exudates (Campbell *et al*., 1997). Xylans, polysaccharides made from units of xylose, represent 21% of the total dry weight of corn stover (Huang *et al*., 2009), explaining the higher rates of D-xylose utilization in S-treated soils. D-mannitol is a constituent of corn stover (Chen *et al*., 2007), which can lead to a higher presence of this carbon source in SI-treated soil and then explain its higher utilization under stover incorporation. Here, it must be taken in consideration that Biolog EcoPlates™ data reflect the responses of only the cultivable, heterotrophic, fast growing portion of the soil bacterial community. In any case, it has been extensively proven that Biolog™ community-level physiological profiles provide an insight into the functional capacity of bacterial communities and enable the establishment of comparisons between microbial communities (Preston-Mafham *et al*., 2002).

The SQI is very useful for management purposes as it can integrate the responses of many different parameters, thus providing an overall representation of soil status (Mijangos *et al*., 2010). Here, the SQI was used to provide an integrative analysis of the different microbial parameters studied here. According to this index, the incorporation of stover posed an overall positive effect on soil microbial communities. This is not surprising as soil microbial communities depend on the carbon and energy provided by the organic matter entering the soil ecosystem.

There are not many studies on the impact of stover management on the structure and composition of soil microbial communities. Johnson *et al*. (2013) performed a fatty acid methyl ester (FAME) analysis to study the effect of stover incorporation on microbial community composition, reporting no clear differences between treatments involving a high rate *versus* a low rate of stover incorporation. Lehman *et al*. (2014) reported a reduction in fungi-to-bacteria ratio under stover removal, as reflected by FAME patterns. After 15 years of study, DGGE analysis yielded no differences in the composition of bacterial dominant taxa as a result of stover removal (Sheibani *et al*., 2013). Through the analysis of phospholipid fatty acid (PLFA) and DGGE profiles, Orr *et al*. (2015b) reported that a reduction in the amount of residue incorporated to the soil surface did not alter the overall microbial community structure.

To our knowledge, there are no reports on the application of metabarcoding analysis to determine the effects of corn stover management on soil microbial communities. Our metabarcoding results did not show any significant differences in terms of genetic diversity between SI-treated and SR-treated soil microbial communities. Yet,

Bayesian parametric estimation of richness showed higher values of fungal extrapolated richness in SI-treated plots, but differences were not statistically significant. In addition, for both bacterial and fungal communities, the ordination (NMDS) based on OTU composition (Bray-Curtis dissimilarity matrices) of individual samples did not form well-defined clusters for the applied treatments, indicating the lack of clear differences in microbial community composition between both treatments. Furthermore, stover management did not have an impact on soil protist community composition (no differences were observed among taxa at phylum, class and order rank between treatments). Similarly, dominant bacterial and fungal taxa were not affected by treatments. However, some differences were found among rare taxa at different taxonomic levels. In relation to fungal communities, stover removal yielded a significantly higher relative abundance of *Mucorales*, while affecting negatively the relative abundance of *Wallemiales* (Table 4.S2). The *Wallemiales* order contains xerophilic fungi that have been isolated from food and soil (Zalar *et al*., 2005). The relative abundance of the bacterial order *Desulfurellales* was higher in SI-treated soil. The *Desulfurellales* order comprises just one family (*Desulforaceae*) and includes hydrocarbon-degrading sulfate-reducing bacteria (Kleindienst *et al*., 2014). Then, the abovementioned higher relative abundance values in SI-treated plots could be due to the fact that, in general, these plots presented a higher content of soil sulfur: 0.89 mg kg^{-1} DW soil in SI-treated soil (*versus* 0.65 mg kg^{-1} DW soil in SR-treated soil). In turn, stover removal increased the relative abundance of two *Acidobacteria* orders (*Acidobacteria group 15 - JG37-AG-116* and *Acidobacteria group 1*). The composition of microbial communities can have a considerable impact on soil functioning and, in particular, on the soil food web dynamics (Morriën, 2016). Awareness of the role that rare microorganisms play in community function is increasing as these taxa may, for instance, confer resilience to the soil ecosystem (Jousset *et al*., 2017). Nevertheless, microbial dominant taxa did not show any significant differences between treatments, suggesting that significant changes in soil functionality have most likely not been produced as a consequence of stover removal.

4.5. Conclusion

This study provides insights regarding the benefits of corn stover incorporation (as compared to stover removal) on soil quality. Incorporation of stover enhanced soil nitrogen and organic carbon pools, which correlated positively with the increased values of soil microbial activity and biomass. Although soil structural diversity and the composition of dominant taxa were not affected by stover management, stover incorporation stimulated soil microbial activity, induced changes in the composition of soil prokaryotic and eukaryotic rare taxa, and affected soil functional diversity (by enhancing D-xylose and D-mannitol utilization rates). Our findings suggest that corn stover incorporation is a beneficial agronomic practice for soil quality.

4.6. Supplementary information

Table 4.S1. Effect of treatments (SI: stover incorporation; SR: stover removal) on carbon substrate utilization patterns obtained with Biolog EcoPlates™.

		Abs (595 nm)		
Carbon Source		**SR**	**SI**	**p**
Amines /amides	Phenylethylamine	0.72	1.13	0.32
	Putrescine	1.75	1.26	0.15
Amino acids	L-Arginine	1.84	1.94	0.81
	L-Asparagine	2.33	2.12	0.29
	L-Phenylalanine	0.41	0.80	0.45
	L-Serine	2.05	2.00	0.75
	L-Threonine	0.32	0.29	0.94
	Glycyl-L-Glutamic Acid	0.15	0.46	0.14
Carbohydrates	C-Cellobiose	1.91	1.87	0.90
	D-Lactose	1.10	1.29	0.59
	Methyl-D-Glucoside	1.87	1.71	0.56
	D-Xylose	0.16	1.09	0.04 *
	i-Erythritol	0.20	0.39	0.17
	D-Mannitol	1.76	2.29	0.03 *
	N-Acetyl-D-glucosamine	1.90	2.00	0.69
Carboxylic acids	D-Glucosaminic acid	0.55	0.94	0.61
	D-Galactonic acid lactone	1.58	1.84	0.51
	D-Galacturonic acid	1.94	1.99	0.83
	2-Hydroxy benzoic acid	0.28	0.23	0.92
	4-Hydroxy benzoic acid	1.88	2.04	0.41
	Hydroxy butyric Acid	0.39	0.50	0.62
	Traconic acid	0.43	0.46	0.88
	Keto Butyric acid	0.19	0.25	0.87
	D-Malic acid	1.76	1.73	0.92
Miscellaneous	Pyrubic Acid Methyl Ester	1.44	1.40	0.72
	Glucose-1-Phosphate	1.32	1.47	0.39
	D,L-Glycerol Phosphate	0.38	0.50	0.09
Polymers	Tween 40	1.18	1.46	0.16
	Tween 80	1.59	1.66	0.86
	Cyclodextrin	0.82	0.97	0.79
	Glycogen	1.50	1.62	0.86

*Significant ($p<0.05$) differences between treatments according to Student's t-test.

Table 4.S2. Effect of treatments (SI: stover incorporation; SR: stover removal) on the abundance of soil bacterial, fungal and protist taxa.

BACTERIA TAXA	**Relative Abundance**		
Phylum	**SR**	**SI**	**Significance ($p<0.05$)**
Proteobacteria	0.41180	0.39482	ns
Acidobacteria	0.15899	0.13294	ns
Bacteroidetes	0.10258	0.10585	ns
Planctomycetes	0.04997	0.05016	ns
Actinobacteria	0.06089	0.07984	ns
Gemmatimonadetes	0.03775	0.03266	ns
Chloroflexi	0.04621	0.04944	ns
Verrucomicrobia	0.03218	0.03105	ns
Candidate division WS3	0.01952	0.01789	ns
Nitrospirae	0.01048	0.00864	ns
Firmicutes	0.00892	0.01109	ns
Chlorobi	0.00474	0.00387	ns
Candidate division SPAM	0.00519	0.00429	ns
Candidate division TM6	0.00266	0.00216	ns
Armatimonadetes	0.00495	0.00395	ns
Candidate division OD1	0.00412	0.00393	ns
Chlamydiae	0.00269	0.00231	ns
Candidate division BRC1	0.00146	0.00124	ns
JL-ETNP-Z39	0.00125	0.00126	ns
Candidate division OP11	0.00115	0.00077	ns
Cyanobacteria	0.00253	0.02440	ns
Elusimicrobia	0.00146	0.00157	ns
Candidate division TM7	0.00098	0.00060	ns
SM2F11	0.00077	0.00060	ns
Candidate division OP3	0.00101	0.00090	ns
WCHB1-60	0.00032	0.00028	ns
BD1-5	0.00034	0.00034	ns
Thermotogae	0.00015	0.00013	ns
Fibrobacteres	0.00043	0.00056	ns
Spirochaetes	0.00038	0.00033	ns
Candidate division GN04 (TA06)	0.00012	0.00005	ns
Lentisphaerae	0.00005	0.00005	ns
MVP-21	0.00003	0.00004	ns
BHI80-139	0.00005	0.00005	ns
Deinococcus-Thermus	0.00002	0.00001	ns
PAUC34f	0.00001	0.00000	ns
Candidate Division Caldithrix	0.00001	0.00002	ns
GOUTA4	0.00000	0.00001	ns
Candidate division NC10	0.00005	0.00003	ns
Candidate division SR1	0.00003	0.00004	ns
Synergistetes	0.00000	0.00000	ns
Tenericutes	0.00001	0.00001	ns
Class			
Acidobacteria (class)	0.14051	0.11918	ns
Alphaproteobacteria	0.12859	0.12081	ns
Gammaproteobacteria	0.12844	0.12724	ns
Betaproteobacteria	0.07618	0.06962	ns
Deltaproteobacteria	0.07184	0.07069	ns
Sphingobacteria	0.04613	0.04575	ns

Cytophagia	0.04466	0.04768	ns
Gemmatimonadetes (class)	0.03774	0.03265	ns
Actinobacteria (class)	0.03596	0.04504	ns
OPB35 soil group	0.02205	0.02052	ns
Planctomycetacia	0.02112	0.02414	ns
Nitrospirales	0.01048	0.00864	ns
Phycisphaerae	0.01226	0.01119	ns
Holophagae	0.01016	0.00775	ns
Chloroflexi Subdivision 2	0.01557	0.01713	ns
OM190	0.01068	0.00963	ns
RB25	0.00830	0.00599	ns
Thermoleophilia	0.01179	0.01791	ns
Flavobacteria	0.01093	0.01175	ns
Acidimicrobiia	0.00710	0.00853	ns
Bacilli	0.00743	0.00928	ns
Chlorobia	0.00321	0.00219	ns
Chloroflexi Subdivision 10	0.00493	0.00533	ns
Opitutae	0.00390	0.00417	ns
Spartobacteria	0.00371	0.00364	ns
Chloroflexi Subdivision 8 - TK10	0.00416	0.00404	ns
Chloroflexi Subdivision 6	0.00356	0.00344	ns
Anaerolineae	0.00497	0.00422	ns
Ktedonobacteria	0.00293	0.00245	ns
Pla3 lineage	0.00249	0.00180	ns
Armatimonadetes Group 5	0.00249	0.00192	ns
Caldilineae	0.00317	0.00360	ns
Chlamydiae (class)	0.00267	0.00228	ns
Thermomicrobia	0.00284	0.00388	ns
Rubrobacteria	0.00280	0.00403	ns
Verrucomicrobiae	0.00206	0.00230	ns
Pla4 lineage	0.00221	0.00215	ns
Chloroflexi (Class)	0.00308	0.00410	ns
Ignavibacteria	0.00154	0.00168	ns
Clostridia	0.00112	0.00142	ns
Armatimonadetes Group 4	0.00165	0.00145	ns
MB-A2-108	0.00157	0.00216	ns
Cyanobacteria (class)	0.00252	0.02440	ns
Elusimicrobia (class)	0.00145	0.00156	ns
Chtomonadetes	0.00057	0.00035	ns
vadinHA49	0.00059	0.00073	ns
BD7-11	0.00024	0.00018	ns
Rhodothermaceae incertae sedis	0.00021	0.00009	ns
OP3 subdivision I (NPL-UPA2)	0.00030	0.00021	ns
VC2.1 Bac22	0.00051	0.00040	ns
TA18	0.00021	0.00022	ns
Thermotogae (class)	0.00015	0.00013	ns
Armatimonadetes Group 1	0.00015	0.00015	ns
Erysipelotrichi	0.00030	0.00035	ns
Fibrobacteria	0.00043	0.00056	ns
S-BQ2-57 soil group	0.00023	0.00018	ns
Chloroflexi Subdivision 5 - SAR202 clade	0.00034	0.00043	ns
Candidatus Methylacidiphilum	0.00018	0.00019	ns
Spirochaetes (class)	0.00029	0.00029	ns
SPOTSOCT00	0.00005	0.00003	ns
Lentisphaeria	0.00005	0.00005	ns

Deinococci	0.00002	0.00001	ns
TakashiAC-B11	0.00009	0.00019	ns
Bacteroidia	0.00001	0.00000	ns
JTB23	0.00004	0.00002	ns
Nitriliruptoria	0.00003	0.00004	ns
SM1A07	0.00001	0.00001	ns
028H05-P-BN-P5	0.00001	0.00002	ns
Order (100 more abundant)			
Xanthomonadales	0.10281	0.09425	ns
Acidobacteria group 6 (DA023)	0.08400	0.07640	ns
Sphingomonadales	0.05055	0.03758	ns
Sphingobacteriales	0.04612	0.04572	ns
Cytophagales	0.04462	0.04763	ns
Nitrosomonadales	0.03769	0.03248	ns
Rhizobiales	0.03541	0.04054	ns
Myxococcales	0.03510	0.03590	ns
Gemmatimonadales	0.03099	0.02639	ns
Acidobacteria group 4	0.02839	0.01954	ns
Rhodospirillales	0.02828	0.02825	ns
Planctomycetales	0.02109	0.02407	ns
Burkholderiales	0.02004	0.02261	ns
GR-WP33-30	0.01598	0.01346	ns
TRA3-20	0.01211	0.00995	ns
Flavobacteriales	0.01093	0.01175	ns
Caulobacterales	0.01052	0.00865	ns
Micrococcales	0.00944	0.01230	ns
Acidobacteria group 5	0.00899	0.00675	ns
Acidobacteria group 3	0.00861	0.00790	ns
Acidobacteria group 22	0.00797	0.00566	ns
Bacillales	0.00741	0.00925	ns
Nitrospinaceae order incertae sedis	0.00737	0.00868	ns
WD2101 soil group	0.00722	0.00688	ns
Acidimicrobiales	0.00710	0.00853	ns
Frankiales	0.00679	0.00759	ns
Micromonosporales	0.00648	0.00746	ns
Solirubrobacterales	0.00607	0.00927	ns
Acidobacteria group 7	0.00606	0.00427	ns
AKIW543	0.00568	0.00857	ns
Sh765B-TzT-29	0.00561	0.00444	ns
Acidobacteria group 17 (DA023)	0.00532	0.00500	ns
SC-I-84	0.00517	0.00383	ns
Marinicella Order Incertae Sedis	0.00461	0.00445	ns
Legionellales	0.00413	0.00347	ns
KD4-96	0.00412	0.00430	ns
Acidobacteria group 10	0.00409	0.00346	ns
Opitutales	0.00380	0.00407	ns
Propionibacteriales	0.00379	0.00556	ns
Pseudomonadales	0.00376	0.00571	ns
Chthoniobacterales	0.00371	0.00364	ns
S085	0.00356	0.00344	ns
S0134 terrestrial group	0.00331	0.00274	ns
Pseudonocardiales	0.00329	0.00394	ns
Chlorobiales	0.00321	0.00219	ns
Caldilineales	0.00315	0.00356	ns
Phycisphaerales	0.00291	0.00289	ns

Rubrobacterales	0.00280	0.00403	ns
Bdellovibrionales	0.00264	0.00297	ns
Chlamydiales	0.00263	0.00222	ns
Acidobacteria group 11	0.00240	0.00163	ns
BD2-11 terrestrial group	0.00240	0.00202	ns
Chloroflexales	0.00238	0.00264	ns
Anaerolineales	0.00232	0.00198	ns
JG30-KF-CM45	0.00231	0.00318	ns
Verrucomicrobiales	0.00206	0.00229	ns
JG30-KF-CM66	0.00198	0.00160	ns
Corynebacteriales	0.00198	0.00252	ns
Rhodobacterales	0.00195	0.00378	ns
Syntrophobacterales	0.00189	0.00170	ns
Ignavibacteriales	0.00154	0.00168	ns
CCM11a	0.00136	0.00082	ns
Desulfurellales	0.00124	0.00165	*
Streptomycetales	0.00108	0.00148	ns
NKB5	0.00107	0.00110	ns
Clostridiales	0.00105	0.00134	ns
AT425-EubC11 terrestrial group	0.00104	0.00150	ns
Rickettsiales	0.00100	0.00093	ns
Streptosporangiales	0.00094	0.00121	ns
Ktedonobacterales	0.00086	0.00072	ns
Acidobacteria group 15 - JG37-AG-116	0.00084	0.00058	*
Alteromonadales	0.00081	0.00041	ns
Lineage IIb	0.00073	0.00071	ns
Gitt-GS-136	0.00071	0.00095	ns
Rhodocyclales	0.00068	0.00016	ns
Chtomonadetes (order)	0.00057	0.00035	ns
Desulfuromonadales	0.00053	0.00046	ns
Oceanospirillales	0.00053	0.00076	ns
Enterobacteriales	0.00047	0.00045	ns
Fibrobacterales	0.00043	0.00056	ns
AKYG1722	0.00042	0.00051	ns
Acidobacteria group 18 (BPC102)	0.00042	0.00032	ns
Acidobacteriales (Acidobacteria group 1)	0.00040	0.00018	*
KI89A clade	0.00040	0.00025	ns
SHA-109	0.00033	0.00025	ns
mle1-8	0.00033	0.00028	ns
Lineage IIa	0.00031	0.00037	ns
MLE1-12	0.00028	0.00025	ns
Acidobacteria group 2 - DA052	0.00027	0.00010	ns
Erysipelotrichales	0.00026	0.00033	ns
Acidobacteria group 25	0.00025	0.00023	ns
Pla1 lineage	0.00022	0.00013	ns
Kineosporiales	0.00018	0.00023	ns
Thermotogales	0.00015	0.00013	ns
AT-s3-28	0.00013	0.00012	ns
Acidobacteria group 9 - BPC015	0.00013	0.00016	ns
B1-7BS	0.00011	0.00012	ns
S-70	0.00010	0.00005	ns
JH-WHS99	0.00010	0.00002	ns
Acidobacteria group 20 (BPC102)	0.00010	0.00009	ns
EC3	0.00009	0.00003	ns

FUNGI TAXA	Relative Abundance		
Phylum	**SR**	**SI**	**Significance (p<0.05)**
Ascomycota	0.3158	0.2733	ns
Basidiomycota	0.0501	0.0387	ns
Chytridiomycota	0.0176	0.0190	ns
Cryptomycota	0.0047	0.0098	ns
Glomeromycota	0.0033	0.0029	ns
Zoopagales	0.0026	0.0028	ns
Blastocladiomycota	0.0015	0.0048	ns
Entomophthoromycota	0.0014	0.0003	ns
Mucorales	0.0011	0.0003	*
Mortierellales	0.0010	0.0010	ns
Kickxellales	0.0010	0.0012	ns
LKM15	0.0005	0.0004	ns
Harpellales	0.0005	0.0013	ns
Endogonales	0.0004	0.0005	ns
Neocallimastigomycota	0.0000	0.0000	ns
Mucoromycotina Incertae Sedis	0.0000	0.0003	ns
Class			
Sordariomycetes	0.09979	0.08100	ns
Dothideomycetes	0.06196	0.06223	ns
Tremellomycetes	0.02896	0.02099	ns
Chytridiomycetes	0.01720	0.01698	ns
Agaricomycetes	0.00967	0.00517	ns
Eurotiomycetes	0.00754	0.01048	ns
Pezizomycetes	0.00637	0.00978	ns
Ustilaginomycetes	0.00426	0.00515	ns
Glomeromycetes	0.00326	0.00288	ns
Cryptomycota Incertae Sedis	0.00245	0.00618	ns
LKM11	0.00215	0.00327	ns
Microbotryomycetes	0.00189	0.00189	ns
Blastocladiomycetes	0.00149	0.00480	ns
Leotiomycetes	0.00147	0.00339	ns
Entomophthorales	0.00142	0.00027	ns
Laboulbeniomycetes	0.00042	0.00044	ns
Saccharomycetes	0.00038	0.00043	ns
Monoblepharidomycetes	0.00034	0.00021	ns
Pucciniomycetes	0.00023	0.00042	ns
Exobasidiomycetes	0.00020	0.00007	ns
Pezizomycotina Incertae Sedis	0.00007	0.00005	ns
Classiculomycetes	0.00006	0.00000	ns
Wallemiomycetes	0.00006	0.00009	ns
Agaricostilbomycetes	0.00005	0.00008	ns
Orbiliomycetes	0.00003	0.00000	ns
Archaeorhizomycetes	0.00002	0.00001	ns
Schizosaccharomycetes	0.00001	0.00000	ns
Neocallimastigomycetes	0.00000	0.00000	ns
Entorrhizomycetes	0.00000	0.00001	ns
Taphrinomycetes	0.00000	0.00001	ns
Lecanoromycetes	0.00000	0.00001	ns
Order			
Pleosporales	0.06101	0.06186	ns
Microascales	0.02443	0.02116	ns
Hypocreales	0.01220	0.01577	ns

Pezizales	0.00637	0.00978	ns
Ustilaginales	0.00426	0.00515	ns
Spizellomycetales	0.00356	0.00466	ns
Onygenales	0.00351	0.00439	ns
Glomerales	0.00266	0.00271	ns
Sordariales	0.00250	0.00115	ns
Tremellales	0.00225	0.00157	ns
Helotiales	0.00147	0.00339	ns
Blastocladiales	0.00128	0.00461	ns
Rhizophydiales	0.00106	0.00099	ns
Chytridiales	0.00102	0.00152	ns
Dothideomycetes Incertae Sedis	0.00092	0.00033	ns
Cladochytriales	0.00089	0.00066	ns
Cystofilobasidiales	0.00069	0.00093	ns
Pyxidiophorales	0.00042	0.00044	ns
Arachnomycetales	0.00039	0.00039	ns
Magnaporthales	0.00034	0.00018	ns
Monoblepharidales	0.00034	0.00021	ns
Saccharomycetales	0.00026	0.00041	ns
Diaporthales	0.00019	0.00010	ns
Pucciniales	0.00016	0.00035	ns
Diversisporales	0.00016	0.00001	ns
Eurotiales	0.00013	0.00014	ns
Chaetothyriales	0.00012	0.00214	ns
Xylariales	0.00010	0.00035	ns
Paraglomerales	0.00009	0.00000	ns
Malasseziales	0.00009	0.00002	ns
Classiculales	0.00006	0.00000	ns
Rhizophlyctidales	0.00006	0.00006	ns
Geminibasidiales	0.00006	0.00007	ns
Agaricostilbales	0.00005	0.00007	ns
Sordariomycetes Incertae Sedis	0.00005	0.00005	ns
Agaricales	0.00003	0.00001	ns
Orbiliales	0.00003	0.00000	ns
Botryosphaeriales	0.00003	0.00001	ns
Sporidiobolales	0.00002	0.00003	ns
Archaeorhizomycetales	0.00002	0.00001	ns
Gomphales	0.00002	0.00000	ns
Ophiostomatales	0.00002	0.00000	ns
Trichosphaeriales	0.00001	0.00001	ns
Cantharellales	0.00001	0.00001	ns
Boletales	0.00000	0.00001	ns
Boliniales	0.00000	0.00000	ns
Platygloeales	0.00000	0.00000	ns
Polyporales	0.00000	0.00000	ns
Archaeosporales	0.00000	0.00001	ns
Lobulomycetales	0.00000	0.00005	ns
Entorrhizales	0.00000	0.00001	ns
Wallemiales	0.00000	0.00002	*
Trechisporales	0.00000	0.00000	ns
Georgefischeriales	0.00000	0.00001	ns
Taphrinales	0.00000	0.00001	ns
Lecanorales	0.00000	0.00001	ns
Acrospermales	0.00000	0.00000	ns

PROTIST TAXA	Relative Abundance		
Phylum	**SR**	**SI**	**Significance ($p<0.05$)**
Cercozoa	0.14790	0.14026	ns
Peronosporomycetes	0.03161	0.03171	ns
Tubulinea	0.02115	0.02228	ns
Ochrophyta	0.02012	0.01696	ns
Ciliophora	0.02078	0.02053	ns
Discosea	0.01243	0.01118	ns
Gracilipodida	0.00718	0.00682	ns
Schizoplasmodiida	0.00360	0.00369	ns
Heterolobosea	0.00246	0.00238	ns
Labyrinthulomycetes	0.00541	0.00407	ns
Apicomplexa	0.00236	0.00458	ns
Euglenozoa	0.00313	0.00306	ns
Hyphochytriomycetes	0.00201	0.00167	ns
WIM5	0.00171	0.00161	ns
LKM74	0.00131	0.00181	ns
Protalveolata	0.00130	0.00115	ns
Cavosteliida	0.00106	0.00107	ns
MAST-12	0.00048	0.00039	ns
Bicosoecida	0.00052	0.00057	ns
LEMD255	0.00066	0.00072	ns
Heterophryidae	0.00057	0.00036	ns
Acanthocystidae	0.00052	0.00036	ns
CV1-B1-93	0.00006	0.00006	ns
Dinoflagellata	0.00020	0.00022	ns
Prymnesiophyceae	0.00024	0.00048	ns
Cryptomonadales	0.00007	0.00010	ns
Alveolata Incertae Sedis	0.00003	0.00009	ns
Opalinata	0.00003	0.00001	ns
Jakobida	0.00002	0.00002	ns
Fractovitelliida	0.00002	0.00002	ns
Retaria	0.00001	0.00002	ns
Class			
Glissomonadida	0.03582	0.03408	ns
Phytomyxea	0.02614	0.02367	ns
Cercomonadidae	0.02609	0.02620	ns
Intramacronucleata	0.02036	0.02000	ns
Imbricatea	0.01763	0.01247	ns
Euamoebida	0.01281	0.01460	ns
Chrysophyceae	0.01267	0.00929	ns
Halophytophthora	0.00881	0.01802	ns
Vampyrellidae	0.00860	0.01181	ns
Thecofilosea	0.00837	0.00921	ns
Longamoebia	0.00812	0.00775	ns
Leptomyxida	0.00452	0.00474	ns
Flabellinia	0.00404	0.00319	ns
Xanthophyceae	0.00397	0.00471	ns
Thraustochytriaceae	0.00346	0.00356	ns
Pythium	0.00335	0.00223	ns
Arcellinida	0.00285	0.00199	ns
Diatomea	0.00255	0.00189	ns
Tetramitia	0.00246	0.00238	ns
Conoidasida	0.00219	0.00423	ns

Euglenida	0.00204	0.00167	ns
Hyphochytriales	0.00201	0.00167	ns
Kinetoplastea	0.00094	0.00122	ns
Filamoeba	0.00091	0.00088	ns
Eustigmatophyceae	0.00088	0.00104	ns
CCW10	0.00087	0.00094	ns
Cercozoa Incertae Sedis	0.00087	0.00062	ns
LEMD267	0.00084	0.00048	ns
Syndiniales	0.00083	0.00069	ns
RT5iin19	0.00073	0.00337	ns
MPE1-14	0.00054	0.00043	ns
Foraminifera	0.00052	0.00028	ns
WIM 1 lineage	0.00051	0.00063	ns
Postciliodesmatophora	0.00040	0.00051	ns
MAST-12C	0.00040	0.00039	ns
Phalansterium	0.00040	0.00027	ns
Telaepolella	0.00039	0.00049	ns
Metromonadea	0.00036	0.00048	ns
Heterophrys	0.00032	0.00019	ns
Prymnesiales	0.00024	0.00048	ns
Flamella	0.00022	0.00030	ns
Dinophyceae	0.00020	0.00022	ns
Chromerida	0.00015	0.00010	ns
CH1-2B-3	0.00014	0.00010	ns
Novel Clade Gran-5	0.00011	0.00006	ns
Novel Clade 10	0.00009	0.00005	ns
Amphitremida	0.00008	0.00012	ns
RM2-SGM58	0.00007	0.00004	ns
Perkinsidae	0.00005	0.00012	ns
Colpodellida	0.00005	0.00005	ns
Opalinea	0.00003	0.00000	ns
LG08-10	0.00003	0.00006	ns
Andalucia	0.00002	0.00002	ns
Order			
Heteromita	0.02651	0.02515	ns
Cercomonas	0.01910	0.01843	ns
Silicofilosea	0.01564	0.01051	ns
Conthreep	0.01006	0.00958	ns
Centramoebida	0.00788	0.00742	ns
Spirotrichea	0.00761	0.00605	ns
Tribonematales	0.00373	0.00469	ns
Chromulinales	0.00353	0.00259	ns
Cryomonadida	0.00294	0.00254	ns
Litostomatea	0.00269	0.00436	ns
Ochromonadales	0.00245	0.00210	ns
BOLA868	0.00243	0.00366	ns
Dactylopodida	0.00239	0.00194	ns
Bacillariophytina	0.00225	0.00179	ns
Platyreta germanica	0.00200	0.00122	ns
Eocercomonas	0.00198	0.00199	ns
Gregarinasina	0.00196	0.00383	ns
Amb-18S-462	0.00191	0.00115	ns
Glaeseria	0.00190	0.00312	ns
Heteronematina	0.00131	0.00121	ns
Allantion	0.00117	0.00103	ns

Vannellida	0.00113	0.00089	ns
Amb-18S-1124	0.00112	0.00113	ns
Nudifila	0.00108	0.00139	ns
Echinamoebida	0.00099	0.00078	ns
E-A1	0.00092	0.00144	ns
Eustigmatales	0.00088	0.00104	ns
Difflugina	0.00085	0.00028	ns
Phryganellina	0.00085	0.00084	ns
Proleptomonas	0.00083	0.00096	ns
Metakinetoplastina	0.00079	0.00114	ns
Spongomonadida	0.00040	0.00005	ns
Heterotrichea	0.00039	0.00051	ns
Polymyxa	0.00036	0.00027	ns
Leptomyxa	0.00026	0.00048	ns
Vaucheriales	0.00024	0.00002	ns
Chrysophyceae Incertae Sedis	0.00022	0.00043	ns
Woronina	0.00021	0.00002	ns
Bodomorpha	0.00020	0.00036	ns
Syndiniales Group II	0.00018	0.00015	ns
Cryptosporida	0.00018	0.00039	ns
DSGM-50	0.00017	0.00018	ns
LG21-05	0.00015	0.00015	ns
Stygamoebida	0.00013	0.00009	ns
Globothalamea	0.00010	0.00006	ns
Naegleria	0.00008	0.00007	ns
Prokinetoplastina	0.00007	0.00009	ns
Tetramitus	0.00007	0.00015	ns
Marimonadida	0.00006	0.00002	ns
Metopion	0.00006	0.00010	ns
Colpodella	0.00005	0.00004	ns
Vahlkampfia	0.00004	0.00007	ns
A31	0.00004	0.00011	ns
Cavernomonas	0.00003	0.00003	ns
Piroplasmorida	0.00002	0.00003	ns
Euglenophyceae	0.00002	0.00003	ns

*Significant differences between means by Student’s t-test.

5| ENMIENDAS LÍQUIDAS FERMENTADAS

5. ENMIENDAS LÍQUIDAS FERMENTADAS

NOTA INTRODUCTORIA: El presente Capítulo está enfocado a la descripción y discusión de los resultados obtenidos en una serie de ensayos llevados a cabo al objeto de evaluar los efectos de la aplicación de enmiendas orgánicas obtenidas a partir de la fermentación de residuos orgánicos agrícolas. Como se menciona en el Capítulo 3, los ensayos se diferenciaron en base al cultivo estudiado: un cultivo hortícola, la lechuga, y un cereal, el maíz. Siguiendo esta diferenciación, este capítulo expone en primer lugar los resultados de los ensayos obtenidos para el cultivo hortícola (5.1) y, posteriormente, los del cereal (5.2).

5.1. Commercial and farm fermented liquid organic amendments to improve soil quality and lettuce yield

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Abstract

The anaerobic decomposition of organic wastes might lead to the formation of organic-byproducts which can then be successfully used as organic fertilizers. This study evaluated the impact of the application of two fermented liquid organic amendments (commercial vs. farm-made) at two doses of application (optimal vs. suboptimal), compared to mineral fertilization, on lettuce growth and soil quality. To this purpose, two experiments were conducted at microcosm- and field-scale, respectively. In the microcosm experiment, organically amended soils resulted in lower lettuce yield than minerally fertilized soil but, in contrast, they enhanced microbial activity and biomass, thus leading to an improvement in soil quality. The fertilization regime (organic vs. inorganic) significantly affected soil microbial composition but did not have any significant effect on structural or functional prokaryotic diversity. In the field experiment, at the optimal dose of application, organically-amended soils resulted in comparable lettuce yield to that displayed by minerally fertilized soils. The application of organic amendments did not result in an enhanced microbial activity and biomass, compared to mineral fertilization, but led to a higher soil prokaryotic diversity. Among the organically-amended plots, the optimal application dose resulted in a higher lettuce

yield and soil microbial activity and biomass, but led to a decline in soil prokaryotic diversity, compared to the suboptimal application dose. Our results indicate that commercial and farm-made fermented liquid organic amendments possess the potential to ameliorate soil quality while sustaining crop yield. Given the strong influence of other factors (*e.g.*, type of soil, dose of application) on the effects exerted by such amendments on soil quality and fertility, we recommend that an exhaustive characterization of both the amendments and the recipient soils should be carried out prior to their application, in order to better ensure their potential beneficial effects.

5.1.1. Introduction

Since the Green Revolution, agricultural intensification has been sustained by indiscriminate inputs of synthetic agrochemicals and an overuse of water, leading to a degradation of the environment, including the soil ecosystem. The soil ecosystem supports a multitude of functions and ecosystem services and, then, its conservation is of utmost importance (CEC, 2006). Accordingly, there is a growing interest in developing sustainable agricultural practices that protect the integrity of the soil ecosystem while producing healthy and abundant crops (Diacono and Montemurro, 2010; Bommarco *et al.*, 2013).

The use of organic wastes and agricultural by-products as soil amendments is gaining much interest as an alternative or complement to synthetic mineral fertilizers (Riva *et al.*, 2016). Besides, the integration of such wastes into agricultural systems as valuable assets provides an environmentally sound approach to the reduction and reuse of organic waste (Chojnacka and others 2019), supporting the waste minimization scenario advocated by the European Commission (2018). In this sense, organic amendments not only supply a wide range of nutrients, but can also increase the pool of soil organic carbon which, in turn, might improve soil physicochemical characteristics, stimulate soil microbial communities and, hence, enhance soil quality and fertility (Fließbach *et al.*, 2007; Lal, 2016).

However, organic waste-derived amendments may also entail potential drawbacks resulting from their composition (they often contain harmful constituents such as pathogens, heavy metals, organic contaminants, emerging contaminants, etc.) (Park *et al.*, 2011; Urra *et al.*, 2019a) and/or an inappropriate application, which may, for instance, lead to nutrient runoff and eutrophication, emission of greenhouse gases, soil acidification

or salinization, etc. (Thangarajan *et al*., 2013; Alvarenga *et al*., 2015). Hence, it is necessary to adopt waste management strategies (*e.g.*, pretreatment technologies) which can minimize potential drawbacks while increasing the agricultural suitability of these organic waste-derived amendments (Montemurro *et al*., 2009).

Among these technologies, the fermentation or anaerobic decomposition of organic wastes has been reported to hygienize raw organic wastes, effectively reducing the load of potential pathogens and organic contaminants (Mohring *et al*., 2009; Goberna *et al*., 2011; Martín *et al*., 2015). This widely used biological process entails the stabilization of the organic waste in the absence of oxygen, leading to the formation of energy-rich biogas and an organic by-product known as digestate, which may be further separated into a liquid and a solid fraction (Tambone *et al*., 2009; Nkoa *et al*., 2014). The former fraction is often rich in soluble nutrients, like ammonium and potassium, and contains biologically-stabilized organic carbon, being thus a promising soil amendment with a high fertilizing potential (Tambone *et al*., 2019). Nevertheless, the potential effects that these amendments can exert on agricultural soil quality depend upon the composition and characteristics of the in-feed organic waste (Nkoa, 2014), which can be very wide-ranging and diverse.

Many studies have been carried out on the agronomic potential of this type of organic amendments (Möller and Müller, 2012; Riva *et al*., 2016). However, to our knowledge, insufficient work has been done to elucidate the effects of fermented liquid organic amendments on soil quality and, in particular, on soil microbial communities as key players in soil functional sustainability. In order to address this gap, we evaluated, at microcosm and field scale, the effects of the application of two fermented organic amendments (commercial *vs*. farm-made) at two application doses (optimal *vs*. suboptimal) on soil quality and lettuce yield, as compared to mineral nitrogen-phosphorus-potassium (NPK) fertilizer. The composition of the in-feed biomass was identical for both amendments and consisted of molasses, milk whey, wheat bran, and leaf litter. We hypothesized that the application of at the optimal application dose (optimal according to the crop nitrogen requirement) will result in a more active and diverse soil microbial community, compared to NPK-fertilization, leading to an improved soil quality and adequate crop yield.

5.1.2. Materials and methods

5.1.2.1. Amendment characterization

Fermented liquid organic amendments are prepared through the anaerobic decomposition of farm-derived organic byproducts. According to their origin, two fermented liquid organic amendments were tested here at both microcosm- and field scale: (1) a commercial amendment bought from a local company (VITAVERIS SC, Spain), which assists local farmers in the preparation of organic amendments utilizing farm-derived byproducts; (2) a farm-made amendment prepared by a local farmer according to the guidelines provided by the aforementioned company, as follows: 3 kg of molasses were thoroughly mixed with 50 L of cow milk whey in a 60 L-polyethylene container. Subsequently, 2.8 kg of a mixture consisting of 29% (w/w) oak leaf litter visually colonized by fungal mycelium, 57% (w/w) wheat bran and 14% (w/w) molasses was introduced to the 60 L-polyethylene container. Afterwards, 2 kg of basaltic dust was added to the container. Finally, the container was hermetically sealed for fermentation, allowing the gas to be released through a septum (Chontal *et al*., 2019), and maintained as such for 30 days. The physicochemical characterization of both the commercial and farm-made amendments is displayed in Table 5.S1.

5.1.2.2. Experimental design

In order to study the impact of the application of the abovementioned organic amendments on lettuce (*Lactuca sativa* L. var. Batavia) yield and soil quality, both a microcosm- and a field-scale experiment were carried out. The experimental factors studied in both experiments were: (i) *origin of amendment*: commercial *vs.* farm-made; (ii) *amendment dose*: optimal (adjusted according to the nitrogen demand of lettuce plants in our region, *i.e.* 150 kg N ha^{-1}) vs. suboptimal (400 L of the liquid amendment pre-diluted to 5% ha^{-1}). This suboptimal dose (suboptimal from the point of view of the N needs of lettuce plants) was tested here as it is the dose recommended by the manufacturer, *i.e.* VITAVERIS SC. A mineral NPK control was included for comparison purposes: 150 kg N ha^{-1} as NH_4NO_3 (34.4%), 50 kg P ha^{-1} as P_2O_5 (18%), and 200 kg K ha^{-1} as K_2O (60%).

5.1.2.2.1. Microcosm experiment

Our experimental soil was collected from the top 30 cm of an agricultural field, located in Haro (La Rioja, Spain, 42°35'47.5"N 2°52'13.3"W), which had not been treated with organic amendments for at least 20 years. After collection, the soil was thoroughly mixed, sieved to <4 mm, air-dried at 30 °C and subjected to physicochemical characterization according to standard methods (MAPA, 1994). The soil was a sandy loam, with a pH of 8.5, an organic matter (OM) content of 1.18%, a total N content of 0.08%, an Olsen P content of 10.2 mg kg^{-1}, and an extractable K^+ content of 91 mg kg^{-1}. Polyethylene pots (3,000 cm^3) were filled with 2.5 kg dry weight (DW) of soil, kept at 20ºC for 10 days for preconditioning, and finally supplemented with the fermented liquid organic amendments. Lettuce seedlings were grown in these pots for 8 weeks under the following controlled conditions in a growth chamber: light intensity = 100 μmol photon m^{-2} s^{-1}; photoperiod = 14/10 h light/darkness; and temperature = 24/20ºC day/night. Following a completely randomized factorial design, each treatment was replicated three times. Pots were watered to field capacity three times a week. After 8 weeks, lettuce plants were harvested and their fresh and dry weights recorded. Dry weight was determined by drying in an oven at 70ºC until reaching a constant mass. Finally, all the soil in the pots was collected for analysis (see below).

5.1.2.2.2. Field experiment

The field experiment was carried out in San Vicente de Arana (Araba, Spain, 42°45'15.7"N 2°21'05.4"W) at an altitude of 825 m above sea level. The area is characterized by a humid, cool maritime Mediterranean climate (mean annual rainfall = 972 mm; mean temperature = 10.6 °C). The soil is a clay loam with a pH of 8.4, an OM content of 1.79%, a total N content of 0.15%, an Olsen P content of 18.7 mg kg^{-1}, and an extractable K^+ content of 160 mg kg^{-1}. Experimental plots of 15 m^2 were arranged following a completely randomized design with six replicates. The fermented liquid organic amendments were applied manually to the soil surface. Lettuce seedlings were planted manually at 50 x 40 cm inter- and intra-row spacing (5 plants m^{-2}). Plots were irrigated with a sprinkler 2-3 times per week depending on the weather conditions. Weed control was performed manually during the first month. Plants from the two central rows of each plot were harvested after 8 weeks and, then, lettuce yield (FW and DW) was determined. Soil sampling was conducted just prior to plant harvest, by randomly

collecting 6 soil samples from each plot at a depth of 0-30 cm and thoroughly mixing them together to obtain a composite sample.

5.1.2.3. Soil parameters

Upon arrival at the laboratory, soil samples from both experiments (microcosm and field experiment) were divided into two parts: one part, intended for the determination of physicochemical parameters, was dried at 30 °C and then sieved to <2 mm; the other part, intended for the determination of biological parameters, was sieved fresh to <2 mm and stored at 4 °C until analysis. Samples for molecular analyses were stored at -20 °C.

Soil pH, organic C, nitrate, ammonium and total N, P and K contents were determined according to standard methods (MAPA, 1994). Regarding soil biological parameters, soil respiration was determined following ISO 16072 Norm (2002). β-glucosidase, arylsulphatase and alkaline phosphatase activities were determined according to Dick *et al*. (1996) and Taylor *et al*. (2002). Urease activity was measured following Kandeler and Gerber (1988). Potentially mineralizable N (N_{PM}) was measured as described by Powers (1980). Microbial biomass C (Cmic) was measured following Vance *et al*. (1987). Community-level physiological profiles (CLPPs) of soil cultivable heterotrophic bacteria were determined with Biolog EcoPlates™ following Epelde *et al*. (2008).

DNA extraction was carried out from three aliquots, each corresponding to 0.25 g DW soil, from each sample using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA). Prior to DNA extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) (Kowalchuk *et al*., 1997). The quantity and quality of DNA was determined with a ND-1000 Spectrophotometer (Thermo-Scientific, Wilmington, DE). For the estimation of the abundance of 16S rRNA gene fragments for total bacteria and 18S rRNA gene fragments for total fungi, qPCR measurements were carried out following the reaction mixtures, primers and PCR conditions described in Epelde *et al*. (2014).

To study the diversity and composition of soil prokaryotic communities, amplicon libraries were prepared using a dual indexing approach with sequence-specific primers targeting the V4 hypervariable region of the 16S rRNA gene (Urra *et al*., 2019b). Sequencing of the libraries was then carried out using an Illumina MiSeq V2 platform and pair-ended 2×250 nt at Tecnalia (Spain). Merging of the read paired ends, quality filtering (primer trimming, removal of singletons and chimeric sequences) and clustering

into operational taxonomic units (OTUs) were performed following Lanzén *et al*. (2016). Taxonomic assignments were carried out using CREST and SilvaMod v128 (Lanzén *et al*., 2012; https://github.com/lanzen/CREST).

5.1.2.4. Statistical analysis

The statistical significance of the differences in the effects of organic amendments and NPK fertilizer on crop yield and soil parameters was determined according to the pooled-variances *t*-test (for equal variances) or Welch's *t*-test (for unequal variances). Regarding the organically-amended soils, the main effects and interactions of the two experimental factors (*origin of amendment, amendment dose*) were evaluated: *origin of amendment* was a fixed factor with two levels: commercial and farm-made; *amendment dose* was a fixed factor with two levels: optimal and suboptimal. In this sense, differences among experimental factors and their interactions were tested by means of two-way ANOVA and Duncan's multiple range test (when the interaction effect was significant) using the package *agricolae* in R software (version 3.3.2). The relationships among the experimental factors were further analyzed by performing redundancy analyses (RDA) and variation partitioning analyses with Canoco 5.0 (Ter Braak and Šmilauer, 2012).

R package *vegan* was used for the determination of α-diversity indices, multivariate statistics and visualization of 16S rRNA amplicon sequencing data (Oksanen *et al*., 2015). OTU distributions were transformed into relative abundances using function *decostand*. Prokaryotic community composition was compared between samples by calculating Bray-Curtis dissimilarity matrices, which were further used to perform non-metric multidimensional scaling (NMDS) with function *metaMDS*. Permutational analyses of variance (PERMANOVA) were performed to assess the impact of the experimental factors on prokaryotic community composition, using *adonis* function. Pairwise analysis on the relative abundances of taxons (at family level) representing more than 0.5% of the total reads were performed by one-way ANOVA.

5.1.3. Results

5.1.3.1. Effect of treatments on crop yield

Regarding the microcosm experiment, significantly ($p=0.003$) higher values (62% higher) of crop yield were found in NPK-fertilized plots than in organically-amended pots (13.0 *vs.* 8.0 g DW lettuce^{-1} for NPK-fertilized and organically-amended pots, respectively).

Actually, crop yield in NPK-fertilized plots was 120 and 28% higher than in plots fertilized with the suboptimal (5.1 g DW lettuce^{-1}) and optimal (10.1 g DW lettuce^{-1}) dose of the fermented liquid organic amendment, respectively. Within the organically-amended pots, *amendment dose* ($p<0.001$) was the most influential factor affecting crop yield (the optimal dose produced 71% more lettuce biomass than the suboptimal dose), while the *origin of amendment* did not result in any significant differences.

On the other hand, in the field experiment, no differences were observed between the two fertilization regimes (NPK *vs.* organically-amended) regarding crop yield. Differences among treatments were nevertheless observed within the organically-amended plots, in which the application of the optimal dose exhibited significantly ($p=0.022$) higher crop yield than the suboptimal dose (the optimal dose resulted in 27% more lettuce biomass than the suboptimal dose).

5.1.3.2. Effect of treatments on soil physicochemical properties

Nitrate content was the only parameter that exhibited statistically significant differences between the organically-amended and NPK-fertilized soils, both in the microcosm and field experiment (Table 5.1). In the microcosm experiment, NPK fertilization led to significantly ($p<0.001$) much higher values (a 10-fold increase) of soil nitrate content than the application of organic amendments, regardless of the origin and dose of the amendment. In the field experiment, differences in soil nitrate content were smaller but still statistically significant ($p=0.017$): NPK-fertilization resulted in an 85% increase in soil nitrate content compared to organic fertilization. However, this difference was due to the treatment with the suboptimal dose of the organic amendment, since the treatment with the optimal dose did result in values of soil nitrate content similar to those observed in NPK fertilized-soils.

Regarding differences among organically-amended plots, the redundancy analysis exhibited a significant effect of the experimental factors on the values of soil physicochemical parameters, but only for the microcosm experiment (Figure 5.1A) and not for the field experiment (Figure 5.1B). In the microcosm experiment, the variation partitioning analysis further revealed that the observed significant effect was due to the experimental factor *amendment dose*, which explained 46% of the total variance ($F=9.1$, $p=0.002$), while the experimental factor *origin of amendment* did not exert any significant effect and explained only 2% of the total variance. Indeed, in the microcosm experiment, several soil physicochemical parameters were significantly affected by the *amendment*

dose: soil pH was significantly reduced by the application of the optimal dose of the organic amendment, as compared to the suboptimal dose; conversely, soil OM and nitrate contents were significantly enhanced by the application of the optimal dose of the organic amendment, as compared to the suboptimal dose (Table 5.1). As for the field experiment, the only parameter exhibiting statistically significant differences among organically-amended plots was soil nitrate content, which increased in plots treated with the optimal dose of the amendment, as compared to the suboptimal dose. No statistically significant differences were observed for total N, P and K^+ contents.

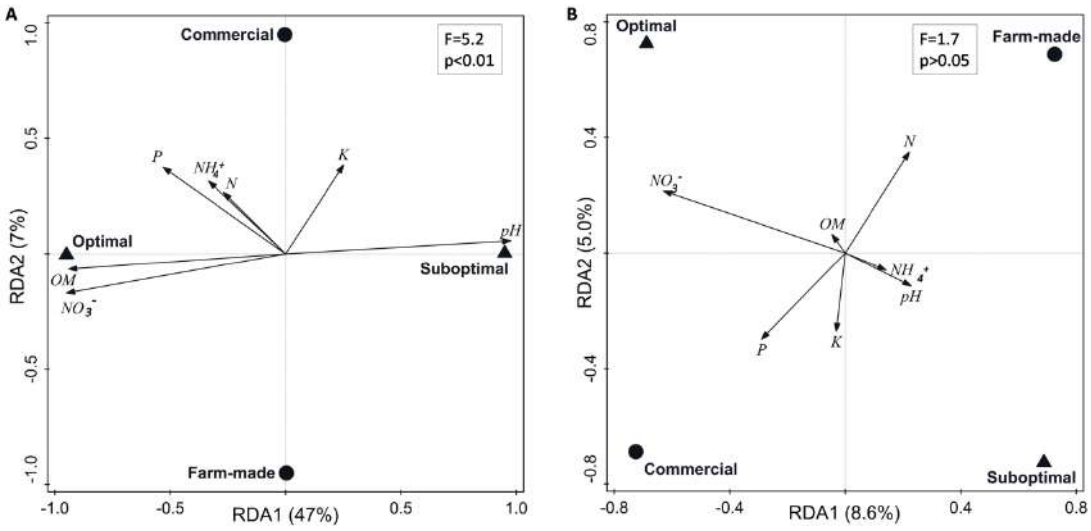

Figure 5.1. Biplot of the redundancy analysis (RDA) performed on soil physicochemical parameters as response variables. (A) Microcosm experiment. (B) Field experiment.

Table 5.1. Effect of organic amendments on soil physicochemical properties. F: Farm-made amendment; C: Commercial amendment; NPK: mineral control; O *vs.* M: organically-amended *vs.* mineral control. Differences based on pooled variances *t*-test or Welch´s *t*-test. For the organically-amended soils, interaction among factors was tested by three-way ANOVA, where A: origin of amendment; D: amendment dose; Ap: time of application. Mean values (n=3) ± SD. *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

MICROCOSM EXPERIMENT

		pH	OM %	Ntot %	Nitrate mg kg^{-1}	Ammonium mg kg^{-1}	P g kg^{-1}	K g kg^{-1}
F	**Optimal**	8.44 ± 0.05	1.00 ± 0.03	0.07 ± 0.01	8.3 ± 0.9	0.3 ± 0.3	0.25 ± 0.03	2.9 ± 1.1
	Subptimal	8.73 ± 0.03	0.79 ± 0.06	0.08 ± 0.01	2.9 ± 0.7	0.2 ± 0.1	0.20 ± 0.02	3.3 ± 0.3
C	**Optimal**	8.47 ± 0.03	1.00 ± 0.04	0.08 ± 0.01	6.9 ± 0.6	0.5 ± 0.3	0.25 ± 0.03	3.5 ± 1.2
	Subptimal	8.73 ± 0.02	0.76 ± 0.06	0.07 ± 0.01	2.7 ± 0.6	0.3 ± 0.2	0.24 ± 0.01	3.9 ± 0.1
	NPK	8.51 ± 0.01	1.00 ± 0.03	0.08 ± 0.01	51.7 ± 2.8	0.2 ± 0.1	0.25 ± 0.01	4.1 ± 0.2
O *vs.* M		***ns***	***ns***	***ns***	***	***ns***	***ns***	***ns***
A		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
D		***	***	*ns*	***	*ns*	*ns*	*ns*
A*D		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

FIELD EXPERIMENT

		pH	OM %	Ntot %	Nitrate mg kg^{-1}	Ammonium mg kg^{-1}	P g kg^{-1}	K g kg^{-1}
F	**Optimal**	8.55 ± 0.02	1.94 ± 0.05	0.16 ± 0.00	7.0 ± 1.5	2.6 ± 0.3	0.56 ± 0.03	12.9 ± 0.8
	Subptimal	8.54 ± 0.05	1.89 ± 0.09	0.15 ± 0.01	4.6 ± 2.0	2.5 ± 0.3	0.58 ± 0.03	13.4 ± 0.5
C	**Optimal**	8.52 ± 0.04	1.90 ± 0.09	0.15 ± 0.01	10.7 ± 4.5	2.4 ± 0.3	0.60 ± 0.02	13.4 ± 0.5
	Subptimal	8.56 ± 0.03	1.93 ± 0.08	0.15 ± 0.01	5.1 ± 1.2	2.6 ± 0.3	0.59 ± 0.05	13.4 ± 0.9
	NPK	8.50 ± 0.05	1.91 ± 0.17	0.16 ± 0.02	12.7 ± 3.0	2.6 ± 0.1	0.60 ± 0.03	13.9 ± 0.3
O *vs.* M		***ns***	***ns***	***ns***	*	***ns***	***ns***	***ns***
A		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
D		*ns*	*ns*	*ns*	***	*ns*	*ns*	*ns*
A*D		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

5.1.3.3. Effect of treatments on soil microbial communities

The application of organic amendments resulted in a significant increase in several microbial activity and biomass parameters with respect to the application of NPK (Table 5.2). This effect was nevertheless observed only in the microcosm experiment (not in the field experiment), in which the application of the organic amendments resulted in an increase of soil respiration, arylsulphatase activity and potentially mineralizable N values, as well as in all the microbial biomass parameters studied here (*i.e.* microbial biomass C, total bacteria and total fungi). In regard to the field experiment, the fertilization regime did not result in any significant differences in soil microbial activity or biomass.

Concerning differences among the organically-amended soils, the redundancy analysis and variation partitioning revealed that both experimental factors (origin and dose of amendment) significantly explained the observed variation in microbial activity and biomass parameters, in both the microcosm and field experiment (Figure 5.2). In the microcosm experiment, the factor *amendment dose* accounted for 46% of the total variance (F=11.4, p=0.002), while the factor *origin of amendment* accounted for 8.5% (F=2.8, p=0.02) of the variance. Similarly, in the field experiment, the factors *amendment dose* and *origin of amendment* accounted for 12.3% (F=4.4, p=0.006) and 7.6% (F=3.1, p=0.006) of the variance, respectively. As abovementioned for soil physicochemical parameters, in the field experiment, the experimental factors explained a much smaller percentage of the total variation of the response variables (*i.e.* microbial activity and biomass parameters) than in the microcosm experiment (Figure 5.2). The factor *amendment dose* resulted in significant differences for 6 and 5 microbial parameters (out of the 9 microbial parameters studied here) in the microcosm and field experiment, respectively. In both experiments, the optimal dose of amendment resulted in a significant increase of alkaline phosphatase, potentially mineralizable N, total bacteria and total fungi values, with respect to the suboptimal dose. In addition, in the microcosm experiment, significant differences were also revealed according to the factor *amendment dose*: higher values of β-glucosidase activity were observed in soils treated with the optimal dose of the amendment; by contrast, higher values of urease activity were observed in soils treated with the suboptimal dose of the amendment (Table 5.2). Regarding the field experiment, values of arylsulphatase activity were higher in soils treated with the optimal dose of the amendment, compared to the suboptimal dose.

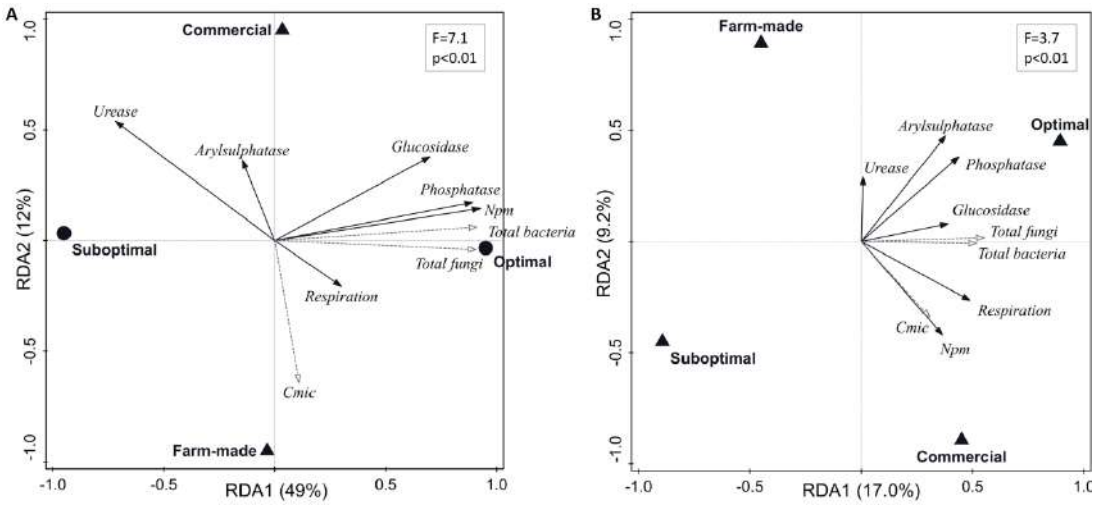

Figure 5.2. Biplot of the redundancy analysis (RDA) performed on microbial activity and biomass parameters as response variables. (A) Microcosm experiment. (B) Field experiment. Solid arrows: activity parameters; dotted arrows: biomass parameters; N_{PM}: potentially mineralizable N; Cmic: microbial biomass C; Total bacteria: 16S rRNA gene copy number; Total fungi: and 18S rRNA gene copy number.

On the other hand, in the microcosm experiment, the factor *origin of amendment* was responsible for significant differences in three microbial activity parameters (*i.e.* β-glucosidase, urease, potentially mineralizable N): values of these parameters were significantly higher in soils treated with the commercial vs. the farm-made amendment. In relation to the field experiment, potentially mineralizable N was the only parameter significantly altered by the factor *origin of amendment*: similar, values of this parameter were significantly higher in soils treated with the commercial vs. the farm-made amendment.

Table 5.2. Effect of organic amendments on soil microbial activity and biomass. F: Farm-made amendment; C: Commercial amendment; NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control. Differences based on pooled variances *t*-test or Welch´s *t*-test. For the organically-amended soils, interaction among factors was tested by two-way ANOVA, where A: origin of amendment; D: amendment dose. Letters address significant differences among treatments according to one-way ANOVA and Duncan's MRT when factor interaction was significant. N_{PM}: potentially mineralizable N; Cmic: microbial biomass C; *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

MICROCOSM EXPERIMENT

		MICROBIAL ACTIVITY						MICROBIAL BIOMASS		
		Respiration	**Arylsulphatase**	**Alkaline Phosphatase**	**β-Glucosidase**	**Urease**	**N_{PM}**	**Cmic**	**Bacterial gene abundance**	**Fungal gene abundance**
		µg C g DW soil^{-1} h^{-1}	mg ρ-nitrophenol kg DW soil^{-1} h^{-1}	mg ρ-nitrophenol kg DW soil^{-1} h^{-1}	mg N-NH_4^+ kg DW soil^{-1} h^{-1}	mg N-NH_4^+ kg DW soil^{-1} h^{-1}	mg N-NH_4^+ kg DW soil^{-1}	mg C kg^{-1} DW soil	x 10^{10} copies g DW soil^{-1}	x 10^{8} copies g DW soil^{-1}
F	**Optimal**	1.9 ± 0.2	32 ± 2	227 ± 11	106 ± 1^{B}	11 ± 0^{C}	41 ± 3^{A}	273 ± 42	4.5 ± 0.8	1.8 ± 0.5
	Subptimal	1.8 ± 0.1	33 ± 0	151 ± 1	102 ± 5^{B}	15 ± 1^{B}	9 ± 1^{C}	238 ± 25	2.2 ± 0.5	0.5 ± 0.3
C	**Optimal**	1.8 ± 0.3	33 ± 1	264 ± 47	126 ± 8^{A}	13 ± 3^{BC}	38 ± 4^{A}	233 ± 26	4.7 ± 0.7	1.7 ± 0.4
	Subptimal	1.7 ± 0.3	34 ± 1	156 ± 5	100 ± 1^{B}	21 ± 1^{A}	21 ± 3^{B}	219 ± 45	2.4 ± 0.3	0.5 ± 0.0
	NPK	1.3 ± 0.1	24 ± 3	171 ± 10	97 ± 3	14 ± 2	7 ± 4	201 ± 29	1.7 ± 0.3	0.5 ± 0.1
O *vs.* M		**	***	***ns***	***ns***	***ns***	**	*	***	*
A		*ns*	*ns*	*ns*	*	**	*	*ns*	*ns*	*ns*
D		*ns*	*ns*	***	***	***	***	*ns*	***	***
A*D		*ns*	*ns*	*ns*	**	*	**	*ns*	*ns*	*ns*

FIELD EXPERIMENT

		MICROBIAL ACTIVITY						MICROBIAL BIOMASS		
		Respiration	**Arylsulphatase**	**Alkaline Phosphatase**	**β-Glucosidase**	**Urease**	**N_{PM}**	**Cmic**	**Bacterial gene abundance**	**Fungal gene abundance**
		µg C g DW soil^{-1} h^{-1}	mg ρ-nitrophenol kg DW soil^{-1} h^{-1}	mg ρ-nitrophenol kg DW soil^{-1} h^{-1}	mg N-NH_4^+ kg DW soil^{-1} h^{-1}	mg N-NH_4^+ kg DW soil^{-1} h^{-1}	mg N-NH_4^+ kg DW soil^{-1}	mg C kg^{-1} DW soil	x 10^{8} copies g DW soil^{-1}	x 10^{6} copies g DW soil^{-1}
F	**Optimal**	1.7 ± 0.2	109 ± 6	335 ± 20^{A}	162 ± 14	159 ± 7.5	79 ± 10^{B}	214 ± 24	3.1 ± 0.5	4.3 ± 0,8
	Subptimal	1.7 ± 0.2	99 ± 8	278 ± 16^{B}	146 ± 14	134 ± 31	39 ± 16^{C}	201 ± 17	2.2 ± 0.3	2.5 ± 0.7
C	**Optimal**	2 ± 0.2	104 ± 6	300 ± 13^{B}	160 ± 10	128 ± 26	101 ± 22^{A}	227 ± 22	3.0 ± 0.6	5.1 ± 0,3
	Subptimal	1.8 ± 0.3	95 ± 9	298 ± 22^{B}	154 ± 16	138 ± 27	76 ± 19^{B}	229 ± 22	2.8 ± 0.3	3.4 ± 0,9
	NPK	2.1 ± 0.4	98 ± 9	309 ± 12	168 ± 17	141 ± 33	67 ± 5	214 ± 15	3.1 ± 0.9	4.8 ± 0.2
O *vs.* M		***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***
A		*ns*	*ns*	*ns*	*ns*	*ns*	***	*ns*	*ns*	*ns*
D		*ns*	**	***	*ns*	*ns*	***	*ns*	*	*
A*D		*ns*	*ns*	**	*ns*	*ns*	***	*ns*	*ns*	*ns*

Functional diversity of soil heterotrophic bacterial communities was estimated from the CLPPs obtained with Biolog EcoPlates™. In particular, the average well color development (AWCD), the number of utilized substrates (NUS) and the Shannon's index (H') were calculated for soil samples from both the microcosm and field experiment. No significant differences were observed for any of these parameters between organically-amended and NPK-fertilized soils (neither in the microcosm nor in the field experiment) (Table 5.3). Significant differences were nevertheless observed among organically-amended soils, but only in the microcosm experiment. Actually, the application of the optimal dose resulted in significantly higher values for all abovementioned functional diversity parameters, with respect to the suboptimal dose (Table 5.3).

Table 5.3. Effect of organic amendments on functional (CLPPs) and structural (genetic) prokaryotic diversity. F: Farm-made amendment; C: Commercial amendment; NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control. Differences based on pooled variances *t*-test or Welch´s *t*-test. For the organically-amended soils, interaction among factors was tested by two-way ANOVA, where A: origin of amendment; D: amendment dose. Letters address significant differences among treatments according to one-way ANOVA and Duncan's MRT when factor interaction was significant. NUS: number of used substrates; AWCD: average well color development; H': Shannon's index; J': Pielou's evenness. *ns*: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

		MICROCOSM EXPERIMENT					
		FUNCTIONAL DIVERSITY			**GENETIC DIVERSITY**		
		NUS	**AWCD**	**H'**	**Rarefied richness**	**H'**	**J'**
F	**Optimal**	17.1 ± 2.0^{A}	0.5 ± 0.1^{A}	3.4 ± 0.3	5866 ± 365	6.7 ± 0.1	0.78 ± 0.01
	Subptimal	5.4 ± 1.8^{C}	0.1 ± 0.0^{C}	2.1 ± 0.5	6491 ± 64	7.2 ± 0.0	0.79 ± 0.00
C	**Optimal**	12.4 ± 1.3^{B}	0.3 ± 0.1^{B}	2.9 ± 0.1	5704 ± 360	6.8 ± 0.2	0.78 ± 0.01
	Subptimal	7.2 ± 1.8^{C}	0.2 ± 0.1^{C}	2.5 ± 0.4	6325 ± 60	7.1 ± 0.0	0.79 ± 0.00
	NPK	7.4 ± 3.7	0.2 ± 0.1	2.3 ± 0.6	6286 ± 182	7.2 ± 0.1	0.80 ± 0.00
O *vs.* M		***ns***	***ns***	***ns***	***ns***	***ns***	***ns***
A		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
D		***	***	**	**	***	*ns*
A*D		*	*	*ns*	*ns*	*ns*	*ns*
		FIELD EXPERIMENT					
		FUNCTIONAL DIVERSITY			**GENETIC DIVERSITY**		
		NUS	**AWCD**	**H'**	**Rarefied richness**	**H'**	**J'**
F	**Optimal**	25 ± 2	1.2 ± 0.2	4.5 ± 0.1	5030 ± 92^{BC}	6.9 ± 0.1	0.79 ± 0.01
	Subptimal	24 ± 3	1.1 ± 0.2	4.4 ± 0.2	5138 ± 186^{AB}	7.0 ± 0.1	0.80 ± 0.01
C	**Optimal**	24 ± 1	1.1 ± 0.1	4.4 ± 0.1	4802 ± 178^{C}	6.7 ± 0.2	0.78 ± 0.02
	Subptimal	26 ± 1	1.3 ± 0.1	4.5 ± 0	5301 ± 257^{A}	7.0 ± 0.1	0.80 ± 0.01
	NPK	26 ± 2	1.3 ± 0.2	4.5 ± 0.1	4770 ± 572	6.4 ± 0.9	0.73 ± 0.10
O *vs.* M		***ns***	***ns***	***ns***	***ns***	*****	*****
A		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
D		*ns*	*ns*	*ns*	***	***	***
A*D		*ns*	*ns*	*ns*	*	*ns*	*ns*

Regarding prokaryotic structural diversity, 16S rRNA amplicon sequencing resulted in 4,237,334 and 3,136,252 prokaryotic reads for the microcosm and field experiment, respectively. These reads were clustered into 13,898 and 12,866 OTUs, at the 3% dissimilarity level, after quality filtering and removal of singletons. Given that the number of reads correlated significantly ($p<0.001$) with OTU richness in both the microcosm and field experiment, rarefied richness estimates were used, along with Shannon's index (H') and Pielou's evenness (J'), for the comparison of the α-diversity among the studied soil samples. Differences between organically-amended and NPK-fertilized soils were observed only in the field experiment, where organically-amended soils exhibited significantly greater values of H' and J' than NPK-fertilized soils. Within the organically-amended soils, in both experiments, the application of the optimal dose resulted in significantly lower values of rarefied richness and H', compared to the suboptimal dose. In addition, in the field experiment, significantly lower values of J' were observed in soils treated with the optimal dose of the amendment.

The effect of the experimental factor *amendment dose* on prokaryotic functional and structural diversity can also be observed in the redundancy analysis (Figure 5.3). The analysis was significant for both experiments (microcosm and field experiment), and the variance partitioning analysis revealed that *amendment dose* was the only experimental factor that significantly explained the variance of the response variables (*i.e.* functional and structural diversity parameters), explaining 67 and 16% of the total variance for the microcosm and field experiment, respectively. On the other hand, the factor *origin of amendment* explained less than 1% of the total variance in both experiments.

Regarding community composition, the NMDS (based on Bray Curtis dissimilarities in terms of OTU composition) separated organically-amended from NPK-fertilized samples in the microcosm experiment (Figure 5.4A), but not in the field experiment (Figure 5.4B). This effect was further confirmed by PERMANOVA analysis ($F=2.7$, $p=0.01$). Among the organically-amended soils, *amendment dose* was the only significant factor according to PERMANOVA for both experiments ($F=9.1$, $p<0.001$ and $F= 2.1$, $p=0.007$ for microcosm and field experiment, respectively). Indeed, prokaryotic community composition was significantly influenced by the dose of the amendment regardless of the *origin of amendment* (farm-made *vs.* commercial).

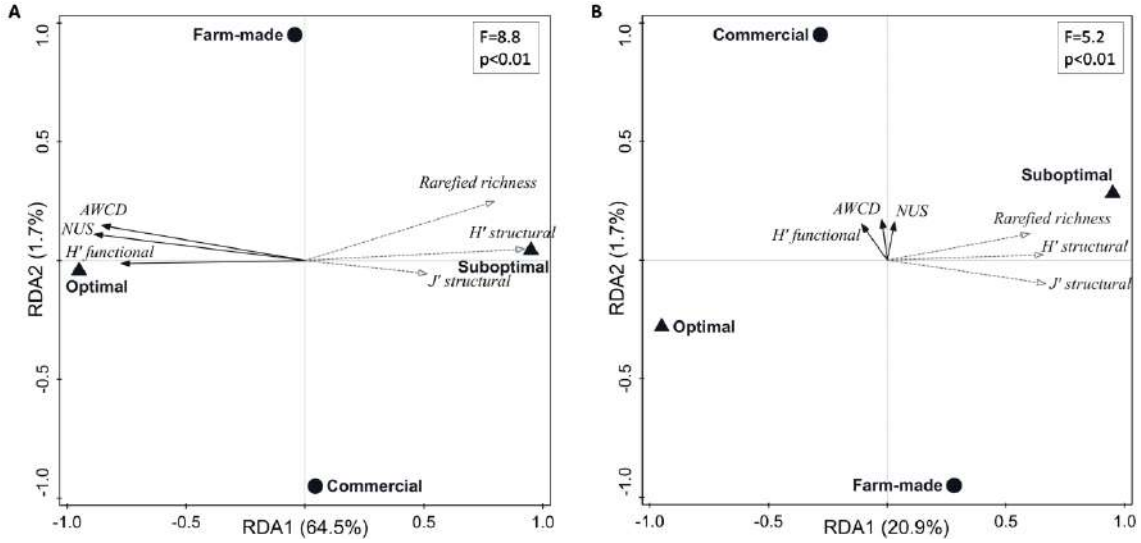

Figure 5.3. Biplot of the redundancy analysis (RDA) performed on functional and structural diversity parameters as response variables. (A) Microcosm experiment. (B) Field experiment. Solid arrows: functional diversity parameters; dotted arrows: structural diversity parameters; NUS: number of used substrates; AWCD: average well color development; H': Shannon's index; J': Pielou's evenness.

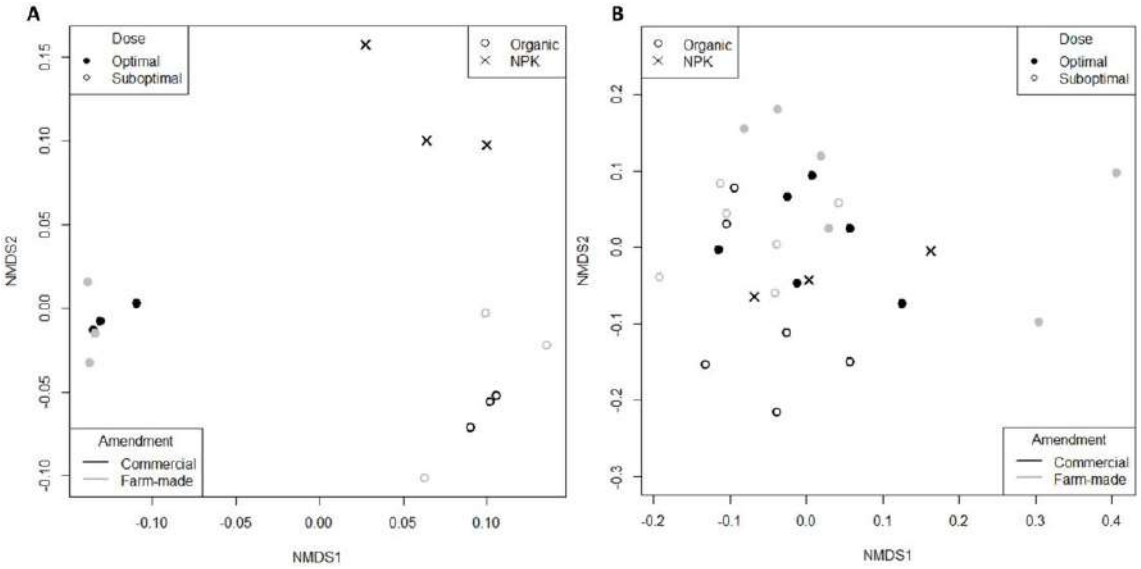

Figure 5.4. Non-metric multidimensional scaling (NMDS) analysis representing patterns of prokaryotic community composition. Bray-Curtis dissimilarities of community composition, based on relative OTU abundances from prokaryotic 16S rRNA amplicon data, are represented as distance in the diagram. (A) Microcosm experiment. (B): Field experiment.

In relation to the taxonomic classification, 99% of the 16S rRNA sequences were classified into phylum rank for both the microcosm and field experiment. At a deeper taxonomical resolution, 74 and 67% of the sequences were classified to family rank for the microcosm and field experiment, respectively. At this family level, the relative abundances of the 30 more dominant taxa (representing more than 0.5% of the total reads)

were evaluated so as to determine the differences in abundance profiles among the studied treatments (Figure 5.5, Table 5.S2). Significant differences in the relative abundances of taxa in organically-amended *vs.* NPK-fertilized soils were only observed for the microcosm experiment, where 8 taxa (constituting *ca.* 19% of the relative abundance of the whole prokaryotic community at the order level) exhibited significant differences (Table 5.S2): three of them (*i.e.* Chitinophagaceae, Sphingomonadaceae, Comamonadaceae) showed higher abundance values in organically-amended soils, accounting for 14.2% of the total prokaryotic community at family rank (compared to 9.0% for NPK-fertilized soils), while the remaining 5 (*i.e.* Rhodospirillaceae, Planctomycetaceae, Bryobacteraceae, Methylobacteriaceae, Streptomycetaceae) exhibited higher abundances in NPK-fertilized soils, accounting for 8.0% of the total community (compared to 5.1% for organically-amended soils). In the field experiment, statistically significant difference regarding the relative abundance of dominant taxa, representing more than 0.5% of the total reads, were not observed (Figure 5.5, Table 5.S2).

Among the organically-amended soils, *amendment dose* was the experimental factor which resulted in the most significant differences: 20 and 15 taxa (constituting 29 and 22% of the relative abundance of the whole prokaryotic community at family rank for the microcosm and field experiment, respectively) exhibited significant differences between soils treated with the optimal *vs.* the suboptimal dose in the microcosm and field experiment, respectively (Table 5.S2). In the microcosm experiment, 9 of these 20 taxa exhibited higher abundance values at the optimal dose, accounting for 20.6% of the total prokaryotic community (compared to 4.6% for soils amended with the suboptimal dose). The remaining 11 taxa were more abundant after the application of the suboptimal dose, accounting for 22.1% of the total prokaryotic community (compared to 13% for soils amended with the optimal dose). In the field experiment, 9 out of 15 taxa were significantly more abundant after the application of the optimal dose, accounting for 17% of the prokaryotic community (compared to 10% for the suboptimal dose), while the remaining 6 taxa showed higher abundance values under the suboptimal dose treatment, accounting for the 9% of the prokaryotic community (compared to 7% for the optimal dose).

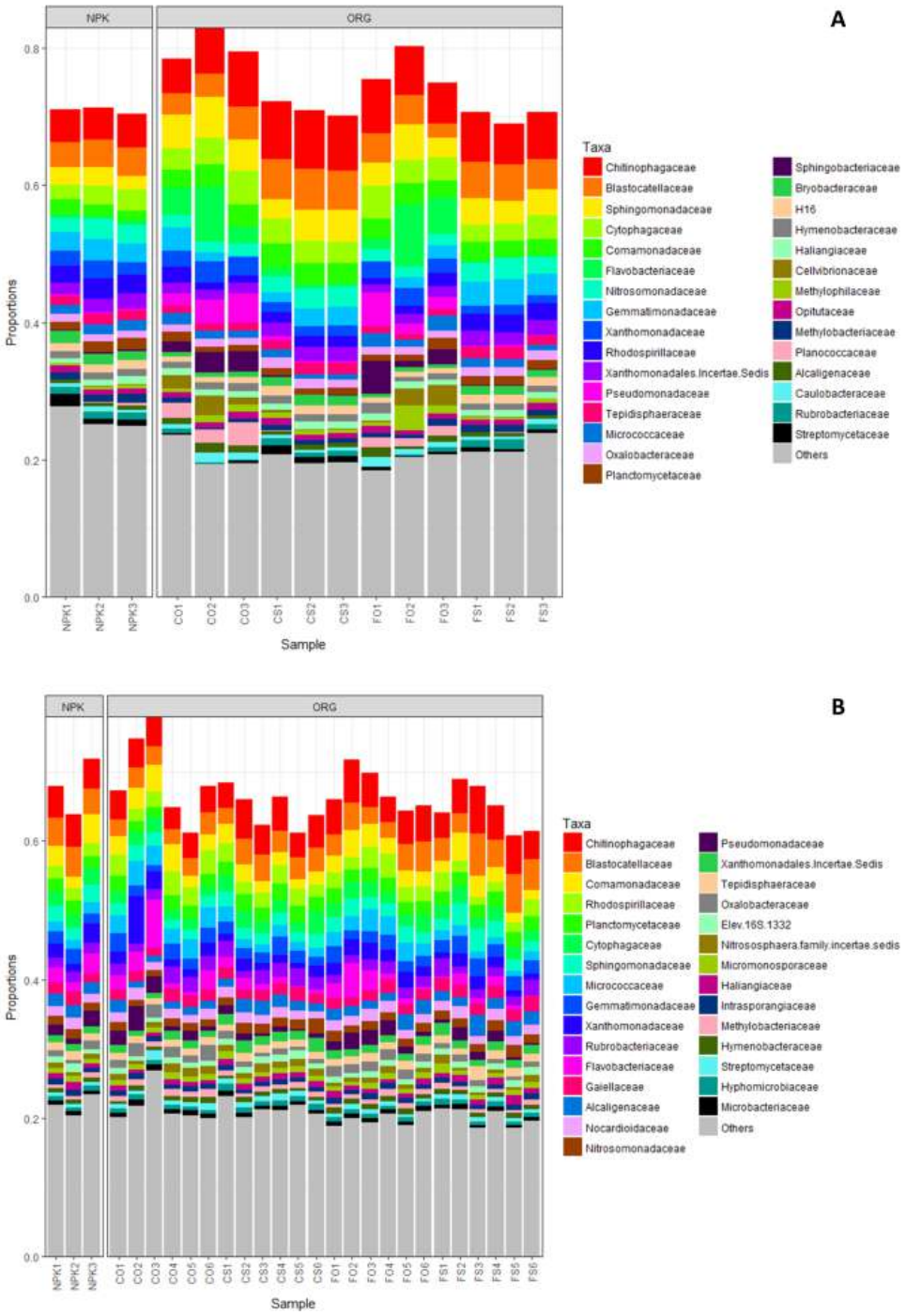

Figure 5.5. Barplots representing the relative abundance of the 30 more abundant prokaryotic taxa at family level. (A) Microcosm experiment. (B) Field experiment. NPK: mineral control; ORG: soils amended with fermented liquid organic amendment.

In the microcosm experiment, the experimental factor *origin of amendment* resulted in significant differences in taxon abundance for just one taxon, Comamonadaceae, which accounted for 3.1 and 2.6% of the prokaryotic community after the application of the commercial and farm-made amendment, respectively. The factor *origin of amendment* resulted in more significant differences in the field experiment, were

7 taxa at family rank were significantly affected (Table 5.S2): the abundance of 5 taxa was significantly enriched by the farm-made amendment (constituting 13% of the prokaryotic community, compared to 10% for the commercial amendment). The remaining 2 taxa exhibited higher abundance in soils treated with the commercial amendment, accounting for 1.7% of the total prokaryotic community (compared to 1.3% for the farm-made amendment).

5.1.4. Discussion

The anaerobic decomposition of organic wastes leads to a reduction of the in-feed biomass through the mineralization of the more labile C fractions and their conversion to biogas (Demirel and Scherer, 2008). After such mineralization, the presence of less labile organic molecules and valuable inorganic nutrients (NPK) in the anaerobically decomposed biomass justifies its use as soil amendment and fertilizer (Tambone *et al*., 2010). Many authors have praised the agronomic properties of this type of amendments as substitutes or complements to synthetic mineral fertilizers. Sogn *et al*. (2018) evaluated the fertilizer value of anaerobically-digested organic amendments, compared to synthetic mineral fertilizer, at the microcosm scale, and reported significantly higher values of wheat yield after the application of digestates in a sandy soil, and similar values in silt- and loam-soils. In another microcosm experiment, Walsh *et al*. (2012) reported similar or higher grass biomass after fertilization with a liquid digestate, compared to mineral fertilizer. At the field scale, Riva *et al*. (2016) concluded that the use of the liquid fraction of a digestate could substitute mineral fertilization for maize production. Similarly, in a field experiment conducted by Alburquerque *et al*. (2012), the application of liquid digestate resulted in watermelon yield values comparable to those obtained with mineral fertilization. Relevantly, in all these studies, the amount of total N supplied by the organic amendments and the mineral fertilizers was matched. Following the same trend, similar crop yields were achieved in organically-amended (optimal dose) *vs.* NPK-fertilized soils in our field experiment.

In the microcosm experiment, lower lettuce yields were found in organically-amended pots, compared to NPK-fertilized pots, even when the N supply was matched. This finding has also been evidenced by other authors such as Quakernack *et al*. (2012) and Wolf *et al*. (2014), who attributed the lower crop yields found in digestate-amended soils to the slow mineralization of the organic N fraction supplied by the amendment. In

this regard, the amount of organic N within the amendment can be reduced by lengthening the fermentation period, which entails increased levels of ammonium (Nkoa, 2014). In our microcosm experiment, the soil was alkaline and sandy and, therefore, the ammonium provided by the amendment could be poorly adsorbed to the soil matrix and then be lost through ammonia volatilization. Also, the utilization of fermented organic amendments may entail nutritional imbalances and/or shortcomings of other essential nutrients such as P and K^+, as suggested by Alburquerque *et al*. (2012). Here, the greater amount of nutrients and OM in our experimental field soil, compared to the microcosm soil, possibly compensated for the lack of phytoavailable nutrients present in the fermented liquid organic amendments. In both experiments (microcosm and field experiment), the suboptimal dose of application of the amendment resulted in significantly lower lettuce yields than those obtained with both the optimal dose of application of the amendment and the mineral fertilizer. Differences in this regard are not surprising since the suboptimal dose of application clearly lacks the required nutrient contents (above all, N) for lettuce plants.

Changes in soil physicochemical parameters following the application of the organic amendments *vs.* mineral fertilizer were only evidenced for the soil nitrate content, which was higher in NPK-fertilized soils in both the microcosm and field experiment (Table 5.1). In the field experiment, this difference was only observed under the suboptimal dose treatment amendment application. In agreement with our findings, Chantigny *et al*. (2007) detected similar nitrate contents in soils fertilized with NPK and those fertilized with organic amendments when total N contents had been matched. On the other hand, in our microcosm experiment, significant differences in nitrate content between organically-amended and NPK-fertilized soils were found regardless of the amendment application dose. The anaerobic decomposition of organic materials containing organic N normally leads to increased levels of soluble inorganic N, mainly ammonium (Möller and Stinner, 2009), which often accounts for 45-80% of the total N present in the liquid phase of the anaerobically-treated organic materials (Möller and Müller, 2012). When ammonium reaches the soil, it can be (i) rapidly oxidized into nitrate by the activity of soil microorganisms (Alburquerque *et al*., 2012), (ii) absorbed by plant root cells, or (iii) adsorbed on negatively charged soil particles. The observed differences in nitrate content between organically-amended and NPK-fertilized soils in our microcosm experiment may be due to the sandy texture of the microcosm soil, resulting in a much lower number of binding sites for the sorption of ammonium (Sogn *et al*.,

2018), which may also result in its loss through ammonia volatilization. In the organically-treated soils, the optimal dose of application led to enhanced nitrate levels in both experiments, compared to the suboptimal dose, which is not surprising given that the application of the optimal dose entails a much greater addition of total N to the soil.

In the microcosm experiment, apart from nitrate levels, values of soil pH and OM content were significantly impacted by the factor *amendment dose*, whereby the optimal dose led to a decrease in pH and an increase in OM content, as compared to the suboptimal dose. Soil pH values may have been altered directly by the application of the organic amendments (the pH of the commercial and farm-made amendments were 4.2 and 4.4, respectively), or indirectly through the condensation of organic acids and/or the mineralization of the ammonium provided by the amendments (Coelho *et al*., 2018). Ammonium mineralization could be responsible for both the enhanced nitrate content and the reduced pH in soils amended with the optimal dose of application. Regarding soil OM content, as described above, the anaerobic decomposition of organic materials leads to the degradation of the more labile organic C fractions, resulting in the presence of a more stable or biochemically protected organic C that can then contribute to soil OM turnover (Tambone *et al*., 2019). The increase in soil OM derived from the application of the optimal dose of the amendment can be directly attributed to a higher input of organic C. Furthermore, the application of the optimal dose of the amendment resulted in higher lettuce yields than the suboptimal dose which may, in turn, lead to an increase in the amount of root exudates (Geisseler and Scow, 2014), thus enhancing the soil organic C pool.

When it comes to assessing soil ecosystem quality and functionality, soil microbial parameters cannot be neglected, since soil microorganisms play a critical role in soil functioning and the delivery of crucial ecosystem services (Garbisu *et al*., 2011; Burges *et al*., 2015). Consequently, soil microbial parameters have become indispensable tools for the evaluation of the effects of disturbances on soil quality, owing to their sensitivity, fast response and ecological relevance (Fließbach *et al*., 2007; Barrutia *et al*., 2011). Significant increases in soil microbial activity and biomass following the application of organic amendments have been evidenced in many studies (Odlare *et al*., 2008; Odlare *et al*., 2011; Alburquerque *et al*., 2012; Siebielec *et al*., 2018). Insam *et al*. (2015) concluded that the application of digested organic materials to agricultural land is likely to enhance soil microbial activity and biomass, compared to mineral fertilization, thus beneficially affecting soil quality and fertility. Here, the application of fermented

liquid organic amendments also resulted in increased soil microbial activity and biomass, with respect to mineral fertilization (Table 5.2). In the microcosm experiment, values of soil respiration, arylsulphatase activity, potentially mineralizable N, microbial biomass C, total bacteria and total fungi were significantly higher in organically-amended *vs*. NPK-fertilized soils. Soil heterotrophic microorganisms rely on the C and nutrients supplied by the OM entering the soil ecosystem, and then this stimulation of microbial activity and biomass can be attributed to increased C and nutrients provided by the amendments (Insam *et al*., 2015). Due to the poor nature of the microcosm experimental soil, the introduction of organic compounds and available nutrients (provided by the organic amendment) could have induced the microbial immobilization of N, boosting microbial biomass and activity (Johansen *et al*., 2013; Tsachidou *et al*., 2019), and resulting in the lower lettuce yields observed in organically-amended soils.

In contrast, none of the studied microbial activity and biomass parameters were significantly altered by the fertilization regime in the field experiment. Other studies have also found no stimulatory effects of fermented organic amendments on soil microbial communities in terms of biomass or activity, when compared to mineral fertilization (Andruschkewitsch *et al*., 2013; Coelho *et al*., 2020). Coelho *et al*. (2020) conducted a two-year field study comparing the effects of four different anaerobic digestates and a mineral fertilizer on soil microbial abundance, and reported no significant effect of the fertilization regime on the abundance of soil bacterial, archaeal and fungal communities. In any case, this lack of differences was attributed to the limited duration of the experiment (Coelho *et al*., 2020). Here, we speculate that the absence of significant differences in microbial activity between organically-amended and NPK-fertilized soils could be explained by a priming effect derived from the addition of mineral N to the soil during NPK fertilization, which may have promoted the mineralization of the indigenous OM present in the soil (Tambone and Adani, 2017). Moreover, the high crop yields displayed under mineral fertilization may have provided an extra amount of organic C through the release of root exudates and, then, stimulate microbial proliferation in the rhizosphere (Baudoin *et al*., 2003; Bais *et al*., 2006).

Differences in microbial activity and biomass were also evidenced within the organically-amended soils, where both factors *amendment dose* and *origin of amendment* exerted a significant effect on microbial activity and biomass in the microcosm and field experiment (Figure 5.2). Particularly, the factor *amendment dose* was determinant in shaping the activity and biomass of soil microbial communities: the optimal dose of

amendment resulted in a substantial increase of several microbial parameters, namely, alkaline phosphatase activity, potentially mineralizable N and the total abundance of bacteria and fungi, compared to the suboptimal dose, in both the microcosm and field experiment. This increment was most likely due to the greater amount of organic C and nutrients derived from the application of a higher dose of amendment, since both the quantity and quality of organic C are crucial in determining the activity and abundance of soil microorganisms (Diacono and Montemurro, 2010). In addition, in the microcosm experiment, the optimal dose led to an increase of β-glucosidase activity (an enzyme related to the C biogeochemical cycle), again probably owing to the higher amount of organic C supplied by the optimal dose. In the field experiment, arylsulphatase activity (an enzyme involved in the mineralization of organic sulphur and the release of plant available S) increased after the application of the optimal dose of amendment, compared to the suboptimal dose, once more due to the greater input of organic substrates susceptible for enzymatic hydrolysis.

The factor *origin of amendment* exerted a significant effect on some microbial activity parameters, but not on microbial biomass parameters. The commercial amendment led to enhanced values of potentially mineral N in both experiments, as well as higher values of urease activity in the microcosm experiment, compared to the farm-made amendment. Both parameters (potentially mineral N, urease activity) reflect the capacity of the soil to supply plant-available N. In this sense, it has been stated (Sikora and Szmidt, 2001) that the C/N ratio has a key role for the dynamics of organic N mineralization in soil. Lower C/N ratios are often associated to a higher soil microbial activity (Tambone and Adani, 2017). Hence, the observed differences in microbial activity may be due to the lower C/N ratio exhibited by the commercial amendment.

The supply of different carbon substrates and nutrients provided by the fermented liquid organic amendments may not only impact soil microbial activity and biomass, but also soil functional and structural diversity owing to an increase in the number of ecological niches and the promotion of ecological interactions (Tian *et al*., 2017). Soil biodiversity is essential for several crucial soil processes, as well as for soil fertility and functional stability (Harrison *et al*., 2014; Isbell *et al*., 2015; Bender *et al*., 2016). In consequence, the assessment of the effects of agricultural practices on soil biodiversity is fundamental to ensure its conservation. In this regard, the long-term utilization of mineral fertilizers has been reported to negatively affect soil microbial diversity (Fließbach *et al*., 2007; Geisseler and Scow, 2014). On the contrary, many studies have addressed the

positive impact of organic amendments on soil microbial structural (Sapp *et al*., 2015; Gu *et al*., 2019) and functional (Frąc *et al*., 2012; Chou *et al*., 2017) diversity. In particular, the sometimes referred to as "bio-extracts", liquids (often of a yellowish-brown colour) derived from the fermentation of plant and animal residues, are known to contain a great variety of microorganisms (together with minerals, hormones, enzymes, organic substances, etc.) whose abundance has been reported to decrease with fermentation time (Sermkiattipong and Tangthong, 2015). Here, we assessed soil microbial (bacterial) functional diversity through the utilization of CLPPs with Biolog EcoPlates™, which illustrate the ability of the culturable portion of the bacterial heterotrophic community to metabolize C substrates. Contrary to the aforementioned studies, in our experiments (microcosm, field), no significant effect of the fertilization regime (organically amended *vs*. NPK-fertilized) was evidenced for any of the functional diversity parameters studied here (Table 5.3). However, in the microcosm experiment, when comparing NPK-fertilized *vs*. organically-amended soils at the optimal dose of application, a significant increase in AWCD, NUS and H' values was observed, possibly owing to the greater amount and diversity of C substrates, as reported by Gomez *et al*. (2006) and Frąc *et al*. (2012). In the field experiment, no differences in functional diversity were observed among the organically-amended soils. On the other hand, in the microcosm experiment, the optimal dose of amendment led to a significant increase of microbial functional diversity, as compared to the suboptimal dose (Figure 5.3). The greater amount of different organic substrates and nutrients is probably responsible for the enhanced values of soil microbial activity, biomass and functional diversity (Epelde *et al*., 2009). Yet, it must be taken in consideration that these differences only reflect the responses of the cultivable, heterotrophic, fast growing fraction of soil bacterial communities, and not the functional ability of the entire microbial community (Ros *et al*., 2008).

Pertaining to structural prokaryotic diversity (16S rRNA metabarcoding analysis), the application of organic amendments increased richness (H') and evenness (J') values, compared to mineral fertilization. Other authors (Sapp *et al*., 2015; Ji *et al*., 2018) also reported the stimulation of soil microbial diversity after the application of fermented organic amendments. As already discussed, the higher availability of different nutrients and organic C substrates may increase the number of ecological niches and, hence, biodiversity. The stimulation of copiotrophic organisms derived from the addition of exogenous OM has been addressed as a potential explanation for this increase in microbial diversity (Hartman *et al*., 2015). Conversely, in the microcosm experiment, no effect of

the fertilization regime in prokaryotic structural diversity was observed. In the respect, it must be taken into account that the high crop yield obtained under NPK fertilization soils most likely promoted the release of root exudates and, thus, the diversity of organic substrates. Further, the supply of organic C may lead to a selective enrichment, which may be reflected in an increase of microbial biomass and a reduction of structural diversity (Goldfarb *et al*., 2011). In organically-amended soils, the optimal dose led to reduced levels of prokaryotic structural diversity, compared to the suboptimal dose, in both experiments (Figure 5.3). This negative correlation between the dose of amendment and the prokaryotic structural diversity may be due to the abovementioned selective enrichment (Goldfarb *et al*., 2011) or to the incorporation of potentially toxic elements as discussed by Daquiado *et al*. (2016).

Regardless of the effects on microbial diversity, the application of organic amendments can induce significant shits in soil microbial community composition (Pershina *et al*., 2015), which may be relevant as they may alter soil functioning (Morriën, 2016). In a long-term experiment on the effect of organic *vs*. mineral farming, Bonanomi *et al*. (2016) reported the stimulation of soil ecosystem functioning under organic management, which was attributed to changes in soil microbial composition. In our microcosm experiment, the ordination analysis (NMDS) displayed well-differentiated clusters for the organically-amended and NPK-fertilized soils, evidencing a significant effect of fertilization regime on prokaryotic community composition. As a matter of fact, in the microcosm experiment, the fertilization regime resulted in significant differences on the relative abundance of 8 taxa at family level, which accounted for almost one-fifth of the whole prokaryotic community. Organically-amended soils displayed enhanced abundances of Chitinophagaceae, Sphingomonadaceae and Comamonadaceae, whose ability to metabolize recalcitrant organic compounds has been suggested (Zhang *et al*., 2018). Given that the anaerobic decomposition of organic materials entails the degradation of the labile organic C fractions, the presence of more recalcitrant organic C within the amendment themselves may explain the enriched abundances of these taxa in the organically-amended soils, compared to NPK-fertilized soil. Wolińska *et al*. (2018) searched for taxonomic indicators of soil fatigue (the exhaustion of the soil through depletion of nutrients essential for plant growth) and, among the 118 bacterial families identified by the authors, 10 were found to be sensitive to agricultural land use by a reduction in the number of OTUs. Interestingly, the three bacterial taxa exhibiting here an enhanced abundance in organically-amended soils are present in the list of those 10

soil fatigue indicators (Wolińska *et al*., 2018), but none of the taxa enriched in the NPK-fertilized soils is present in such list. On the other hand, in our field experiment, differences in community composition between organically-amended and NPK-fertilized soils were not significant according to PERMANOVA ($p>0.05$). As differences in microbial composition are often attributed to alterations in soil physicochemical properties (Das *et al*., 2017; Wang *et al*., 2017), the observed lack of differences in the field experiment may be due to the absence of substantial differences in soil physicochemical parameters between organically-amended and NPK-fertilized soils, or to a high degree of ecological stability of the indigenous soil prokaryotic community (Allison and Martiny, 2008).

Regarding organically-amended soils, in both experiments, the factor *amendment dose* significantly affected soil prokaryotic community composition. The observed differences between the optimal and suboptimal dose of application may arise from the higher nitrate content detected in the soil treated with the former, since nitrate level has been reported to be a key factor shifting the prokaryotic composition on agricultural soils (Liu *et al*., 2018; Gu *et al*., 2019). Similarly, in the microcosm experiment, both the increased OM values and the reduced pH values might be responsible for the observed differences in prokaryotic composition. Soil pH has been frequently reported to substantially affect microbial composition (Rousk *et al*., 2010). Moreover, crop yield can also play an important role shifting soil microbial community composition (Sapp *et al*., 2015). The optimal dose of application resulted in the enrichment of 9 taxa in both experiments (3 of them, *i.e.* Xanthomonadaceae, Pseudomonadaceae and Micrococcaceae were found in both experiments). Interestingly, in the microcosm experiment, 5 bacterial families (*i.e.* Flavobacteriaceae, Pseudomonadaceae, Sphingobacteriaceae, Cellvibrionaceae, Planococcaceae) accounted for 14 and 1% of the prokaryotic community in soils amended with the optimal and suboptimal dose, respectively. Such enhancement could be directly related to the application of the organic amendments, as all taxa were initially present in the amendments themselves, except for Planococcaceae (Figure 5.S1). Regarding the field experiment, Flavobacteriaceae, Oxalobacteraceae and Micrococcaceae were the most enriched taxa under the optimal dose treatment. Representatives of Micrococcaceae family have been associated with the degradation of recalcitrant organic compounds (Storey *et al*., 2018). Members of Oxalobacteraceae have been reported to act as saprophytic bacteria with the capacity to metabolize a wide range of organic molecules, and to be strongly influenced by plant exudates (Green *et al*., 2006,

2007). The enrichment of these taxa may directly result from a greater input of organic substrates at the optimal dose of application, or indirectly, due to the greater release of plant exudates arising from the higher crop yield values.

5.1.5. Conclusions

The microcosm and field experimental soil were different, and so it is not surprising that different conclusions were gathered regarding the effect of fermented liquid organic amendments vs. NPK-fertilization on lettuce yield and soil quality. In the microcosm experiment, the application of fermented liquid organic amendments resulted in a substantial improvement of soil quality, as evidenced by the higher values of soil microbial biomass, activity and functional diversity, compared to NPK fertilization. Nevertheless, values of lettuce crop yield were lower in organically-amended soils. In the field experiment, organically-amended and NPK-fertilized treatments resulted in comparable lettuce yields when the N supply was matched (*i.e.* with the optimal dose of the amendment). Further, amendment application led to enhanced soil prokaryotic α-diversity, compared to NPK fertilization, but did not result in a stimulation of soil microbial activity and biomass. Soil parameters were not significantly influenced by the origin of the amendment (commercial vs. farm-made) in none of the experiments. Eventually, our results suggest that the potential benefits exerted by fermented liquid organic amendments strongly depend on the amendment dose and the type of soil. Further studies are required to better understand the benefits and limitations of fermented liquid organic amendments on both crop yield and soil quality, especially in the long-term.

5.2. Impact of the application of commercial and farm-made fermented liquid organic amendments on corn yield and soil quality

Urra, J., Mijangos, I., Epelde, L., Alkorta, I., Garbisu, C., accepted with revisions in ***Applied Soil Ecology***.

Abstract

Organic amendments are being increasingly applied to agricultural soils as alternatives or complements to inorganic fertilizers. Organic amendments can increase the content of soil organic matter, concomitantly improving soil physical, chemical and biological properties. We evaluated the effect of the application of commercial *vs.* farm-made fermented liquid organic amendments, compared to mineral (NPK) fertilization, on corn yield and nutritional status, as well as on soil physicochemical and microbial properties as indicators of soil quality. In particular, we assessed the effect of two doses (optimal *vs.* suboptimal) and times of amendment application (basal dressing *vs.* basal+top dressing) for two consecutive growing seasons. Values of corn grain yield were lower in organically-amended plots than in those under NPK fertilization. At the optimal dose, the application of organic amendments led to significantly higher values of microbial activity (soil respiration; enzyme activities; potentially mineralizable N) and biomass (microbial biomass C, bacterial and fungal biomass), leading to an improvement in soil quality. The optimal application dose resulted in an enhanced soil nutrient pool. Conversely, soil properties were not significantly affected by the origin of the amendment (commercial *vs.* farm-made) or the time of application (basal dressing *vs.* basal+top dressing). 16S rRNA metabarcoding analysis revealed no significant changes in the diversity and composition of the soil prokaryotic communities between organically-amended and NPK-fertilized soils. It was concluded that the application of fermented liquid organic amendments at the optimal dose (according to the N requirements of corn) can be a beneficial agronomic practice for agricultural soil quality, posing a suitable alternative to mineral fertilization.

5.2.1. Introduction

Intensive agriculture has resulted in a decrease in soil quality by adversely affecting soil biota, reducing soil organic matter (OM) content and polluting the environment (Diacono and Montemurro, 2010; Das *et al.*, 2014). Furthermore, agricultural intensification, along

with animal husbandry and poultry breeding, has led to the generation of large amounts of wastes that need to be properly managed (He *et al*., 2019). In the last years and decades, the application of OM in the form of crop residues, manure, slurry, compost, sewage sludge, etc. into soil has gained much interest as a sustainable approach for the reutilization of these byproducts as soil amendments and as a realistic, cost-effective and environmentally sound alternative to landfill disposal (Mondini and Sequi, 2008; Diacono and Montemurro, 2010; Urra *et al*., 2018; Chojnacka *et al*. 2019). Organic amendments represent a valuable source of nutrients for impoverished agricultural soils, with the potential to act as alternatives or complements to mineral fertilizers (Riva *et al*., 2016; Aranguren *et al*., 2018; Ji *et al*., 2018). Organic amendments can replenish depleted soil organic carbon (C), thus stimulating soil biological activity and improving soil structure, water-holding capacity, etc. and concomitantly enhancing crop yield (Lal, 2008; Powlson *et al*., 2014; Hernández *et al*., 2016). Organic amendments can positively affect soil microbial activity, biomass and diversity, the latter being presumably linked to functional redundancy and ecological stability (Stockdale *et al*., 2013; Larney *et al*., 2016), as well as to the provision of ecosystem services (Tilman, *et al*., 2006; Delgado-Baquerizo *et al*., 2016). The long-term use of mineral fertilizers can negatively influence soil microbial diversity (Fließbach *et al*., 2007). In turn, the application of organic amendments has been reported to positively impact the diversity and composition of soil microbial communities (Chaudhry *et al*., 2012), with concomitant beneficial effects on soil health (Fließbach *et al*., 2007).

However, the improper application and overuse of organic amendments (especially those derived from raw organic waste) to soil may entail important drawbacks as they can, for instance, contain a variety of contaminants (McBride *et al*., 1997; Rizzo *et al*., 2013; Zhu *et al*., 2013; Petrie *et al*., 2014; Martín *et al*., 2015; Urra *et al*., 2019b,c), thus posing a potential risk to human and ecosystem health. We must guarantee that the application of organic amendments addresses soil constraints without introducing risks to human and ecosystem health (Castán *et al*., 2016). In this sense, the fermentation or anaerobic decomposition of OM can effectively reduce the levels of organic contaminants present in the amendments (Ghattas *et al*., 2017; Martín *et al*., 2015) while resulting in the production of energy (biogas) and adequate soil amendments with high fertilization potential (Riva *et al*., 2016; Tambone and Adani, 2017). Furthermore, fermented amendments can be adapted to plant nutrient needs through the addition of substrates with various elemental compositions during the anaerobic digestion (Möller, 2018). The

impact of fermented amendments on soil quality depends on the composition and properties of the applied biomass which, in turn, depends on the fermentation process itself (Nkoa, 2014).

The objective of this study was to assess, during two consecutive growing seasons, the impact of two different preparations (commercial *vs.* farm-made) of fermented liquid organic amendments at two doses (optimal *vs.* suboptimal) and times (basal dressing *vs.* basal+top dressing) of application on: (i) corn yield and nutritional status, and (ii) soil physicochemical and microbial parameters as indicators of soil quality. There is still limited information at field scale on the changes induced by the application of fermented liquid organic amendments at different doses and times of application on soil prokaryotic diversity and composition. We hypothesized that, after two years of application, both fermented liquid organic amendments (commercial *vs.* farm-made) at the optimal dose (established according to the N needs of corn) will positively impact soil microbial communities, enhancing their diversity and stimulating microbial activity and biomass, and will have a more positive effect on soil quality than the mineral (NPK) fertilizer.

5.2.2. Materials and methods

5.2.2.1. Fermented liquid organic amendments

Two fermented liquid organic amendments were tested in this study: (1) a *commercial amendment* bought from a local company (VITAVERIS SC, Spain) which acts regularly as a consultant for farmers who want to prepare this type of liquid organic amendments by themselves using byproducts; and (2) *farm-made amendment:* it was prepared by a local farmer according to the guidelines provided by VITAVERIS SC., using the same in-feed biomass, as follows: 3 kg of molasses were thoroughly mixed with 50 L of cow milk whey in a 60 L-polyethylene container. Subsequently, 2.8 kg of a mixture consisting of 29% (w/w) oak leaf litter visually colonized by fungal mycelium, 57% (w/w) wheat bran and 14% (w/w) molasses was introduced to the 60 L-polyethylene container. Afterwards, 2 kg of basaltic dust was added to the container. Finally, the container was hermetically sealed for fermentation, allowing the gas to be released through a septum, and maintained as such for 30 days. The fermented liquid organic amendments were subjected to physicochemical characterization (Table 5.S1) according to standard methods (MAPA, 1994).

5.2.2.2. Experimental design and treatments

To study the effect of the application of the organic amendments on crop yield and soil quality, a field experiment was conducted during two consecutive seasons (2015 and 2016) in San Vicente de Arana (Araba, Spain, 42°45'16.4"N, 2°21'07.4"W), at an altitude of 825 m above sea level. This area is characterized by a humid, cool maritime Mediterranean climate, with a mean rainfall of 340 mm and a daily average temperature of 13.6ºC during the corn-growing period (Figure 5.S2). A physicochemical characterization of the experimental soil (Table 5.4) was performed according to standard methods (MAPA, 1994).

Table 5.4. Physicochemical characterization of the experimental soil.

Coarse sand (%)	3.2
Fine sand (%)	17.1
Silt (%)	51.7
Clay (%)	28.0
Classification (USDA)	Silty clay loam
pH 1:2.5	8.5
OM (%)	1.42
Olsen P (mg kg^{-1})	23.1
K^+ (mg kg^{-1})	135.7
N-total (%)	0.11
N-NO_3^- (mg kg^{-1})	1.89
N-NH_4^+ (mg kg^{-1})	0.25

In two consecutive growing seasons, corn seeds belonging to the 400 FAO (var. Anjou 456) cycle were planted in late spring at a density of 66,666 plants ha^{-1} with a distance of 75 cm between rows. Experimental plots of 15 m^2 were arranged following a completely randomized factorial design with three replicates. Three experimental factors were tested: (i) *origin of amendment:* commercial *vs.* farm-made; (ii) *amendment dose:* optimal (adjusted according to the N demand of corn in our geographical area, *i.e.* 150 kg N ha^{-1}) *vs.* suboptimal (400 L of the liquid amendment pre-diluted to 5% ha^{-1}). This suboptimal dose (suboptimal from the point of view of the N needs of corn) was tested as it is the dose recommended by VITAVERIS SC for this type of fermented liquid organic amendments; and (iii) *time of application:* basal dressing (one sole application of the whole dose of the amendment before sowing) vs. basal+top dressing (the dose was divided in four identical applications throughout the crop growing stages: before sowing, 30, 45 and 60 days after sowing). The liquid organic amendments were applied manually to the soil surface prior to sowing. A control treatment, consisting of mineral (NPK) fertilization applied at the typical corn fertilization rate in our region, was included for

comparison purposes: 50 kg N ha^{-1} as NH_4NO_3 (34.4%), 60 kg P ha^{-1} as P_2O_5 (18%), and 100 kg K ha^{-1} as K_2O (60%), applied as basal dressing; and 100 kg N ha^{-1} as NH_4NO_3 (34.4%) one month after sowing. The plots were irrigated with a sprinkler 2-3 times per week depending on the weather conditions. Weed control was performed manually during the first month of the crop growing period. Treatments are summarized in Table 5.5.

Table 5.5. Scheme of the studied experimental factors.

Origin of amendment	Amendment dose	Time of application
Commercial	Optimal	Basal dressing
Commercial	Optimal	Basal + Top dressing
Commercial	Suboptimal	Basal dressing
Commercial	Suboptimal	Basal + Top dressing
Farm-made	Optimal	Basal dressing
Farm-made	Optimal	Basal + Top dressing
Farm-made	Suboptimal	Basal dressing
Farm-made	Suboptimal	Basal + Top dressing
Mineral (NPK) control		

The corn was harvested in late November. The grain yield was measured by harvesting 10 plants from the two central rows. Grain yield data were converted (on a dry mass basis) to kg ha^{-1}. Regarding the nutritional parameters of corn grain, ash, crude protein, crude fiber, crude fat and starch contents were determined by near-infrared reflectance spectroscopy (NIRS) at regions 400-2500 nm using a NIRSystems 6500 Scanning Monochromator (Foss NIRSystems, Silver Spring, MD, USA) following Fassio *et al*. (2009).

5.2.2.3. Soil parameters

5.2.2.3.1. Physicochemical parameters

Sampling was conducted just before to crop harvest by randomly collecting 6 soil samples from each plot at a depth of 0-30 cm and mixing them together to obtain a composite sample. Then, soil samples were dried at 30°C and sieved to <2 mm. The following soil physicochemical parameters were determined according to standard methods (MAPA, 1994): pH, Olsen P, extractable K^+, nitrate, ammonium, total N and organic C.

5.2.2.3.2. Microbial parameters

For the microbial parameters, the same composite samples were sieved to <2 mm and then stored fresh, for less than a month, at 4°C until analysis. Samples for molecular analyses were stored at -20°C. Soil respiration was determined following ISO 16072 Norm (2002). Urease activity was measured following Kandeler and Gerber (1988). β-glucosidase, arylsulphatase and alkaline phosphatase activities were determined according to Dick *et al*. (1996) and Taylor *et al*. (2002). Potentially mineralizable N (P_{MN}) was measured as described by Powers (1980), and microbial biomass C (Cmic) was measured following Vance *et al*. (1987).

For the molecular analysis, DNA extraction was carried out from three technical replicates, each corresponding to 0.25 g dry weight (DW) soil from each sample, using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA). Prior to DNA extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk *et al*., 1997). The amount of DNA was determined with a ND-1000 Spectrophotometer (Thermo-Scientific, Wilmington, DE). To estimate the abundance of 16S rRNA gene fragments for total bacteria and 18S rRNA gene fragments for total fungi, real-time quantitative PCR (qPCR) measurements were carried out following the reaction mixtures, primers and PCR conditions described in Epelde *et al*. (2014).

5.2.2.3.3. DNA metabarcoding

To study the prokaryotic community diversity and composition of the organic amendments and fertilized soils (organically-amended *vs.* NPK-fertilized), amplicon libraries were prepared using a dual indexing approach with sequence-specific primers targeting the V4 hypervariable region of the 16S rRNA gene (Lanzén *et al*., 2016): 519F (CAGCMGCCGCGGTAA) adapted from Øvreås *et al*. (1997), and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso *et al*. (2012). Libraries were subsequently sent to Tecnalia (Spain), where sequencing was carried out with an Illumina MiSeq V2 platform and pair-ended 2×250 nt. Merging of the read paired ends, quality filtering (primer trimming, removal of singletons and chimeric sequences) and clustering into operational taxonomic units (OTUs) were performed following Lanzén *et al*. (2016). Taxonomic assignments were carried out using CREST and SilvaMod v128 (Lanzén *et al*., 2012; https://github.com/lanzen/CREST).

5.2.2.4. Statistical analysis

We evaluated the impact of organic amendment application on (i) crop yield and nutritional status and (ii) soil quality, compared to NPK fertilization. The main effects and interactions of the three experimental factors (*amendment dose*, *origin of amendment*, *time of application*) were evaluated: *amendment dose* was a fixed factor with two levels: optimal and suboptimal; *origin of amendment* was a fixed factor with two levels: commercial and farm-made; *time of application* was a fixed factor with two levels: basal dressing and basal+top dressing. The statistical significance of the differences in the effects of organic amendments and NPK fertilizer on (i) crop yield and nutritional status, and (ii) soil physicochemical and microbial parameters as indicators of soil quality, was determined according to the pooled-variances *t*-test (for equal variances) or Welch's *t*-test (for unequal variances). After normality and homogeneity testing, differences among experimental factors and their interactions were assessed by means of three-way ANOVA and Duncan's multiple range test (when the interaction effect was significant) using the package *agricolae* in the R software (version 3.3.2). The relationships among the experimental factors were further analyzed by performing redundancy analyses (RDA) and variation partitioning analyses with Canoco 5.0 (Ter Braak and Šmilauer, 2002).

For the assessment of the impact of the experimental factor *amendment dose* on the values of soil microbial activity and biomass parameters, in comparison with values obtained in NPK-fertilized soil, individual values of all the parameters determined here were used for the calculation of the soil quality index (SQI) following Mijangos *et al*. (2010):

$$\mathrm{SQI} = 10^{\log m + \frac{\sum_{i=1}^{n}(\log n_i - \log m)}{n}}$$

where *m* is the reference value (set to 100% for the mean value of each parameter in the NPK-fertilized soil) and *n* corresponds to the measured values for each parameter as a percentage of the reference value. The microbial parameters considered for this calculation were: respiration, enzyme activities (β-glucosidase, arylsulphatase, alkaline phosphatase, urease), P_{MN}, Cmic, and bacterial and fungal gene abundance.

Determination of α-diversity indices, multivariate statistics and visualization of 16S rRNA amplicon sequencing data were performed with R package *vegan* (Oksanen *et al*., 2015). OTU distributions were transformed into relative abundances using

function *decostand*. Bray-Curtis dissimilarity matrices were calculated to compare prokaryotic community composition between samples. These matrices were further used to perform non-metric multidimensional scaling (NMDS) with function *metaMDS*. Permutational analyses of variance (PERMANOVA) were performed to assess the impact of the experimental factors on prokaryotic community composition, using *adonis* function. Pairwise analysis on the relative abundances of every taxon (at order level) representing more than 0.1% of the total reads were performed by means of one-way ANOVA.

5.2.3. Results

5.2.3.1. Plant parameters

5.2.3.1.1. Crop yield

In the first growing season, the application of organic amendments did not result in significant differences in corn grain yield when compared to NPK fertilization (Figure 5.6). Similarly, no significant differences were observed among the organically-amended plots. In contrast, statistically significant differences in corn grain yield were observed after the second growing season, not only between organically-amended and NPK-fertilized plots but also among the organically-amended plots (Figure 5.6). The optimal dose of organic amendments resulted in corn grain yield values similar to those exhibited by the NPK control and much greater (49% higher; $p<0.001$) than those obtained with the suboptimal dose. In addition, the commercial amendment resulted in a 21% increase in corn grain yield in comparison to the farm-made amendment. The basal dressing application yielded 28% more corn grain than the basal+top dressing application.

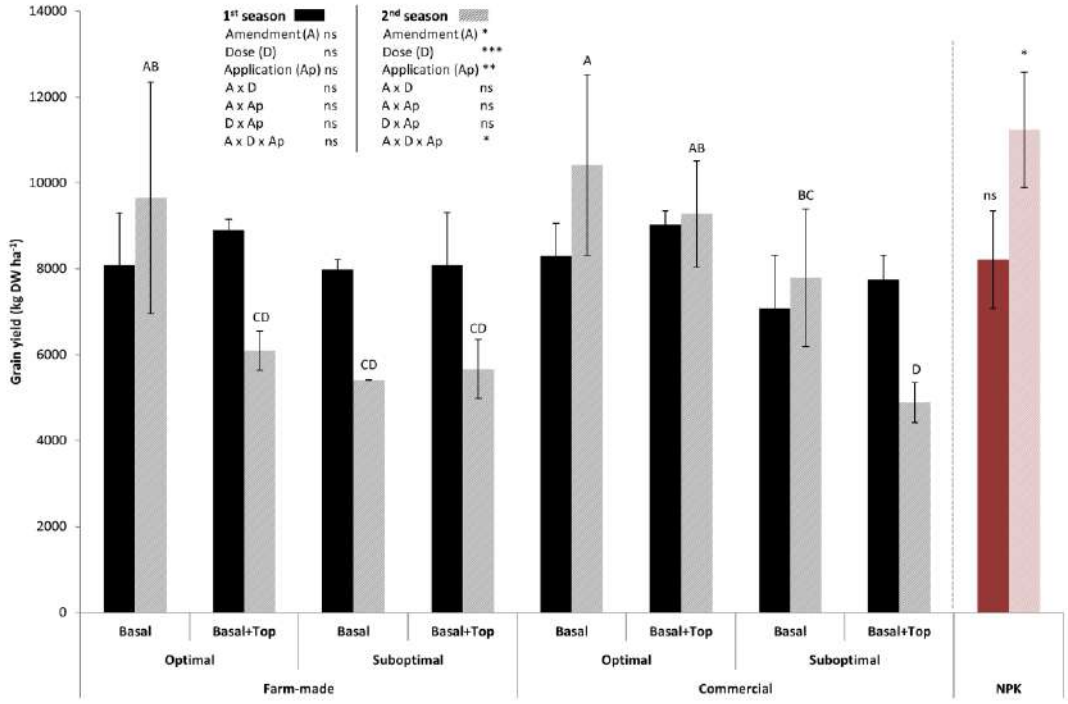

Figure 5.6. Corn grain yields (kg DW ha^{-1}) for the two growing seasons. NPK: mineral control. Differences between mineral control and organic amendment treatments for each year are based on pooled variances *t*-test or Welch´s *t*-test and displayed on the top of the NPK bars as *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001. For the organically-amended soils, interaction among factors was tested by three-way ANOVA. Letters address significant differences among treatments according to one-way ANOVA and Duncan's MRT when experimental factor interaction was significant. Mean values (n=3) ± SD.

5.2.3.1.2. Nutritional parameters

The suboptimal dose resulted in significantly higher values of corn grain starch content than those exhibited by corn grown in NPK-fertilized soils which, on the other hand, yielded higher values of ash and crude protein contents (Table 5.S3). The optimal dose led to similar results to those exhibited by the NPK control for each of the nutritional parameters, except for protein content. The NPK control yielded a 21% increase in crude protein content compared to the optimal dose of organic amendments. Regarding the differences among organically-amended plots, the redundancy analysis showed a significant effect of the experimental factors (Figure 5.7: F=9.9, p<0.01) on corn grain nutritional parameters. The variation partitioning analysis further revealed a significant effect of the three experimental factors, with *amendment dose* being the most relevant factor (it explained 35% of the total variation) followed by the factor *origin of amendment* and the factor *time of application* (10.1 and 8.8% of the total explained variation, respectively).

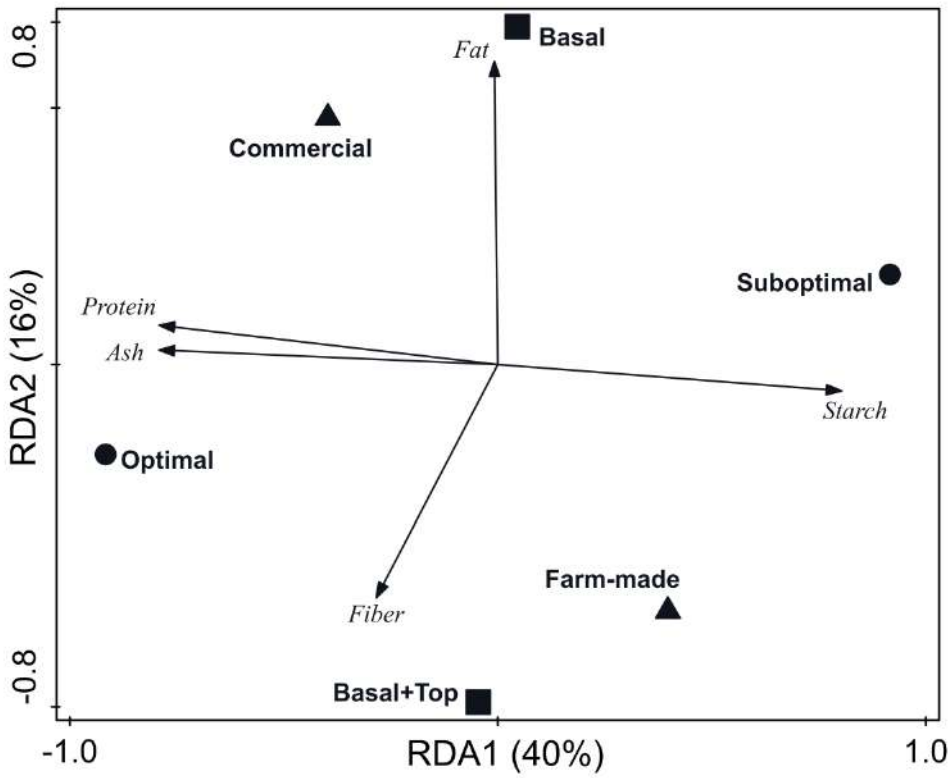

Figure 5.7. Biplot of the redundancy analysis (RDA) performed on corn grain nutritional parameters as response variables displayed by means of the experimental factors.

The interaction between *origin of amendment* and *amendment dose* resulted in significant effects on ash, starch and crude protein contents. Starch content showed higher values when using the farm-made amendment at the suboptimal dose (values were significantly lower with the optimal dose of the commercial amendment). The opposite effect was observed for ash and crude protein contents, showing enhanced values after the application of the optimal dose of the commercial amendment, which were significantly higher than those obtained by the application of the optimal dose of the farm-made amendment, and in both cases significantly higher than those obtained with the suboptimal dose regardless of the *origin of amendment* (Table 5.S3). The crude fiber content was significantly affected by the factors *amendment dose* and *time of application*, with higher values observed when the organic amendment was applied at the optimal dose followed by the basal+top dressing application. The basal dressing application showed higher values of crude fat in comparison to the basal+top dressing application. This parameter was also affected by the *origin of amendment*: the application of the commercial amendment led to significantly higher values than the application of the farm-made amendment (Figure 5.7, Table 5.S3).

5.2.3.2. Soil parameters

5.2.3.2.1. Physicochemical parameters

No statistically significant differences were observed between organically-amended and NPK-fertilized soils for any of the soil physicochemical parameters (Table 5.S4). However, significant differences in some of these parameters were observed among the organically-amended soils depending on the experimental factor. Thus, the variation partitioning and redundancy analysis (Figure 5.8: $F=3.0$, $p<0.01$) revealed that the experimental factors *amendment dose* and *origin of amendment* were both significant in explaining the observed differences in the values of the physicochemical parameters and accounted for 16.1 and 6.2% of the total variation, respectively. The interaction of these two factors was significant for soil nitrate content, where the application of the commercial amendment at the optimal dose resulted in significantly higher values of this parameter than those obtained with the farm-made amendment at the same dose (significantly higher values of soil nitrate content were obtained at the optimal *vs*. the suboptimal dose) (Figure 5.8, Table 5.S4). Values of Olsen P and extractable K^+ were significantly affected by the *amendment dose*: significantly higher values were observed at the optimal *vs.* the suboptimal dose. Regarding the *origin of the amendment*, the application of the commercial amendment resulted in significantly higher values of Olsen P than those obtained with the farm-made amendment. The factor *time of application* did not have a significant effect on the multivariate models but did significantly affect the content of soil ammonium: the basal+top dressing application led to higher values of this parameter than the basal dressing application. No statistically significant differences were observed for soil pH, OM and total N.

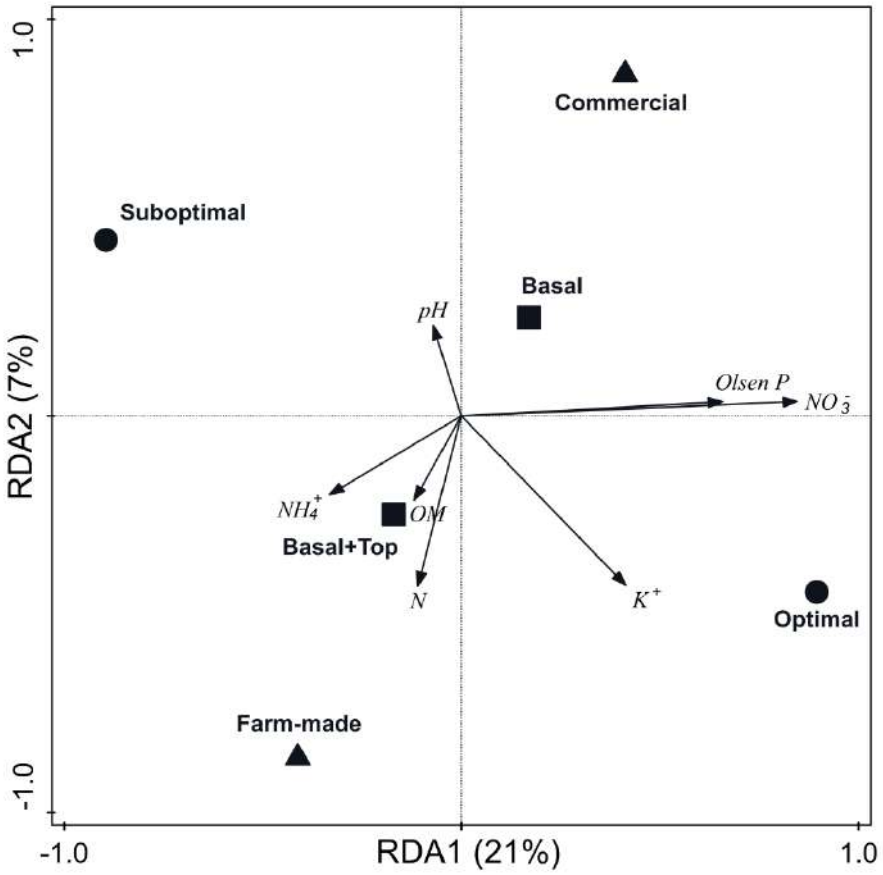

Figure 5.8. Biplot of the redundancy analysis (RDA) performed on soil physicochemical parameters as response variables displayed by means of the experimental factors.

5.2.3.2.2. Microbial parameters

5.2.3.2.2.1. Activity and biomass

In regard to microbial activity and biomass parameters, the application of organic amendments led to numerically higher, but not significantly different, values of all parameters except for Cmic when compared to NPK-fertilized soils (Table 5.6). Nonetheless, the SQI calculated here (an integrative index of all the microbial activity and biomass parameters) was significantly affected by the *amendment dose* ($F=24.4$, $p<0.001$): the application of the optimal dose resulted in a SQI value of 128. This value was 37 and 30% higher than the SQI value obtained with the suboptimal dose of organic amendment and the NPK fertilizer, respectively (Figure 5.S3).

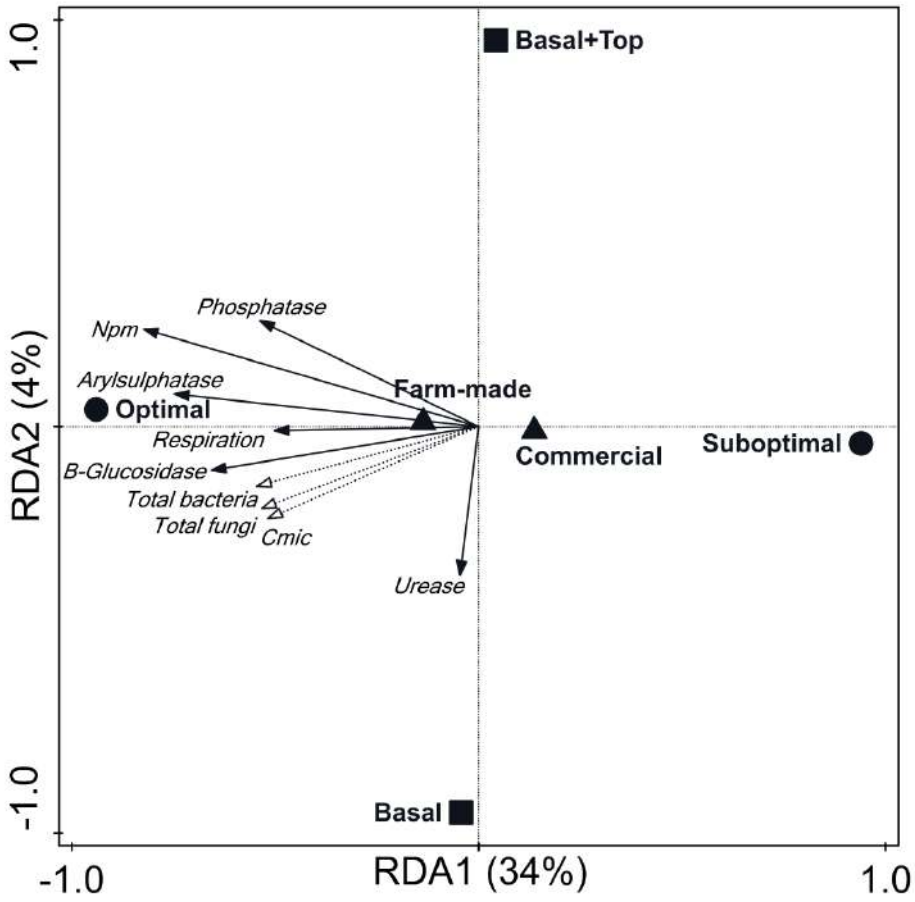

Figure 5.9. Biplot of the redundancy analysis (RDA) performed on microbial activity and biomass parameters as response variables displayed by means of the experimental factors. Solid arrows: microbial activity parameters; dotted arrows: microbial biomass parameters; N_{PM}: potentially mineralizable N; Cmic: microbial biomass C; 16S rRNA and 18S rRNA: gene copy numbers determined by qPCR.

Regarding the differences among the organically-amended soils, the variation partitioning and redundancy analysis (Figure 5.9: $F=4.4$, $p<0.01$) revealed that *amendment dose* was the only experimental factor exhibiting a significant effect on the values of microbial activity and biomass parameters, and explained 33% of the total variation. In contrast, the experimental factors *origin of amendment* and *time of application* did not reveal significant differences (they explained 0 and 1.5% of the total variation, respectively). The factor *amendment dose* exhibited a significant effect on five out of the six studied parameters of microbial activity (*i.e.*, respiration; arylsulphatase, β-glucosidase and alkaline phosphatase activities; N_{PM}), as well as on all the microbial biomass parameters (Cmic, bacterial and fungal abundance). All of these microbial activity and biomass parameters showed significantly higher values at the optimal *vs.* the suboptimal dose (Table 5.6, Figure 5.9).

Table 5.6. Impact of organic amendments on microbial activity and biomass. NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control, differences based on pooled variances *t*-test or Welch´s *t*-test. For the organically-amended soils, interaction among factors was tested by three-way ANOVA, where A: origin of amendment; D: amendment dose; Ap: time of application. Letters address significant differences among treatments according to one-way ANOVA and Duncan's MRT when factor interaction was significant. Mean values (n=3) ± SD.; N_{PM}: potentially mineralizable N; Cmic: microbial biomass C. *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

			ACTIVITY						BIOMASS		
			Respiration	**Arylsulphatase**	**Alkaline Phosphatase**	**β-Glucosidase**	**Urease**	**N_{PM}**	**Cmic**	**Bacterial gene abundance**	**Fungal gene abundance**
			µg C g^{-1} DW soil h^{-1}	mg ρ-nitrophenol kg^{-1} DW soil h^{-1}	mg ρ-nitrophenol kg^{-1} DW soil h^{-1}	mg N-NH_4^+ kg^{-1} DW soil h^{-1}	mg N-NH_4^+ kg^{-1} DW soil h^{-1}	mg N-NH_4^+ kg DW soil $^{-1}$	mg C kg^{-1} DW soil	x 10^9 copies g^{-1} DW soil	x 10^8 copies g^{-1} DW soil
Farm-made	**Optimal**	**Basal**	0.8 ± 0.2	90.5 ± 4.8 ^{A}	129.7 ± 11.5	107.4 ± 16.5	54.7 ± 9.7	14.4 ± 1.9	356 ± 46	6.9 ± 0.0	6.7 ± 1.7
		Basal+Top	0.8 ± 0.1	87.2 ± 3.1 ^{AB}	130.2 ± 8.4	107.2 ± 11.2	50.5 ± 10.8	18.9 ± 4.5	328 ± 99	6.5 ± 1.4	5.8 ± 3.0
	Suboptimal	**Basal**	0.7 ± 0.1	74.4 ± 3.5 ^{D}	111.3 ± 2.8	93.1 ± 8.3	53.1 ± 8.7	2.4 ± 0.4	290 ± 19	5.7 ± 0.3	4.0 ± 0.5
		Basal+Top	0.6 ± 0.1	79.5 ± 7.1 ^{CD}	116.3 ± 6.9	91.2 ± 7.7	51.3 ± 5.6	2.9 ± 1.6	240 ± 58	5.3 ± 1.5	3.1 ± 0.8
Commercial	**Optimal**	**Basal**	0.7 ± 0.0	83.7 ± 3.1 ^{ABC}	112.9 ± 9.5	105.7 ± 5.0	52.2 ± 2.0	8.8 ± 1.2	301 ± 28	6.3 ± 0.3	5.1 ± 0.3
		Basal+Top	0.8 ± 0.1	88.8 ± 4.9 ^{A}	122.5 ± 9.6	107.3 ± 2.8	46.5 ± 5.7	16.0 ± 9.3	307 ± 46	6.5 ± 0.4	5.1 ± 0.5
	Suboptimal	**Basal**	0.7 ± 0.2	80.4 ± 2.8 ^{BCD}	111.8 ± 10.0	99.6 ± 5.2	53.7 ± 7.8	3.2 ± 1.3	291 ± 16	6.1 ± 0.8	5.0 ± 1.6
		Basal+Top	0.6 ± 0.1	76.0 ± 4.2 ^{D}	115.3 ± 4.9	88.8 ± 5.8	45.9 ± 2.0	2.5 ± 0.7	252 ± 59	5.5 ± 0.3	3.9 ± 1.7
		NPK	0.6 ± 0.0	79.8 ± 3.2	112.3 ± 6.1	97.7 ± 8.0	46.5 ± 0.9	4.2 ± 3.1	298 ± 39	6.1 ± 0.3	4.0 ± 0.2
O *vs.* M			***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***
Origin of amendment (A)			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Amendment dose (D)			*	***	**	**	*ns*	***	*	*	*
Time of application (Ap)			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
A x D			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
A x Ap			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
D x Ap			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
A x D xAp			*ns*	*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

5.2.3.2.2.2. Biodiversity

16S rRNA amplicon sequencing resulted in 3,473,317 prokaryotic reads that were clustered into 16,369 OTUs at the 3% dissimilarity level after quality filtering and removal of singletons. The number of reads was significantly correlated with OTU richness ($p<0.001$), indicating that the sequencing effort was insufficient to obtain full coverage of prokaryotic diversity. Rarefied richness estimates, as well as Shannon's index (H') and Pielou's evenness (J'), were used to compare the α-diversity values among the studied soil samples and between the organic amendments themselves. The farm-made amendment showed significantly higher H' and J' values than the commercial amendment (4.3 and 2.8 for H' and 0.56 and 0.36 for J' for the farm-made and commercial amendments, respectively). The application of organic amendments did not lead to statistically significant differences in α-diversity values among the treated soils (Table 5.S5). In fact, none of the experimental factors revealed significant differences in soil α-diversity values nor was there any difference between the organically-amended and NPK-fertilized soils (Table 5.S5).

Regarding prokaryotic community composition, the NMDS (based on Bray-Curtis dissimilarities in terms of OTU composition) did not separate soils treated with organic amendments from those fertilized with NPK (Figure 5.S4). The PERMANOVA analysis revealed no significant differences in prokaryotic community composition between organically-amended and NPK-fertilized soils. Among the organically-amended soils, the *amendment dose* was the only significant factor according to PERMANOVA ($F=1.86$, $p<0.05$): the community composition was significantly influenced by the dose of the amendment regardless of the *origin of amendment* (farm-made *vs.* commercial), the *time of application* (basal dressing *vs.* basal+top dressing) or the interaction among these two experimental factors.

In relation to the taxonomic classification, 77.3% of the 16S rRNA sequences were classified to the order rank, while only 62% of the quality-filtered reads were classified to the family rank. Order was chosen as the level for the analysis of taxonomic classification, as the use of a better taxonomic resolution may not compensate for the loss of information regarding the rarest OTUs. At this order level, the composition of the prokaryotic communities was relatively similar among all studied soils, with the 30 most abundant taxa together representing between 60 and 70% of the total abundance in all cases (Figure 5.10A). Not even one statistically significant difference regarding the

relative abundance of each of these 30 dominant taxa was observed between organically-amended and NPK-fertilized soils (Table 5.S6).

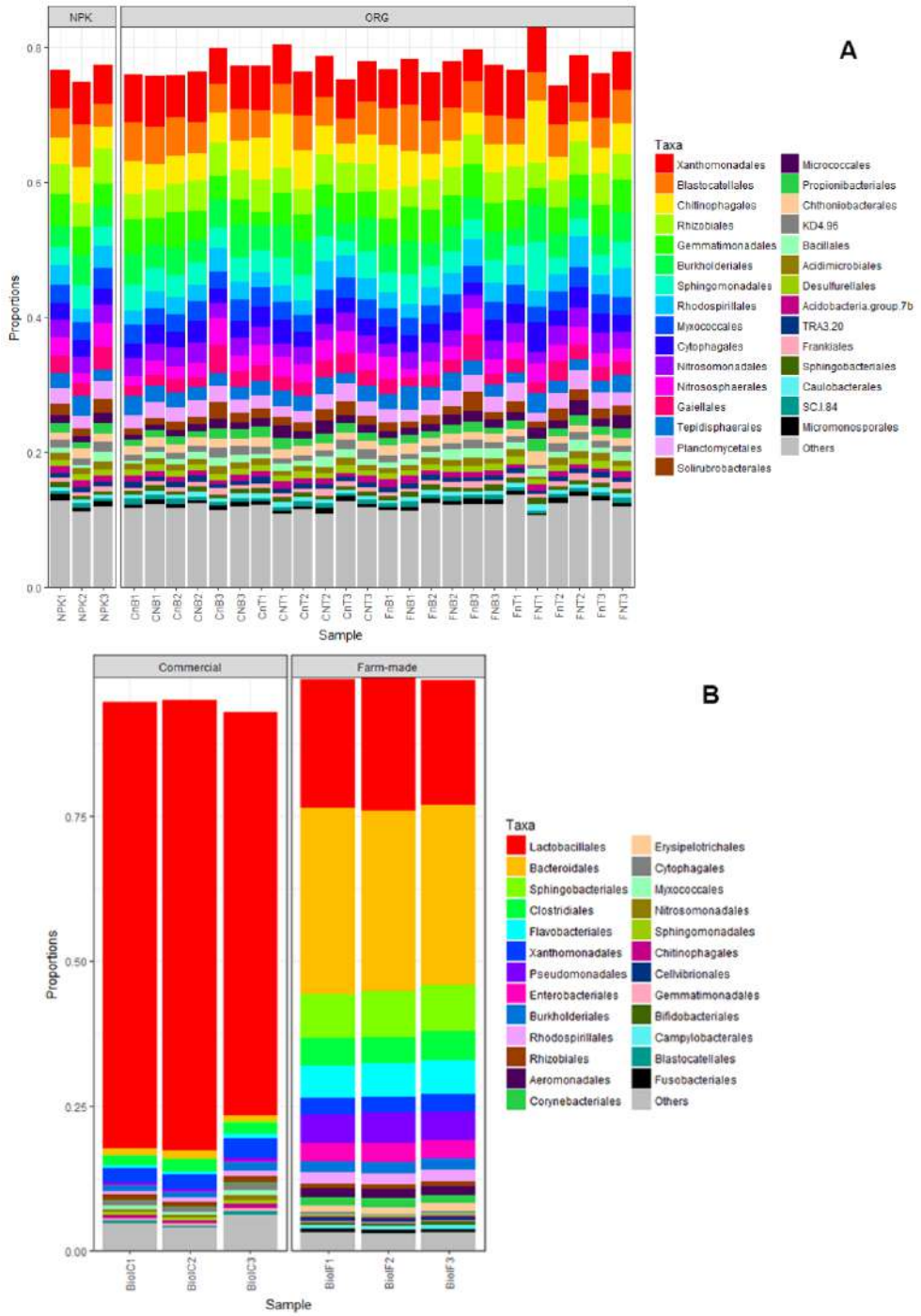

Figure 5.10. Barplots displaying the relative abundance of (A) the 30 most abundant prokaryotic taxa (at order rank) in all studied soils for each individual sample; (B) the 25 most abundant prokaryotic taxa at order rank for both fermented liquid organic amendments prior to soil application. MIN: mineral control soils; ORG: soils amended with fermented liquid organic amendment.

For a more comprehensive analysis, the relative abundance of all taxa representing more than 0.1% of the total reads was studied. *Amendment dose* was the experimental factor which resulted in the most significant differences: 15 taxa (constituting almost 19% of the relative abundance of the whole prokaryotic community at the order level) exhibited significant differences between soils treated with the optimal *vs.* the suboptimal dose (Table 5.7). The abundance of 8 of these taxa was higher at the optimal dose (*i.e.*, Sphingomonadales, Cytophagales, Tepidisphaerales, Micrococcales, Bacillales, Sphingobacteriales, Pseudomonadales, Longimicrobiales), accounting for 14% of the total prokaryotic community (compared to 11% for soils to which the suboptimal dose was applied). The remaining 7 taxa were more abundant after the application of the suboptimal dose and accounted for 8% of the total prokaryotic community (compared to 6% for soils to which the optimal dose was applied).

The factor *origin of amendment* resulted in significant differences in taxon abundance for just one taxon, Solibacterales, which represented 0.5 and 0.4% of the prokaryotic community in soils treated with the farm-made and commercial amendment, respectively. The factor *time of application* led to statistically different abundances for 5 taxa, representing 8% of the soil prokaryotic community. The abundances of 4 of these 5 taxa (*i.e.*, Micrococcales, Propionibacteriales, Corynebacteriales, Rhodobacterales) were significantly higher for the basal+top dressing application, accounting for 3.5% of the prokaryotic community (compared to 2.8% for the basal dressing application). Just one taxon, Blastocatellales, showed a significantly higher abundance in basal *vs.* basal+top dressing application. Blastocatellales accounted for a greater abundance (5.1 and 4.3% for the basal and basal+top dressing application, respectively) than the 4 taxa whose abundance was increased by the basal+top dressing application.

Table 5.7. Significant differences in taxa relative abundance (%) at order rank for the three factors studied here (*i.e.*, origin of amendment, amendment dose, and time of application), based on pooled variances *t*-test. Mean values (n=6) ± SD. Only those orders representing more than 0.1% of the total reads were included in the analysis.

ORIGIN OF AMENDMENT			
Taxa (Order)	**Farm-made**	**Commercial**	**p value**
Solibacterales	0.5 ± 0.1	0.4 ± 0.1	<0.05
AMENDMENT DOSE			
Taxa (Order)	**Optimal**	**Suboptimal**	**p value**
Sphingomonadales	3.9 ± 1.1	3.0 ± 0.5	<0.05
Cytophagales	2.9 ± 0.6	2.4 ± 0.4	<0.05
Tepidisphaerales	2.4 ± 0.5	2.0 ± 0.4	<0.05
Gaiellales	1.9 ± 0.4	2.5 ± 0.9	<0.05
Micrococcales	1.5 ± 0.4	1.2 ± 0.2	<0.05
Solirubrobacterales	1.2 ± 0.3	1.7 ± 0.5	<0.05
Bacillales	1.2 ± 0.3	0.9 ± 0.3	<0.01
Desulfurellales	0.9 ± 0.1	1.0 ± 0.1	<0.05
Acidimicrobiales	0.8 ± 0.1	1.0 ± 0.2	<0.01
Sphingobacteriales	0.7 ± 0.1	0.6 ± 0.1	<0.05
Streptomycetales	0.5 ± 0.1	0.7 ± 0.2	<0.05
Pseudomonadales	0.5 ± 0.1	0.4 ± 0.1	<0.05
Longimicrobiales	0.4 ± 0.2	0.3 ± 0.1	<0.01
Anaerolineales	0.3 ± 0.1	0.3 ± 0.0	<0.05
Clostridiales	0.2 ± 0.1	0.3 ± 0.1	<0.05
TIME OF APPLICATION			
Taxa (Order)	**Basal**	**Basal+Top**	**p value**
Blastocatellales	5.1 ± 0.8	4.3 ± 0.7	<0.01
Propionibacteriales	1.2 ± 0.2	1.4 ± 0.2	<0.05
Micrococcales	1.1 ± 0.3	1.5 ± 0.4	<0.05
Corynebacteriales	0.4 ± 0.1	0.5 ± 0.1	<0.05
Rhodobacterales	0.1 ± 0.0	0.2 ± 0.0	<0.05

The order Lactobacillales accounted for *ca.* 75 and 22% of the prokaryotic community in soils treated with the commercial and farm-made amendment, respectively. With respect to the farm-made amendment, the order Bacteroidales was the most dominant taxon, representing 31% of the prokaryotic community (Figure 5.10B). The application of organic amendments did not have a significant effect on the abundance of these dominant taxa, as the organically-amended soils showed a mean abundance of 0.039% for Lactobacillales and 0.005% for Bacteroidales, whereas the NPK-fertilized soils exhibited abundances of 0.024% and 0.004% (pairwise analyses resulted in $p>0.05$ in both cases), respectively.

5.2.4. Discussion

5.2.4.1. Corn yield and nutritional parameters

Comparable grain yields were obtained in organically-amended *vs.* NPK-fertilized soils when the optimal dose was used (*i.e.*, when matching the N dose provided by both the organic and mineral fertilizer). Many authors (Dawe *et al*., 2003; Montemurro, 2009; Oelofse *et al*., 2015; Sistani *et al*., 2017) have reported comparable crop yields when the nutrient (especially, N) contents of the organic and inorganic fertilizers are matched. The abovementioned maintenance of crop yield can translate into economic revenue for the farmers, since the price of 100 kg corn grain accounts for 17 and 30 € for conventionally- and organically-grown crops, respectively (MAPA, 2020). The price of fertilizer should also be addressed, which stands at 2.6 € kg^{-1} for the NPK fertilizer (MAPA, 2020) and 2.0 € l^{-1} for the commercial fermented liquid organic amendment. As expected, the suboptimal dose (the dose recommended by the company commercializing the fermented liquid organic amendments) resulted in lower corn grain yields, compared with the optimal dose or the NPK fertilizer. On the other hand, the commercial amendment resulted in significantly higher corn grain yields than the farm-made amendment. This difference may be due to the lower C/N ratio of the commercial amendment, which increases its fertilization value (Möller, 2018). With respect to the time of application, the basal dressing application resulted in higher crop yields than the basal+top dressing application, in accordance with Singh *et al*. (2005). Da Silva *et al*. (2005) reported that top dressing fertilization of corn should be applied only in cases of undesirable crop nutrition during the reproductive phase. Regarding the first cropping season, lack of differences in corn yield may be due to the fact that the previous crop was a legume, hence the N uptake by the subsequent corn crop may have been supplied by the residual soil nitrogen pool (especially in those plots where suboptimal dose was used).

The application of organic amendments led to higher values of starch content than NPK fertilization; in turn, NPK fertilization resulted in increased values of crude protein and ash. A negative correlation between starch and protein content has been reported for corn grain (Singh *et al*., 2005) and attributed to a tradeoff between these two components during the filling of the grain, which depends on the N supply. A higher N content can enhance the grain protein content at the expense of starch (Feng *et al*., 1993). These results are, however, biased by the application of the suboptimal dose. Indeed, the higher N supply at the optimal dose resulted in increased protein values compared to those

obtained with the suboptimal dose. Differences in crude protein content between the organic amendment at the optimal dose and the NPK fertilizer were significant for the farm-made amendment. The farm-made amendment exhibited lower values of crude protein, and higher values of starch, than the commercial amendment, which may be due to the higher C/N ratio of the farm-made amendment (13.8 and 3.8 for the farm-made and commercial amendments, respectively). Corn grain crude fat has been reported to be positively correlated with both N supply and protein content (Feng *et al*., 1993; Tallada *et al*., 2009; Ai and Jane, 2016), because a better crop nutrition often leads to a larger germ size (Bhatia and Rabson, 1987) which consists mainly of oil and protein (Ai and Jane, 2016). In our study, no correlation between crude fat and crude protein was found, which agrees with the results exhibited by Singh *et al*. (2005), reporting no correlation between oil and protein content in corn grain after the application of mineral N fertilizer at 5 different rates.

5.2.4.2. Soil physicochemical parameters

Given that organic amendments are known to supply valuable nutrients and OM, their application to soil is a common practice worldwide (Celestina *et al*., 2019). Particularly, due to its importance for soil fertility and functioning, enhancing soil OM is one of the main goals of the application of organic amendments (Lal, 2008). The anaerobic decomposition of organic wastes leads to the degradation of the OM labile fraction, resulting in the presence of a more recalcitrant organic C that can then be further sequestered into the soil matrix (Tambone *et al*., 2019). Here, although statistical significance was not achieved, the application of organic amendments resulted in a slight increase in soil OM content. Given that NPK fertilization resulted in a higher crop yield than that obtained with the suboptimal dose of the organic amendment, an increase in the amount of root exudates (Geisseler and Scow, 2014) probably occurred in NPK-fertilized soils, thus "buffering" the expected difference in soil organic C between organically-amended and NPK-fertilized soils. Changes in soil organic C content are usually slow, but long-term experiments on the effect of organic amendments on soil quality have extensively reported an enhancement of the soil organic C pool (Diacono and Montemurro, 2010).

The anaerobic decomposition of organic wastes normally involves the acidification of the medium owing to the generation of organic acids. The commercial and farm-made amendments tested here had pH values of 4.4 and 4.2, respectively. The

application of these amendments to calcareous alkaline soil, such as the one used in this experiment, can result in lower soil pH values. Moreover, the nitrification of the ammonium applied to soil through the application of these amendments might also lead to soil acidification (Cytryn *et al*., 2012). Surprisingly, no effect on soil pH was observed in any of the treatments studied here.

Total and mineral N contents, as well as those of Olsen P and extractable K^+, did not vary significantly between organically-amended and NPK-fertilized soils. However, differences regarding soil macronutrients were observed among organically-amended soils themselves. Olsen P and extractable K^+ values were significantly higher when applying the optimal *vs.* the suboptimal dose. Fermented amendments (*e.g.*, digestates) often contain higher contents of P and K^+ than those present in composts (Tambone *et al*., 2010). Phosphorus is often adsorbed on the surface of particles and colloids of solid phases (Hjorth *et al*., 2010) but, in digestates, it can be in available form (Börjesson and Berglund, 2007). Potassium, along with ammonium, is mostly soluble and associated with the liquid phase (Masse *et al*., 2005). Thus, it was not surprising to find a higher content of Olsen P and extractable K^+ in soils receiving a higher dose of the organic amendment (*i.e.*, optimal dose). Ammonium can be mineralized into nitrate by the activity of soil microorganisms, and then be taken up by plants. In this sense, it was not surprising that the optimal dose resulted in higher nitrate contents than the ones exhibited by soils amended with the suboptimal dose. The origin of amendment also had a significant effect on soil nitrate and Olsen P contents: higher values were obtained with the commercial *vs.* the farm-made amendment, which can be explained by the higher P content and lower C/N ratio present in the commercial amendment (Table 5.S1).

5.2.4.3. Soil microbial parameters

Microorganisms play a critical role in many soil processes and ecosystem services (Jeffery *et al*., 2010; Burges *et al*., 2015). Consequently, microbial parameters have become an almost imperative tool for the assessment of the effects of disturbances on soil health (Fließbach *et al*., 2007; Pardo *et al*., 2014). Microbial parameters which provide information on the activity, biomass and diversity of microbial communities are very sensitive to changes in soil management (Garbisu *et al*., 2011). In this regard, land application of organic amendments has broadly been reported to significantly increase microbial activity and biomass compared to mineral fertilization (Ginting *et al*., 2003; Aparna *et al*., 2014; Insam *et al*., 2015; Hernández *et al*., 2016; Siebielec *et al*., 2018).

Here, the application of organic amendments resulted in a slight overall increase in microbial activity and biomass compared to NPK fertilization (nonetheless, this increase was not statistically significant for any of the individual parameters of microbial activity or biomass). This lack of statistical significance can be due to a priming effect derived from the addition of mineral N to the soil during NPK fertilization (Tambone and Adani, 2017), and due to the fact that soils amended with the suboptimal and the optimal dose were both simultaneously included in the statistical analysis, leading to high data variability. In fact, when comparing amendment doses separately, the optimal dose led to higher SQI values, compared to the suboptimal dose of the organic amendment or the NPK treatment (Figure 5.S3). The SQI calculated here integrates the response of all the microbial activity and biomass parameters, providing an overview of the status of soil microbial communities (Mijangos *et al*., 2010). In this sense, the application of the organic amendment at the optimal dose increased microbial activity and biomass, compared to NPK fertilization. This increase was most likely due to the organic C provided by the amendment, given that soil heterotrophic microorganisms rely on the C and energy supplied by the pool of OM entering the soil. The anaerobic decomposition of organic wastes entails a loss in the amount of total organic C through the consumption of the labile fraction and its mineralization and conversion to biogas (Demirel and Scherer, 2008). However, part of the OM present in fermented amendments is often recalcitrant and thus more stable when applied to soil (Tambone *et al*., 2019). This stability may lead to a longer-term positive effect on microbial activity and biomass, and to maintain soil fertility for a prolonged period of time (Diacono and Montemurro, 2010).

Differences in microbial activity and biomass were also observed among organically-amended soils. *Amendment dose* was the only factor with a significant impact on microbial activity and biomass. The optimal dose resulted in a significant enhancement of all microbial activity and biomass parameters, except for urease activity, compared to the suboptimal dose. This effect can be explained by the different loads of organic C and nutrients, which are directly related to the amendment dose. Both the quantity and quality of the OM applied to the soil are critical for the activity and abundance of soil microorganisms (Diacono and Montemurro, 2010). Nevertheless, in our case, we speculate that the observed increase in the values of the microbial activity and biomass parameters may be linked to the higher soil N, P and K^+ contents obtained after the application of the optimal dose of the amendment, as these parameters correlated positively (Figure 5.S5). Finally, the optimal dose led to an increase in crop yield, which

may have resulted in a higher amount of root exudates, thus enhancing microbial growth in the rhizosphere (Bais *et al*. 2006).

Mineral fertilization has been shown to negatively affect soil microbial diversity, as compared to the application of organic amendments. After a 10-year field experiment in which mineral N fertilizer was substituted at different ratios (0, 25, 50, 75 and 100%) with an organic amendment, Ji *et al*. (2018) reported an increase in soil bacterial diversity along with the fertilizer substitution ratio. Aparna *et al*. (2014) found an increase in microbial diversity after the application of fermented organic amendments. Sapp *et al*. (2015) reported higher microbial diversity values in soils amended with anaerobically-digested organic waste than with mineral fertilizer. These differences in microbial diversity are often explained by the predominance of copiotrophic microorganisms, which are stimulated by organic inputs (Hartman, *et al*., 2015). Conversely, our metabarcoding results did not show any significant differences in terms of prokaryotic diversity between organically-amended and NPK-fertilized soils, or among organically-amended soils. In response to the supply of organic C, microorganisms can undergo selective enrichment (often reflected as an enhancement in microbial biomass), which may result in no change in, or even reduced, taxonomic diversity in organically-amended *vs.* NPK-fertilized soils (Goldfarb *et al*., 2011). Pershina *et al*. (2015) reported no significant differences in soil α-diversity between organic farming and conventional farming systems. Here, the farm-made amendment itself had a more diverse and uniform prokaryotic community (as reflected by the values of the Shannon's index and Pielou's evenness, respectively), compared to the commercial amendment. Nonetheless, after their application to soil, no differences in microbial diversity driven by the factor *origin of amendment* were observed. This was not surprising because the soil ecosystem is well known for its high biodiversity and then probably "buffered" the differences observed in the amendments.

Application of organic amendments may enhance substrate availability, which may affect soil microbial composition by increasing the number of ecological niches and promoting ecological interactions among soil organisms (Tian *et al*., 2017), which may concomitantly alter soil functioning (Morriën, 2016). The ordination analysis (NMDS) did not form well-defined clusters for the two fertilization regimes (organic *vs.* NPK fertilization), indicating a lack of substantial differences in prokaryotic community composition between organically-amended and NPK-fertilized soils, which was further confirmed by PERMANOVA analysis ($p>0.05$). The application of the organic

amendments did increase the numerically value of the relative abundances of the orders Lactobacillales and Bacteroidales (most abundant taxa in the commercial and farm-made amendments, respectively) by 58 and 36%, respectively, compared to NPK-fertilized soils. However, such increase was not statistically significant (both taxa accounted for less than 0.05% of the prokaryotic community). In fact, no significant differences were observed between organically-amended and NPK-fertilized soils for any of the relative abundances of the 30 most dominant orders (Table 5.S6). This lack of observed differences in microbial community composition could reflect a case of high ecological stability (resistance, resilience) for our soil microbial communities (Allison and Martiny, 2008). Moreover, intrinsic microbial communities of the organic amendments have shown to surrender to native microbial communities in the receiving soil exerting a minimal impact on them (Bastida *et al*., 2008). Johansen *et al*. (2013) found that anaerobically-digested amendments induced only minor and transient changes in soil microbial community composition. Perhaps, our experiment was not performed for a sufficiently long period of time to induce relevant and stable changes in soil microbial communities. In this sense, long-term fertilization may lead to more stable changes in microbial communities (Zhao *et al*., 2014).

Differences in community composition were nevertheless observed among organically-amended soils (the *amendment dose* significantly influenced community composition), as reflected by PERMANOVA. Many authors (Zhong *et al*., 2010; Xun *et al*., 2016; Das *et al*., 2017) have attributed shifts in microbial community composition to changes in soil physicochemical properties, such as organic C, pH and nutrients. Here, the soil OM content was not significantly altered by the *amendment dose*, but the optimal dose did yield higher values of soil nutrient contents compared to the suboptimal dose. Moreover, the optimal dose also resulted in enhanced crop yield, probably leading to an increase in the amount of root exudates, thereby altering soil microbial community composition. Among all taxa (at the order level) representing more than 0.1% of the relative abundance of the prokaryotic community, the *amendment dose* had a significant impact on the relative abundance of 15 of them, which accounted for 19% of the total prokaryotic community abundance (Table 5.7). In this regard, the optimal dose resulted in an enhancement of the abundance of the orders Sphingomonadales and Pseudomonadales, whose abundance in soil has been reported to increase after the application of organic amendments (Starke *et al*., 2016; Bastida *et al*., 2017). Members of both orders have been reported to effectively degrade recalcitrant organic molecules in

the soil environment due to their ability to produce catabolic enzymes (Bastida *et al*., 2016; Viswanathan *et al*., 2017). The abundances of other orders, such as Cytophagales and Sphingobacteriales, were also enhanced by the optimal dose. This enhancement could be directly related to the application of the organic amendments, as both orders were initially present in the amendments themselves. Tian *et al*. (2017) identified these two orders in the liquid phase resulting from the anaerobic digestion of organic residues. Both orders belong to Bacteroidetes phylum, whose members are known to play a major role in OM turnover and carbon cycling in the soil ecosystem (Suleiman *et al*., 2016). In this sense, members of Cytophagales and Sphingobacteriales have been shown to produce a variety of hydrolases and oxidoreductases which can metabolize a wide array of complex organic molecules, such as cellulose, chitin and pectin (Reichenbach, 2006; Chen *et al*., 2015; Tian *et al*., 2015). Furthermore, members of Sphingobacteriales have been reported to contain oligosaccharide fermentation genes (Hester *et al*., 2018). On the other hand, the optimal dose resulted in a decrease in the relative abundances of several orders belonging to the phylum Actinobacteria, such as Gaiellales, Solirubrobacterales, Acidimicrobiales and Streptomycetales. Actinobacteria have been characterized as regulators of the decomposition and synthesis of soil OM (Piao *et al*., 2008). Nevertheless, no significant differences in terms of the relative abundance of the phylum Actinobacteria were observed between the two doses of the organic amendments, because the relative abundance of other taxa belonging to this phylum (*e.g.*, Micrococcales) was higher in soils amended with the optimal dose. Micrococcaceae, a family belonging to the Micrococcales order, has been reported to increase in abundance after the long-term application of organic amendments (Pershina *et al*., 2015).

5.2.5. Conclusions

The application of fermented liquid organic amendments can enhance soil microbial biomass and activity, compared to NPK fertilization. The impact of organic amendments on soil properties (and, hence, soil quality) was not significantly influenced by the origin of the amendment (commercial *vs.* farm-made) or the time of application (basal vs. basal+top dressing). Contrary to our hypothesis, no significant differences in the diversity and composition of prokaryotic communities were observed between organically-amended and NPK-fertilized soils. Our findings indicate that the application of fermented liquid organic amendments can be a beneficial agronomic practice for improving or

maintaining agricultural soil quality and crop productivity when applied at a dose that guarantees the nutrient (nitrogen) requirements of the growing crop.

5.3. Supplementary information

Table 5.S1. Physicochemical characterization of the fermented liquid organic amendments.

	Commercial	**Farm-made**
Dry matter content (%)	15.3	8.0
pH	4.38	4.20
Organic matter (% DW)	67.4	71.2
C/N	3.8	13.8
Total N (% DW)	10.13	3.62
P (g kg^{-1} DW)	63.56	8.18
K (g kg^{-1} DW)	20.9	58.99

Table 5.S2. Effect of the application of liquid organic amendments on the relative abundance (%) of the 30 more abundant prokaryotic taxa at family rank. NPK: mineral control; O vs. M: organically-amended soils *vs.* mineral control. Differences based on pooled variances *t*-test or Welch´s *t*-test: ns: not significant; *: p<0.05; **: p<0.01; ***: p<0.001. A: amendment; D: dose

MICROCOSM EXPERIMENT								
	Farm-made		Commercial		NPK	O *vs.* M	A	D
	Optimal	Suboptimal	Optimal	Suboptimal				
Chitinophagaceae	7.0 ± 1.0	6.7 ± 0.6	6.6 ± 1.5	8.4 ± 0.2	4.8 ± 0.1	**	ns	ns
Blastocatellaceae	3.5 ± 1.2	5.0 ± 0.6	3.8 ± 0.9	5.8 ± 0.1	3.9 ± 0.3	ns	ns	**
Sphingomonadaceae	3.8 ± 1.3	3.7 ± 0.2	5.1 ± 0.7	4.0 ± 1.0	2.4 ± 0.4	**	ns	ns
Cytophagaceae	3.8 ± 0.8	2.8 ± 0.6	4.0 ± 0.9	3.4 ± 0.3	2.6 ± 0.5	ns	ns	ns
Comamonadaceae	2.8 ± 0.3	2.5 ± 0.3	3.1 ± 0.3	3.2 ± 0.3	1.8 ± 0.3	***	*	ns
Flavobacteriaceae	5.3 ± 3.7	0.2 ± 0.1	4.8 ± 2.8	0.7 ± 0.5	0.5 ± 0.1	ns	ns	**
Nitrosomonadaceae	1.7 ± 0.1	2.8 ± 0.4	1.8 ± 0.2	2.7 ± 0.3	2.6 ± 0.4	ns	ns	***
Gemmatimonadaceae	1.2 ± 1.0	3.4 ± 0.3	1.6 ± 1.6	2.2 ± 0.8	2.8 ± 0.3	ns	ns	*
Xanthomonadaceae	2.4 ± 0.3	1.3 ± 0.1	2.7 ± 0.5	1.5 ± 0.0	2.2 ± 0.3	ns	ns	***
Rhodospirillaceae	1.4 ± 0.6	2.5 ± 0.1	1.5 ± 0.7	1.6 ± 0.0	2.7 ± 0.2	*	ns	ns
Xanthomonadales Incertae Sedis	1.3 ± 0.1	2.1 ± 0.2	1.5 ± 0.1	2.1 ± 0.1	2.0 ± 0.2	ns	ns	***
Pseudomonadaceae	2.8 ± 1.8	0.2 ± 0.0	3.2 ± 1.3	0.4 ± 0.2	0.2 ± 0.0	ns	ns	***
Tepidisphaeraceae	0.9 ± 0.2	1.6 ± 0.3	1.1 ± 0.0	1.6 ± 0.2	1.4 ± 0.2	ns	ns	***
Micrococcaceae	1.7 ± 0.6	1.1 ± 0.2	1.3 ± 0.3	0.9 ± 0.2	1.4 ± 0.1	ns	ns	*
Oxalobacteraceae	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	ns	ns	**
Planctomycetaceae	1.1 ± 0.4	1.3 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.5 ± 0.3	*	ns	ns
Sphingobacteriaceae	2.7 ± 2.0	0.1 ± 0.0	2.5 ± 0.8	0.2 ± 0.1	0.2 ± 0.1	ns	ns	**
Bryobacteraceae	0.6 ± 0.1	1.2 ± 0.2	0.7 ± 0.0	1.4 ± 0.2	1.5 ± 0.3	*	ns	***
H16	0.7 ± 0.0	1.3 ± 0.1	0.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	ns	ns	***
Hymenobacteraceae	1.1 ± 0.4	0.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.0	ns	ns	ns
Haliangiaceae	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.3	ns	ns	ns
Cellvibrionaceae	1.9 ± 1.4	0.1 ± 0.1	1.9 ± 0.9	0.2 ± 0.2	0.1 ± 0.1	ns	ns	**
Methylophilaceae	1.8 ± 1.7	0.5 ± 0.2	0.8 ± 0.3	0.7 ± 0.1	0.4 ± 0.0	ns	ns	ns
Opitutaceae	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	ns	ns	ns
Methylobacteriaceae	0.7 ± 0.3	1.0 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	1.1 ± 0.1	*	ns	**
Planococcaceae	1.3 ± 0.2	0.0 ± 0.0	2.5 ± 0.8	0.0 ± 0.0	0.2 ± 0.2	ns	ns	***
Alcaligenaceae	1.0 ± 0.5	0.6 ± 0.1	1.1 ± 0.3	0.7 ± 0.1	0.5 ± 0.1	ns	ns	*
Caulobacteraceae	1.0 ± 0.3	0.5 ± 0.2	1.0 ± 0.3	0.5 ± 0.0	0.6 ± 0.2	ns	ns	**
Rubrobacteriaceae	0.3 ± 0.2	1.0 ± 0.3	0.4 ± 0.2	0.8 ± 0.2	1.0 ± 0.0	ns	ns	**
Streptomycetaceae	0.3 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	1.0 ± 0.3	1.2 ± 0.6	*	ns	**

FIELD EXPERIMENT								
	Farm-made		Commercial		NPK	O *vs.* M	A	D
	Optimal	Suboptimal	Optimal	Suboptimal				
Chitinophagaceae	5.0 ± 0.8	5.0 ± 1.1	3.9 ± 0.4	4.4 ± 0.8	4.5 ± 0.2	ns	*	ns
Blastocatellaceae	3.3 ± 0.9	4.4 ± 1.3	2.6 ± 0.4	3.3 ± 0.6	4.0 ± 0.4	ns	*	*
Comamonadaceae	2.7 ± 0.7	2.3 ± 1.1	2.9 ± 0.9	2.0 ± 0.5	2.9 ± 1.3	ns	ns	*
Rhodospirillaceae	2.5 ± 0.6	1.8 ± 0.6	2.8 ± 0.7	2.3 ± 0.4	1.8 ± 0.1	ns	ns	*
Planctomycetaceae	2.0 ± 0.2	2.0 ± 0.1	1.8 ± 0.4	2.2 ± 0.2	1.9 ± 0.2	ns	ns	ns
Cytophagaceae	2.0 ± 0.4	1.9 ± 0.3	1.7 ± 0.4	2.2 ± 0.7	2.1 ± 0.2	ns	ns	ns
Sphingomonadaceae	2.1 ± 0.3	2.3 ± 0.8	1.7 ± 0.3	1.7 ± 0.3	2.2 ± 0.4	ns	*	
Micrococcaceae	2.4 ± 0.5	1.2 ± 0.3	2.8 ± 0.5	1.5 ± 0.3	1.4 ± 0.2	ns	ns	***
Gemmatimonadaceae	1.7 ± 0.4	2.1 ± 0.4	1.8 ± 0.6	1.7 ± 0.5	1.8 ± 0.4	ns	ns	ns
Xanthomonadaceae	2.0 ± 0.4	1.3 ± 0.5	2.5 ± 2.2	1.2 ± 0.3	2.0 ± 0.9	ns	ns	*
Rubrobacteriaceae	1.5 ± 0.2	1.6 ± 0.6	1.8 ± 0.5	1.9 ± 0.5	1.5 ± 0.1	ns	ns	ns
Flavobacteriaceae	2.0 ± 1.7	0.8 ± 0.6	2.6 ± 2.3	1.0 ± 0.6	2.1 ± 0.8	ns	ns	*
Gaiellaceae	1.6 ± 0.2	1.8 ± 0.2	1.6 ± 0.3	1.7 ± 0.2	1.7 ± 0.4	ns	ns	ns
Alcaligenaceae	1.6 ± 0.5	1.9 ± 0.7	1.1 ± 0.3	1.5 ± 0.4	1.7 ± 0.1	ns	ns	ns
Nocardioidaceae	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	ns	ns	ns
Nitrosomonadaceae	1.2 ± 0.1	1.4 ± 0.2	1.0 ± 0.2	1.5 ± 0.3	1.3 ± 0.1	ns	ns	**
Pseudomonadaceae	1.6 ± 0.8	0.9 ± 0.7	1.8 ± 1.1	0.7 ± 0.4	1.5 ± 0.7	ns	ns	**
Xanthomonadales Incertae Sedis	1.1 ± 0.2	1.3 ± 0.3	1.0 ± 0.2	1.5 ± 0.3	1.1 ± 0.1	ns	ns	**
Tepidisphaeraceae	1.2 ± 0.2	1.3 ± 0.4	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	ns	*	ns
Oxalobacteraceae	1.3 ± 0.2	0.8 ± 0.2	1.6 ± 0.5	0.7 ± 0.2	0.9 ± 0.4	ns	ns	***
Elev-16S-1332	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	0.9 ± 0.3	ns	ns	*
Nitrososphaera family incertae sedis	0.7 ± 0.2	0.7 ± 0.4	0.9 ± 0.3	1.0 ± 0.2	0.7 ± 0.1	ns	*	ns
Micromonosporaceae	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	ns	ns	ns
Haliangiaceae	0.7 ± 0.2	0.9 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	ns	ns	**
Intrasporangiaceae	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	ns	ns	ns
Methylobacteriaceae	0.7 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	ns	ns	*
Hymenobacteraceae	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	ns	*	ns
Streptomycetaceae	0.5 ± 0.2	0.6 ± 0.1	0.8 ± 0.3	0.8 ± 0.2	0.5 ± 0.1	ns	*	ns
Hyphomicrobiaceae	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	ns	ns	*
Microbacteriaceae	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	ns	ns	**

Table 5.S3. Impact of organic amendments on corn grain nutritional parameters. NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control, differences based on pooled variances *t*-test or Welch´s *t*-test. For the organically-amended soils, interaction among factors was tested by three-way ANOVA, where A: origin of amendment; D: amendment dose; Ap: time of application. Mean values (n=3) ± SD. *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

			Ash (%)	Crude fiber (%)	Crude fat (%)	Starch (%)	Crude protein (%)
Farm-made	**Optimal**	**Basal**	1.07 ± 0.02	1.4 ± 0.4	2.9 ± 0.0	61.3 ± 0.7	6.0 ± 0.2
		Basal+Top	1.12 ± 0.04	1.6 ± 0.2	2.5 ± 0.1	60.8 ± 1.1	6.7 ± 0.6
	Subptimal	**Basal**	1.05 ± 0.01	1.3 ± 0.1	2.7 ± 0.2	61.9 ± 0.2	5.7 ± 0.2
		Basal+Top	1.03 ± 0.04	1.2 ± 0.2	2.6 ± 0.2	62.3 ± 1.0	5.6 ± 0.3
Commercial	**Optimal**	**Basal**	1.19 ± 0.07	1.2 ± 0.0	2.9 ± 0.2	58.7 ± 0.9	7.7 ± 1.1
		Basal+Top	1.18 ± 0.04	1.5 ± 0.2	2.6 ± 0.2	59.3 ± 1.0	7.4 ± 0.3
	Subptimal	**Basal**	1.05 ± 0.04	1.0 ± 0.3	3.0 ± 0.2	61.8 ± 0.9	6.0 ± 0.4
		Basal+Top	1.04 ± 0.03	1.4 ± 0.3	2.8 ± 0.0	61.9 ± 0.5	5.4 ± 0.1
		NPK	1.19 ± 0.05	1.2 ± 0.2	2.9 ± 0.4	58.8 ± 1.2	8.5 ± 1.0
O *vs.* M			*****	***ns***	***ns***	*****	*****
Origin of amendment (A)			*	*ns*	*	*	*
Amendment dose (D)			***	*	*ns*	***	***
Time of application (Ap)			*ns*	*	**	*ns*	*ns*
A x D			*	*ns*	*ns*	*	*
A x Ap			*ns*	*ns*	*ns*	*ns*	*ns*
D x Ap			*ns*	*ns*	*ns*	*ns*	*ns*
A x D xAp			*ns*	*ns*	*ns*	*ns*	*ns*

Table 5.S4. Impact of organic amendments on soil physicochemical properties. NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control, differences based on pooled variances *t*-test or Welch´s *t*-test. For the organically-amended soils, interaction among factors was tested by three-way ANOVA, where A: origin of amendment; D: amendment dose; Ap: time of application. Mean values (n=3) ± SD. *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

			pH	OM %	Ntot %	NO_3^- mg kg^{-1}	NH_4^+ mg kg^{-1}	Olsen P mg kg^{-1}	K^+ mg kg^{-1}
Farm-made	**Optimal**	**Basal**	8.5 ± 0.1	1.4 ± 0.3	0.11 ± 0.02	2.9 ± 0.2	2.3 ± 0.4	18.7 ± 2.0	156 ± 11
		Basal+Top	8.5 ± 0.1	1.5 ± 0.1	0.12 ± 0.01	2.7 ± 0.2	2.5 ± 0.1	24.9 ± 9.8	161 ± 37
	Subptimal	**Basal**	8.5 ± 0.1	1.5 ± 0.3	0.12 ± 0.02	2.5 ± 0.0	1.9 ± 0.5	14.5 ± 6.4	140 ± 19
		Basal+Top	8.5 ± 0.1	1.5 ± 0.1	0.11 ± 0.01	2.3 ± 0.4	2.6 ± 0.7	17.1 ± 7.7	108 ± 9
Commercial	**Optimal**	**Basal**	8.5 ± 0.1	1.4 ± 0.2	0.10 ± 0.01	3.7 ± 0.2	1.7 ± 0.2	35.9 ± 1.2	132 ± 7
		Basal+Top	8.5 ± 0.0	1.4 ± 0.2	0.11 ± 0.01	3.7 ± 0.1	2.1 ± 0.3	39.5 ± 19.3	146 ± 20
	Subptimal	**Basal**	8.50 ± 0.1	1.4 ± 0.3	0.10 ± 0.02	2.6 ± 0.3	2.3 ± 0.5	17.5 ± 4.3	122 ± 10
		Basal+Top	8.6 ± 0.0	1.3 ± 0.2	0.10 ± 0.01	2.2 ± 0.2	2.5 ± 0.4	16.9 ± 2.2	118 ± 30
		NPK	8.5 ± 0.1	1.3 ± 0.2	0.10 ± 0.01	3.3 ± 1.1	2.3 ± 0.5	23.2 ± 3.6	140 ± 12
O *vs.* M			***ns***	***ns***	***ns***	***ns***	***ns***	***ns***	***ns***
Origin of amendment (A)			*ns*	*ns*	*ns*	***	*ns*	*	*ns*
Amendment dose (D)			*ns*	*ns*	*ns*	***	*ns*	**	**
Time of application (Ap)			*ns*	*ns*	*ns*	*	*	*ns*	*ns*
A x D			*ns*	*ns*	*ns*	***	*ns*	*ns*	*ns*
A x Ap			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
D x Ap			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
A x D xAp			*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Table 5.S5. Impact of organic amendments on prokaryotic (16S rRNA) α-diversity. NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control. differences based on pooled variances *t*-test or Welch´s *t*-test: *ns*: not significant; *: p<0.05; **: p<0.01; ***: p<0.001. For the organically-amended soils, interaction among factors was tested by three-way ANOVA where A: origin of amendment; D: amendment dose; Ap: time of application. Letters address significant differences among treatments according to one-way ANOVA and Duncan's MRT when factor interaction was significant. Mean values (n=3) ± SD.; RR: rarefied richness. H': Shannon index; J': Pielou's evenness.

			RR	H'	J'
Farm-made	**Optimal**	**Basal**	4002 ± 235	7.09 ± 0.13	0.82 ± 0.01
		Basal+Top	3959 ± 394	7.05 ± 0.22	0.81 ± 0.03
	Subptimal	**Basal**	4055 ± 283	7.10 ± 0.17	0.82 ± 0.01
		Basal+Top	4353 ± 183	7.27 ± 0.09	0.83 ± 0.01
Commercial	**Optimal**	**Basal**	4112 ± 139	7.12 ± 0.04	0.84 ± 0.02
		Basal+Top	3788 ± 159	6.99 ± 0.08	0.81 ± 0.01
	Subptimal	**Basal**	3965 ± 136	7.06 ± 0.05	0.81 ± 0.00
		Basal+Top	4031 ± 236	7.11 ± 0.12	0.82 ± 0.01
		NPK	4021 ± 188	7.12 ± 0.11	0.81 ± 0.01
O *vs.* M			***ns***	***ns***	***ns***
Origin of amendment (A)			*ns*	*ns*	*ns*
Amendment dose (D)			*ns*	*ns*	*ns*
Time of application (Ap)			*ns*	*ns*	*ns*
A x D			*ns*	*ns*	*ns*
A x Ap			*ns*	*ns*	*ns*
D x Ap			*ns*	*ns*	*ns*
A x D xAp			*ns*	*ns*	*ns*

Table 5.S6. Impact of organic amendments on the relative abundance (%) of the 30 most abundant prokaryotic taxa at order rank. NPK: mineral control; O *vs.* M: organically-amended soils *vs.* mineral control, differences based on pooled variances *t*-test or Welch´s *t*-test: ns: not significant; *: p<0.05; **: p<0.01; ***: p<0.001. For the organically-amended soils, interaction among factors was tested by three-way ANOVA where A: origin of amendment; D: amendment dose; Ap: time of application. Mean values (n = 3) ± SD.

	Farm-made				Commercial				NPK	O *vs.*M	A	D	Ap	AxD	AxAp	DxAp	AxDxAp
	Optimal		Suboptimal		Optimal		Suboptimal										
	Basal	Basal+Top	Basal	Basal+Top	Basal	Basal+Top	Basal	Basal+Top									
Xanthomonadales	7.0±0.5	6.4±0.6	5.8±1.2	6.5±0.8	7.2±0.6	6.0±0.2	6.2±0.9	6.3±0.4	5.9±0.2	ns	ns	ns	ns	ns	ns	*	ns
Blastocatellales	5.4±1.3	4.0±1.1	5.0±0.4	4.3±0.6	4.9±0.5	4.6±0.4	5.2±0.9	4.2±0.8	4.6±1.6	ns	ns	ns	*	ns	ns	ns	ns
Chitinophagales	4.4±0.7	5.6±3.2	4.6±1.8	3.5±0.2	4.0±0.2	5.5±2.1	4.5±0.4	5.0±1.9	4.2±1.1	ns	ns	ns	ns	ns	ns	ns	ns
Rhizobiales	3.9±0.8	4.2±0.7	4.4±0.2	4.6±0.5	4.4±0.2	4.1±0.4	4.3±0.6	4.3±0.5	4.4±0.8	ns	ns	ns	ns	ns	ns	ns	ns
Gemmatimonadales	4.6±0.4	3.7±1.2	4.5±0.6	4.1±0.3	3.9±0.4	4.0±0.7	4.6±1.0	3.5±1.3	4.1±0.6	ns	ns	ns	ns	ns	ns	ns	ns
Burkholderiales	4.3±0.7	4.3±1.3	3.6±0.5	3.0±0.5	3.9±0.9	4.7±0.3	4.2±0.3	3.8±1.2	3.5±0.8	ns	ns	ns	ns	ns	ns	ns	ns
Sphingomonadales	3.6±0.7	4.5±2.4	3.0±0.1	2.8±0.3	3.4±0.1	3.9±0.3	3.4±0.5	3.0±0.8	3.0±0.5	ns	ns	*	ns	ns	ns	ns	ns
Rhodospirillales	2.7±0.6	3.7±01.2	3.0±0.9	2.8±0.1	2.6±0.1	3.3±1.0	2.7±0.7	2.6±0.6	2.7±0.7	ns	ns	ns	ns	ns	ns	ns	ns
Myxococcales	2.7±0.1	2.6±0.3	2.6±0.2	2.8±0.0	2.8±0.3	2.7±0.1	2.6±0.0	2.7±0.3	2.8±0.3	ns	ns	ns	ns	ns	ns	ns	ns
Cytophagales	2.7±0.4	3.3±1.0	2.3±0.5	2.7±0.4	2.9±0.3	2.7±0.7	2.2±0.1	2.5±0.5	2.4±0.1	ns	ns	*	ns	ns	ns	ns	ns
Nitrosomonadales	2.5±0.2	2.3±0.6	2.3±0.4	2.9±0.1	2.7±0.2	2.4±0.3	2.5±0.3	2.6±0.1	2.6±0.2	ns	ns	ns	ns	ns	ns	*	ns
Gaiellales	1.5±0.2	1.9±0.3	2.7±1.1	2.3±0.6	1.7±0.5	2.3±0.2	2.5±1.5	2.4±0.6	2.6±0.9	ns	ns	*	ns	ns	ns	ns	ns
Tepidisphaerales	2.7±0.9	2.3±0.7	2.0±0.1	1.7±0.1	2.2±0.4	2.4±0.1	2.3±0.5	1.9±0.4	2.3±0.7	ns	ns	*	ns	ns	ns	ns	ns
Planctomycetales	2.1±0.4	2.1±0.7	2.2±0.3	2.4±0.2	2.2±0.2	2.0±0.3	2.2±0.2	2.1±0.5	2.2±0.4	ns	ns	ns	ns	ns	ns	ns	ns
Solirubrobacterales	1.1±0.1	1.3±0.4	1.9±0.9	1.7±0.2	1.1±0.2	1.4±0.2	1.6±0.8	1.6±0.4	1.7±0.5	ns	ns	*	ns	ns	ns	ns	ns
Micrococcales	1.3±0.2	2.0±0.2	1.2±0.3	1.2±0.1	1.0±0.2	1.6±0.4	1.0±0.4	1.2±0.0	1.2±0.3	ns	ns	**	**	ns	ns	*	ns
Propionibacteriales	1.1±0.2	1.5±0.2	1.2±0.4	1.2±0.1	1.1±0.2	1.4±0.3	1.2±0.3	1.4±0.3	1.3±0.3	ns	ns	ns	ns	ns	ns	ns	ns
Chthoniobacterales	1.4±0.2	1.4±0.6	1.2±0.2	1.0±0.0	1.2±0.1	1.2±0.1	1.3±0.0	1.3±0.2	1.2±0.1	ns	ns	ns	ns	ns	ns	ns	ns
KD4-96	0.9±0.1	0.9±0.3	1.1±0.2	1.0±0.2	1.1±0.2	1.1±0.3	1.1±0.3	1.1±0.2	1.3±0.3	ns	ns	ns	ns	ns	ns	ns	ns
Bacillales	1.3±0.3	1.3±0.3	1.0±0.3	0.9±0.1	1.0±0.1	1.2±0.3	0.8±0.5	0.9±0.2	0.9±0.4	ns	ns	*	ns	ns	ns	ns	ns
Acidimicrobiales	0.8±0.1	0.8±0.3	1.0±0.2	1.1±0.0	0.8±0.1	0.8±0.1	1.0±0.1	1.0±0.2	1.1±0.2	ns	ns	**	ns	ns	ns	ns	ns
Desulfurellales	0.9±0.2	0.8±0.1	0.9±0.1	1.1±0.1	1.0±0.1	0.7±0.1	0.9±0.0	1.0±0.1	0.9±0.1	ns	ns	*	ns	ns	ns	***	ns
Acidobacteriagroup 7b	0.9±0.2	0.8±0.2	0.9±0.3	0.6±0.1	0.8±0.1	1.0±0.2	0.8±0.1	0.7±0.1	0.9±0.1	ns	ns	ns	ns	ns	ns	ns	ns
TRA3-20	0.7±0.0	0.6±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.0	0.8±0.2	0.8±0.1	0.7±0.1	ns	ns	ns	ns	ns	ns	ns	ns
Frankiales	0.6±0.1	0.9±0.3	0.8±0.3	0.7±0.0	0.6±0.1	0.7±0.3	0.7±0.3	0.7±0.1	0.7±0.2	ns	ns	ns	ns	ns	ns	ns	ns
Sphingobacteriales	0.7±0.1	0.8±0.2	0.6±0.1	0.7±0.1	0.8±0.1	0.7±0.1	0.5±0.0	0.7±0.2	0.6±0.1	ns	ns	*	ns	ns	ns	ns	ns
Caulobacterales	0.6±0.0	0.7±0.2	0.6±0.2	0.7±0.1	0.7±0.1	0.6±0.1	0.6±0.0	0.6±0.1	0.5±0.0	ns	ns	ns	ns	ns	ns	ns	ns
SC-I-84	0.8±0.2	0.5±0.2	0.5±0.0	0.5±0.1	0.6±0.1	0.7±0.1	0.7±0.2	0.6±0.2	0.6±0.2	ns	ns	ns	ns	ns	ns	ns	ns
Micromonosporales	0.6±0.1	0.5±0.2	0.6±0.2	0.7±0.0	0.5±0.1	0.5±0.2	0.6±0.1	0.6±0.2	0.7±0.2	ns	ns	ns	ns	ns	ns	ns	ns
Pseudonocardiales	0.6±0.2	0.7±0.0	0.6±0.1	0.5±0.1	0.5±0.0	0.7±0.2	0.5±0.2	0.6±0.1	0.6±0.1	ns	ns	ns	ns	ns	ns	ns	ns

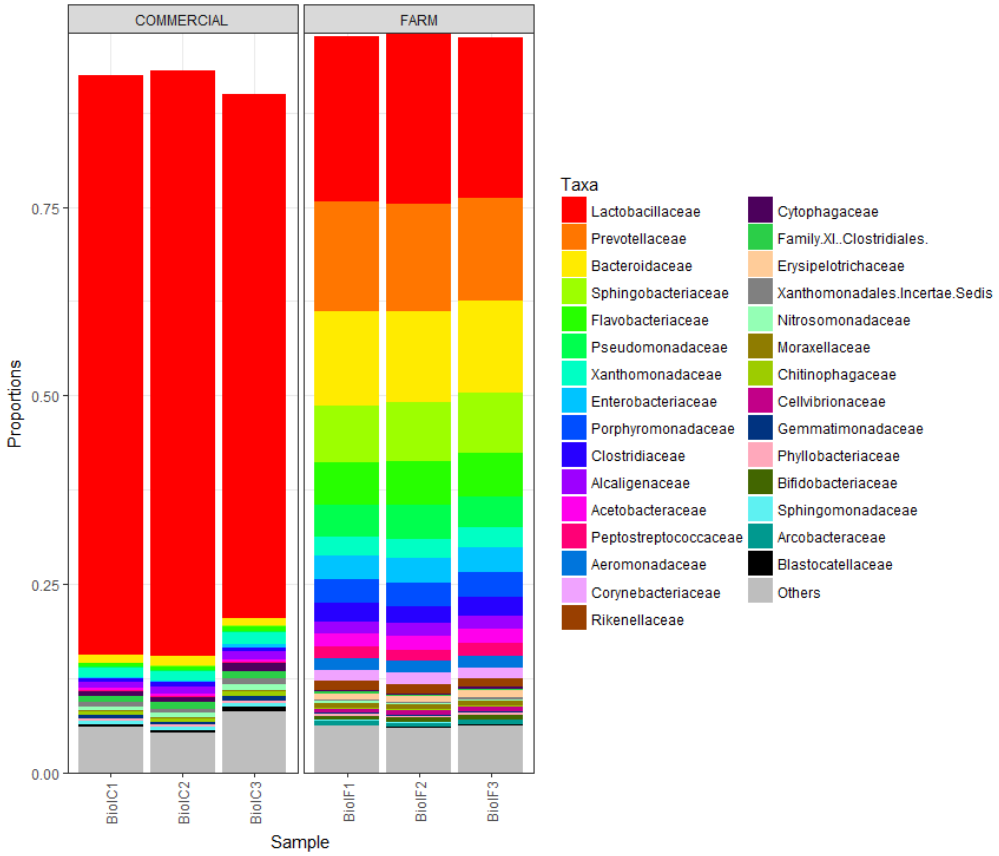

Figure 5.S1. Biological characterization of the fermented liquid organic amendments, represented as a barplot displaying the relative abundance of the 30 more abundant prokaryotic taxa at family level.

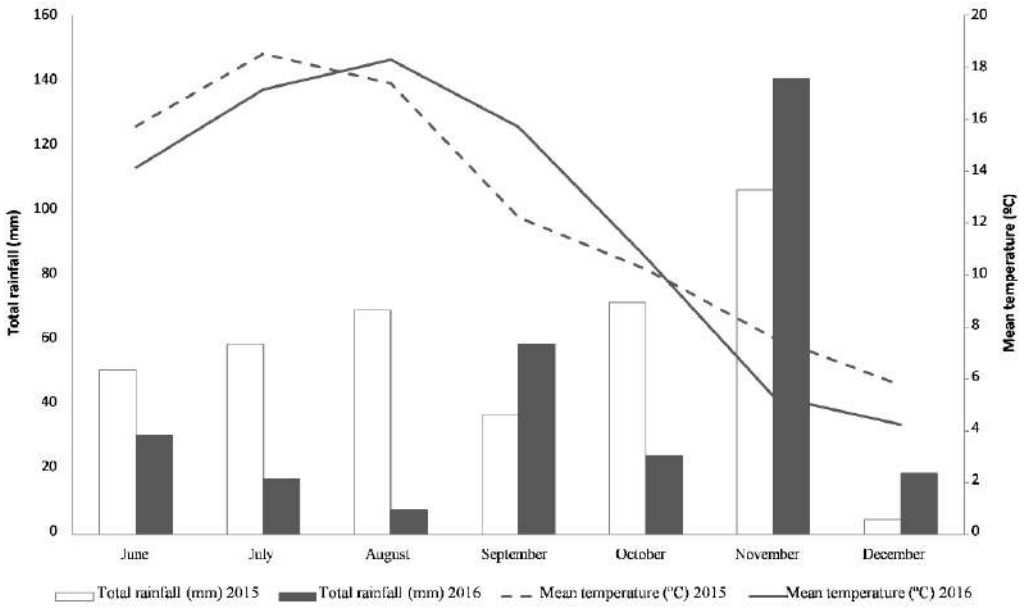

Figure 5.S2. Total rainfall (mm) and mean temperature (ºC) in 2015 and 2016 corn growing seasons in Arana Valley (Basque Country, northern Spain).

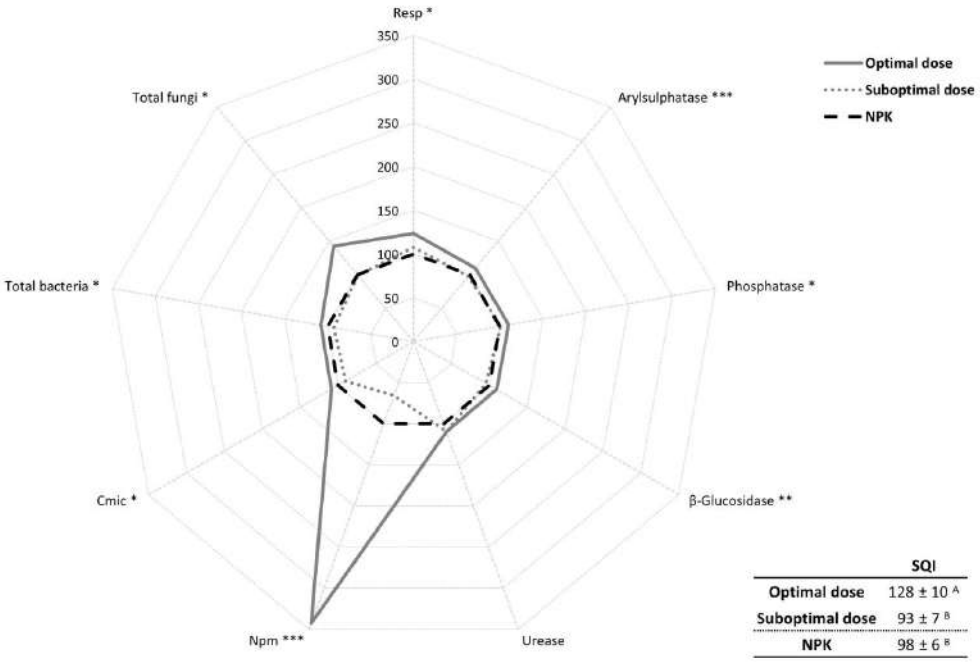

Figure 5.S3. SQI from the values of microbial activity and biomass parameters. A value of 100 corresponds to the mean value obtained for each parameter in the mineral control, while values for the organically-amended soils (optimal and suboptimal dose) were calculated and displayed according to such control value. Statistical differences are displayed by means of one-way ANOVA, where *: p<0.05; **: p<0.01; ***: p<0.001 show differences between treatments for each microbial parameter; small letters display differences between treatments for the SQI value according to one-way ANOVA and Duncan's MRT.

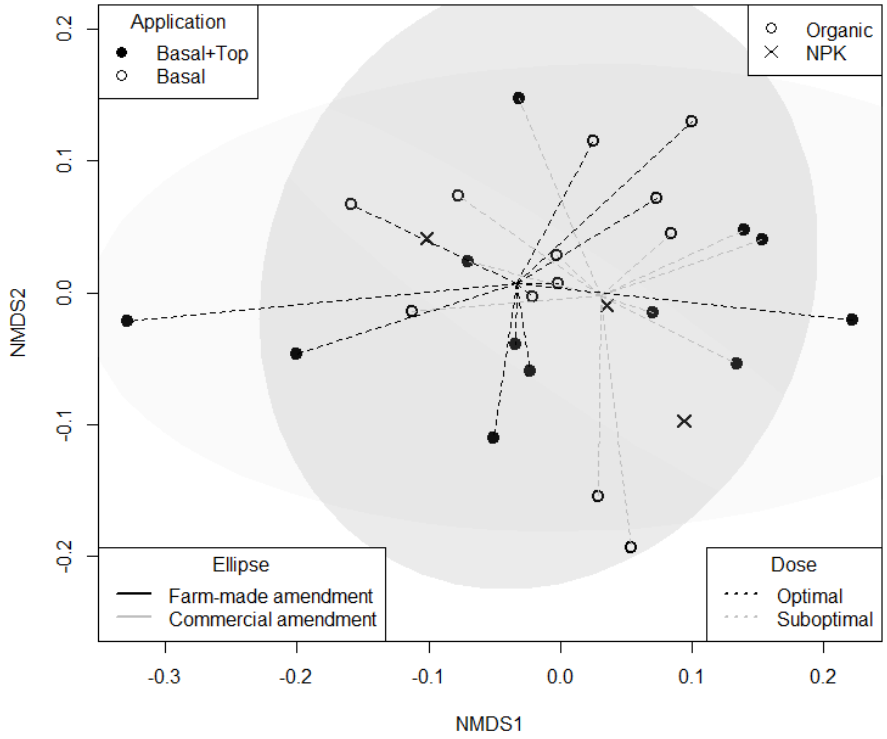

Figure 5.S4. Non-metric multidimensional scaling (NMDS) analysis representing patterns of prokaryotic community composition. Bray-Curtis dissimilarities of community composition,

based on relative OTU abundances from prokaryotic 16S rRNA amplicon data, are represented as distance in the diagram.

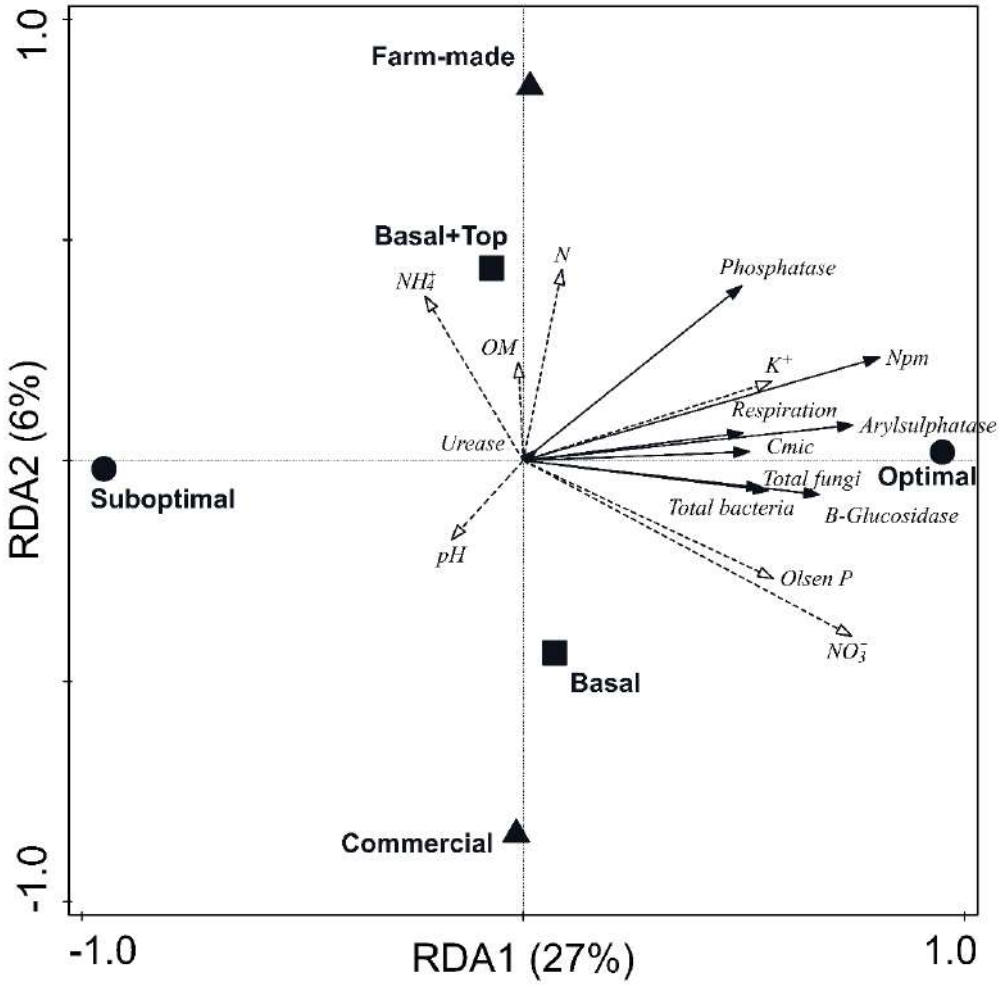

Figure 5.S5. Biplot of the redundancy analysis (RDA) performed on soil physicochemical and microbial activity and biomass parameters as response variables displayed by means of the experimental factors. Solid arrows: microbial parameters; dotted arrows: physicochemical parameters; N_{PM}: potentially mineralizable N; Cmic: microbial biomass C; 16S rRNA and 18S rRNA: gene copy numbers determined by qPCR.

6| THE APPLICATION OF FRESH AND COMPOSTED HORSE AND CHICKEN MANURE AFFECTS SOIL QUALITY, MICROBIAL COMPOSITION AND ANTIBIOTIC RESISTANCE

6. THE APPLICATION OF FRESH AND COMPOSTED HORSE AND CHICKEN MANURE AFFECTS SOIL QUALITY, MICROBIAL COMPOSITION AND ANTIBIOTIC RESISTANCE

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Abstract

Livestock manure-derived amendments can be beneficial for agricultural soil quality, as they can increase the content of soil organic matter and nutrients, stimulate microbial activity and biomass, and enhance crop yield. Here, we studied the impact of six different manure-derived amendments, according to the origin (horse manure-derived *vs.* chicken manure-derived) and type of amendment (fresh *vs.* composted *vs.* bokashi), on agricultural soil quality. To this purpose, an experiment was conducted with lettuce plants, paying special attention to amendment-induced changes in soil microbial properties and the abundance and risk of dissemination of antibiotic resistance genes (ARGs) through horizontal gene transfer (HGT). Soils amended with fresh manure showed higher values of microbial biomass and activity. In particular, fresh chicken manure yielded the highest crop yield of lettuce, but also increased the abundance of ARGs considerably. Genes encoding mobile genetic elements (*tnpA, intI1*) were positively correlated with ARGs, suggesting a risk of dissemination of antibiotic resistance via HGT in agricultural soils, as a result of the application of livestock manure-derived amendments. In order to minimize this risk, we therefore suggest that manure-derived amendments be properly treated and managed prior to their application to agricultural soil.

6.1. Introduction

Agricultural intensification, characterized by an extensive use of fertilizers and pesticides of synthetic origin, has proved to be very successful in terms of crop yield. Nevertheless, agricultural intensification has led to a decline in soil quality by decreasing soil organic matter (OM) content, reducing the soil´s natural fertility, polluting the environment, and negatively affecting the soil biota (Dinesh *et al*., 2010; Lal, 2008).

In consequence, there is currently a great interest in the development of strategies and approaches for a more sustainable agricultural production (Diacono and Montemurro, 2010; Pretty and Bharucha, 2014). In particular, organic amendments appear a suitable alternative to the excessive use of synthetic fertilizers, as they provide OM and nutrients, and can improve both the physicochemical and biological properties of the soil ecosystem (Fließbach *et al*., 2007). Specifically, livestock manure-derived amendments can be very beneficial for agricultural soil quality, as they increase the content of soil OM (thereby, improving porosity, aeration, water holding capacity, structural stability and nutrient availability) and stimulate microbial activity and biomass, thus enhancing crop yield (Das *et al*., 2017; Hernández *et al*., 2016). Livestock manure can be applied directly (fresh) or after being subjected to a composting process. The latter option is commonly preferred, as composting minimizes some chemical and, above all, biological risks associated to the use of organic amendments (*e.g.*, presence of pathogens) (Evanylo *et al*., 2008). The term "bokashi" refers to the Japanese way of composting in which OM is fermented using microbial inocula (Zimmermann and Kamukuenjandje, 2008).

On the other hand, livestock intensive production relies on the use, often misuse or abuse, of antibiotics to prevent and treat infectious diseases, or promote animal growth. However, most antibiotics are poorly absorbed in the animal gut and, hence, a substantial amount is excreted unchanged (Kumar *et al*., 2005). In addition, manure is acknowledged as a reservoir of both antibiotic resistant bacteria and antibiotic resistance genes (ARGs) (Zhu *et al*., 2013). The application of manure to agricultural soil can then lead to dissemination of ARGs in the environment (Marti *et al*., 2013; Wang *et al*., 2015) through, for instance, horizontal gene transfer (HGT) among bacteria mediated by mobile genetic elements (MGEs) such as plasmids, integrons and transposons (Heuer *et al*., 2011), or through changes in the composition of microbial communities (Su *et al*., 2014). Antibiotic resistance is, at the moment, a global concern that urgently requires the implementation of efficient measures to palliate the dissemination of ARGs (Pruden *et al*., 2013). Although composting has been reported to be an effective measure to alleviate such risks during the application of organic amendments to agricultural soil (Gou *et al*., 2018; Qian *et al*., 2017), results are inconsistent (Peng *et al*., 2015; Storteboom *et al*., 2007) and at least one study (Su *et al*., 2014) instead identified an increase in ARG abundance during composting of sewage sludge.

The aim of this work was to study the impact of the application of six different livestock manure-derived amendments, according to the origin (horse manure-derived *vs.*

chicken manure-derived) and type (processing) of amendment (fresh *vs.* composted *vs.* bokashi), on agricultural soil quality. To this aim, an experiment was conducted, under controlled conditions, with lettuce plants, paying special attention to amendment-induced changes in soil microbial parameters that provide information on the biomass, activity and diversity of soil microorganisms. In addition, we investigated the abundance and risk of dissemination of ARGs through HGT. To our knowledge, there are not many studies in which the impact of three different types of amendments (fresh, composted, bokashi) of two origins (horse and chicken manure-derived) on soil physicochemical and microbial properties (and, hence, on soil quality) and the soil resistome is simultaneously evaluated. We hypothesized that both composted and bokashi amendments have a more positive impact on agricultural soil quality, including regarding the presence of ARGs and MGE genes, as compared to fresh manure.

6.2. Materials and methods

6.2.1. Soil and amendment characterization

Topsoil (0-30 cm) was collected from an agricultural field located in Haro (42° 36′ N; 2° 52′ W), Spain. The soil had not been treated with organic amendments for at least 20 years. After collection, the soil was thoroughly mixed, sieved to <4 mm, air-dried at 30 °C and subjected to physicochemical characterization according to standard methods (MAPA, 1994). The soil was a sandy loam, with a pH of 8.5, an OM content of 1.18 %, a total nitrogen (N) content of 0.08 %, a C/N ratio of 8.61, a phosphorous (P) content of 10.2 mg kg^{-1}, a potassium (K^+) content of 91 mg kg^{-1}, an exchangeable calcium (Ca^{2+}) content of 19.7 meq 100 g^{-1}, and a exchangeable magnesium (Mg^{2+}) content of 0.32 meq 100 g^{-1}.

Fresh and composted horse manure was obtained from Bolaleku S.A.T (Mungia, Spain), while fresh and composted chicken manure was provided by Productos Flower S.A. (Lleida, Spain). Two different bokashi amendments (horse manure-derived and chicken manure-derived) were prepared by mixing: (1) 100 kg of the abovementioned soil (sieved to <1 cm), 100 kg of fresh horse manure, 6 kg of pruning waste biochar, 7 kg of wood ash, 8 kg of wheat straw, 4 kg of sugar beet molasses and 0.3 kg of commercial yeast (*Saccharomyces cerevisiae*); and (2) 175 kg of soil (sieved to <1 cm), 175 kg of fresh chicken manure, 11 kg of pruning waste biochar, 10 kg of wood ash, 11 kg of wheat

straw, 4 kg of molasses and 0.3 kg of commercial yeast. For homogenization, mixtures were thoroughly tumbled four times, resulting in homogeneous piles of approximately 0.8 meters. The piles were again tumbled twice a day for the first 5 days. From the 5th day onwards, the pile was tumbled once a day for 10 consecutive days. The main difference between our bokashi and compost amendments refers to the maturing (composting) time, which extends for approximately 6 months for compost and only 2-3 weeks for bokashi. The physicochemical characterization of the amendments (fresh, composted, bokashi) (Table 6.S1) was performed following standard methods (MAPA, 1994).

6.2.2. Experimental design and treatments

To study the effect of amendments on soil quality, a microcosm pot experiment was carried out under controlled conditions. Three types of amendments (*i.e.*, fresh manure, compost and bokashi) from different origin (*i.e.*, horse manure-derived and chicken manure-derived) were used. Then, the following treatments were considered: (1) fresh horse manure; (2) fresh chicken manure; (3) composted horse manure; (4) composted chicken manure; (5) bokashi from horse manure; and (6) bokashi from chicken manure. An unamended control treatment was included for comparison purposes between amended and unamended soil regarding data on microbial community composition and abundance of ARGs.

Prior to amendment application, polyethylene pots (3,000 cm^3) were filled with 2.5 kg dry weight (DW) of the experimental soil (sieved to <4 mm), kept at 20ºC for 10 days for preconditioning, and finally supplemented with the abovementioned manure-derived amendments. The dose of amendment was adjusted in order to equal the amount of nitrogen added (*i.e.*, 150 kg N ha^{-1}) in all treatments. Lettuce (*Lactuca sativa* var. Batavia) seedlings were grown in the pots for 8 weeks under the following controlled conditions (growth chamber): light intensity = 100 μmol photon m^{-2} s^{-1}; photoperiod = 14/10 h light/darkness; and temperature = 24/20ºC day/night. Each treatment was replicated three times in a completely randomized design. Pots were watered to field capacity three times a week.

After 8 weeks, lettuce plants were harvested and their fresh and dry weights recorded. Dry weight was determined by drying in an oven at 70ºC until reaching a constant mass. Dry leaf tissue was analyzed for total N and C using dry combustion and an elemental analyzer (TruSpec CN, LECO, St. Joseph, Michigan). Crude protein, crude

fiber and starch content were determined according to Zasoski and Burau (1977). Metal and mineral concentrations (*i.e.*, P, Ca, Mg, Na, K, S, Cu, Zn, Fe and Mn) were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) after acid digestion. Then, all the soil in the pots (the soil was fully colonized by plant roots) was collected for analysis.

6.2.3. Soil parameters

6.2.3.1. Physicochemical parameters

Immediately after collection, soil was sieved to <2 mm. Then, the following parameters were determined according to standard methods (MAPA, 1994): pH, carbonate, limestone, nitrate, ammonium, total N and organic C. Metal and mineral concentrations were determined by ICP-OES.

6.2.3.2. Microbial parameters

For the microbial characterization, soils were stored fresh (no more than one month) at 4ºC until analysis. Samples for molecular analyses were stored at -20ºC. β-glucosidase, arylsulphatase and alkaline phosphatase enzyme activities were determined according to Dick *et al*. (1996) and Taylor *et al*. (2002). Urease activity was measured following Kandeler and Gerber (1988). Soil respiration and substrate-induced respiration (SIR) were measured following ISO 16072 Norm (2002) and ISO 17155 Norm (2002), respectively. Potentially mineralizable N (PMN) was measured as described by Powers (1980). Microbial biomass carbon (Cmic) was determined following Vance *et al*. (1987).

For the molecular analysis, DNA extraction was carried out from three aliquots, each of them corresponding to 0.25 g DW soil from each sample, using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA). Prior to DNA extraction, soil samples were washed twice in solution of 120 mM K_2PO_4 (pH 8.0) (Kowalchuk *et al*., 1997). The amount of DNA in the samples was determined on a ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE). For the estimation of the abundance of 16S rRNA gene fragments for total bacteria, qPCR measurements were carried out using the primers, reaction mixtures and PCR conditions described in Epelde *et al*. (2014).

6.2.3.3. Quantification of ARGs and MGE genes

Real-time quantitative-PCR (qPCR) measurements were performed for the quantification of ARGs and MGE genes using the primer pairs listed in Table 6.S2. Three tetracycline resistance genes (*tetA*, *tetM* and *tetW*), two sulfonamide resistance genes (*sul1* and *sul2*), one quaternary ammonium compound resistance gene (*qacEΔ1*), and two MGE genes (class 1 integron integrase, *intI1*; *tnpA*, transposase) were determined in all amended soils and the unamended control soil, which served as a control for the calculation of gene relative abundances (see below). These genes were selected due to their relevance in organically amended soil (Chessa *et al*., 2016; Heuer *et al*., 2011; Heuer and Smalla, 2007; Jechalke *et al*., 2014; Peng *et al*., 2015; You *et al*., 2012). Real-time PCR was performed with ABI 7500 Real-Time PCR System (Applied Biosystems) by using SYBR Green with a standardized annealing temperature of 60ºC. The specificity of the amplified products was confirmed by the melting temperature and the dissociation curve in each run. Each sample was analysed in triplicate with a reference pooled sample and a negative control included in each run. The relative abundance of each gene was expressed as fold change (FC) between the target gene (ARG, MGE gene) and the matched reference 16S rRNA gene, relative to the mean value obtained in the unamended control, for every amended and unamended soil sample, following Livak and Schmittgen (2001):

$$\Delta C_T = C_{T\,(\text{target gene})} - C_{T\,(16S\ \text{rRNA gene})}$$

$$\Delta\Delta CT = \Delta C_{T\,(\text{amended soil})} - \Delta C_{T\,(\text{unamended control mean value})}$$

$$FC = 2^{-\Delta\Delta C_T}$$

where C_T is the q-PCR threshold cycle.

6.2.3.4. DNA metabarcoding

In order to reveal differences between the prokaryotic community structure of: (i) the original soil (as collected from the field), (ii) the amendments, and (iii) the amended soils, amplicon libraries were prepared with a dual indexing approach using sequence-specific primers targeting the V4 hypervariable region of the 16S rRNA gene following Lanzén *et al*. (2016): 519F (CAGCMGCCGCGGTAA) adapted from Øvreås *et al*. (1997), and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso *et al*. (2012). Sequencing was carried out in an Illumina MiSeq V2 platform and pair-ended 2x250 nt at Tecnalia, Spain. Merging of the read paired ends, quality filtering (*i.e.*, primer trimming, removal of

singletons and chimeric sequences) and clustering into operational taxonomic units (OTUs) was performed as described by Lanzén *et al*. (2016). Taxonomic assignments were carried out using CREST and SilvaMod v128 (Lanzén *et al*., 2012; https://github.com/lanzen/CREST).

6.2.4. Statistical analysis

The main effects and interactions of the two experimental factors (origin and type of amendment) on agricultural soil quality were studied. Origin was a fixed factor with two levels: horse manure-derived and chicken manure-derived. Type was a fixed factor with three levels: fresh manure, compost and bokashi. The statistical significance of the effects of these factors and their interaction on crop yield, plant nutritional parameters, and soil physicochemical and microbial parameters were tested by means of two-way ANOVA using R. Multivariate analyses of variance (MANOVA) were performed to evaluate the impact of these factors on soil microbial activity and biomass. Regarding ARG and MGE gene abundances, differences in fold change values between amended soils and unamended control soil were tested using one-way ANOVA and Duncan's multiple range test. In order to explore the effect of treatments on soil physicochemical parameters, a principal component analysis (PCA) and a redundancy analysis (RDA) were performed. Furthermore, to assess the significance of the effect of each explanatory factor on the abundance of ARGs and MGE genes, a RDA and a variation partitioning analysis were performed. Multivariate analyses were performed using Canoco 5 (Ter Braak and Smilauer, 2012).

R package *vegan* was used to perform multivariate statistics, diversity indices calculations and visualization of amplicon sequencing data (Oksanen *et al*., 2013). Function *decostand* was used to transform OTU distributions into relative abundances. Community composition between samples was compared using Bray-Curtis dissimilarity matrices, that were further used to perform non-metric multidimensional scaling (NMDS) with function *metaMDS*. Permutational analyses of variance (PERMANOVA) were performed to assess the influence of the investigated factors on soil prokaryotic community composition, using *adonis* function. Venn diagrams were performed to study differences in prokaryotic taxa. FC values for all studied antibiotic resistance genes was plotted through a heatmap with *Morpheus* online tool. Pearson's correlations between the abundance of ARGs and MGE genes were calculated. Pearson's correlations

were also calculated between those prokaryotic families that represented more than 0.1% of the abundance.

6.3. Results

6.3.1. Plant parameters

6.3.1.1. Crop yield

Application of fresh manure resulted in 61 and 45% higher lettuce yields compared to compost and bokashi, respectively, with fresh chicken manure leading to higher yields than the other amendments (Table 6.1; $p<0.01$). Regardless of amendment type (fresh, compost or bokashi), those derived from chicken manure yielded 113% higher yields, compared to those from horse manure.

Table 6.1. Effect of treatments on crop yield and nutritional parameters. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Mean values (n = 3) ± SD.

		Yield (g)	Starch (%)	Protein (%)	Fiber (%)	N (%)	C/N
Horse	**Fresh**	42.6 ± 9.2^{c}	1.9 ± 0.0	11.5 ± 1.5	1.5 ± 0.2	1.8 ± 0.2	21.5 ± 3.0
	Compost	33.8 ± 10.3^{c}	1.9 ± 0.0	11.0 ± 0.8	1.5 ± 0.0	1.8 ± 0.1	22.4 ± 1.8
	Bokashi	41.9 ± 3.7^{c}	1.9 ± 0.0	12.0 ± 2.4	1.5 ± 0.0	1.9 ± 0.4	21.2 ± 4.9
Chicken	**Fresh**	117.7 ± 12.1^{a}	1.9 ± 0.1	10.6 ± 0.4	1.8 ± 0.2	1.7 ± 0.1	24.0 ± 0.6
	Compost	65.7 ± 14.4^{b}	1.9 ± 0.0	12.0 ± 2.9	1.9 ± 0.3	1.9 ± 0.5	21.9 ± 5.1
	Bokashi	68.5 ± 2.6^{b}	1.9 ± 0.0	12.4 ± 1.2	1.7 ± 0.2	2.0 ± 0.2	20.2 ± 1.8
	Origin (O)	**	ns	ns	**	ns	ns
	Type (T)	**	ns	ns	ns	ns	ns
	O x T	**	ns	ns	ns	ns	ns

6.3.1.2. Nutritional parameters

The type of amendment (fresh manure *vs.* compost *vs.* bokashi) did not result in significant differences in plant mineral contents (Table 6.2). By contrast, regarding the origin of the amendment, horse manure-derived amendments led to significantly higher contents of P (21%), K (23%), Mn (66%) and Zn (20%), compared to chicken manure-derived amendments. Finally, chicken manure-derived amendments led to significantly higher values of crude fiber content, compared to horse manure-derived amendments (Table 6.1).

Table 6.2. Effect of treatments on plant metal and mineral contents. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD.

		P (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Na (g kg^{-1})	K (g kg^{-1})	S (g kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})
Horse	**Fresh**	0.26 ± 0.01	0.81 ± 0.21	0.22 ± 0.06	0.11 ± 0.03^{bc}	4.2 ± 0.9	0.15 ± 0.02	2.9 ± 0.3	2.9 ± 1.1	0.45 ± 0.04	2.9 ± 0.1
	Compost	0.31 ± 0.05	0.86 ± 0.12	0.26 ± 0.01	0.11 ± 0.03^{bc}	4.5 ± 0.3	0.18 ± 0.02	3.7 ± 1.5	3.3 ± 0.7	0.52 ± 0.10	3.0 ± 0.3
	Bokashi	0.27 ± 0.04	0.67 ± 0.19	0.19 ± 0.03	0.14 ± 0.07^{ab}	4.1 ± 0.5	0.15 ± 0.02	3.6 ± 2.5	3.7 ± 1.7	0.59 ± 0.38	2.3 ± 0.3
Chicken	**Fresh**	0.21 ± 0.02	0.76 ± 0.09	0.21 ± 0.02	0.19 ± 0.01^{a}	2.9 ± 1.4	0.12 ± 0.02	2.6 ± 0.4	1.6 ± 0.1	0.38 ± 0.01	2.1 ± 0.4
	Compost	0.20 ± 0.02	0.69 ± 0.13	0.19 ± 0.03	0.11 ± 0.03^{bc}	3.6 ± 0.4	0.12 ± 0.01	2.4 ± 0.3	1.6 ± 0.5	0.40 ± 0.02	2.1 ± 0.3
	Bokashi	0.27 ± 0.05	0.84 ± 0.40	0.20 ± 0.06	0.06 ± 0.02^{c}	3.9 ± 1.0	0.16 ± 0.05	2.9 ± 0.8	2.8 ± 1.9	0.44 ± 0.09	2.7 ± 0.8
	Origin (O)	*	ns	ns	ns	*	ns	ns	*	ns	*
	Type (T)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	O x T	ns	ns	ns	**	ns	ns	ns	ns	ns	ns

Table 6.3. Effect of treatments on soil physicochemical properties. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD.

		pH	OM (%)	N (%)	Carbonate (%)	Limestone (%)	Nitrate (mg kg^{-1})	Ammonium (mg kg^{-1})
Horse	**Fresh**	8.5 ± 0.1^{bc}	0.87 ± 0.04	0.08 ± 0.00	35.6 ± 1.7	5.2 ± 0.7	2.0 ± 1.3^{a}	0.31 ± 0.02
	Compost	8.5 ± 0.1^{bc}	0.86 ± 0.01	0.08 ± 0.01	34.1 ± 1.2	7.8 ± 2.1	5.9 ± 1.8^{a}	0.28 ± 0.16
	Bokashi	8.5 ± 0.0^{ab}	0.92 ± 0.13	0.08 ± 0.00	36.9 ± 0.5	7.9 ± 2.5	4.3 ± 1.5^{a}	0.25 ± 0.05
Chicken	**Fresh**	8.4 ± 0.1^{c}	0.77 ± 0.06	0.08 ± 0.00	35.2 ± 0.8	6.5 ± 2.2	4.9 ± 1.7^{a}	0.38 ± 0.25
	Compost	8.4 ± 0.0^{bc}	0.77 ± 0.10	0.07 ± 0.01	35.8 ± 2.1	7.4 ± 1.5	5.3 ± 3.0^{a}	0.19 ± 0.05
	Bokashi	8.6 ± 0.0^{a}	0.91 ± 0.03	0.07 ± 0.01	36.1 ± 0.9	6.6 ± 0.3	4.9 ± 1.8^{a}	0.16 ± 0.13
	Origin (O)	ns	ns	ns	ns	ns	ns	ns
	Type (T)	**	ns	ns	ns	ns	ns	ns
	O x T	**	ns	ns	ns	ns	ns	ns

6.3.2. Soil parameters

6.3.2.1. Physicochemical parameters

The addition of chicken manure-derived bokashi resulted in significantly higher values of soil pH, compared to all the other amended soils except for horse manure-derived bokashi (Table 6.3). Contents of Mg, K and Fe were significantly lower in fresh manure-treated soils, than in compost- and bokashi-treated ones (Tables 4 and 5). On the contrary, fresh manure-treated soils showed significantly higher values of Ni (Table 6.5). The PCA (Figure 6.1A) performed from all soil physicochemical properties separated fresh horse manure-treated soil from all the other treated soils. The RDA (Figure 6.1B) showed a significant (*pseudo-F*: 2.1, $p<0.01$) effect of amendments on soil physicochemical properties.

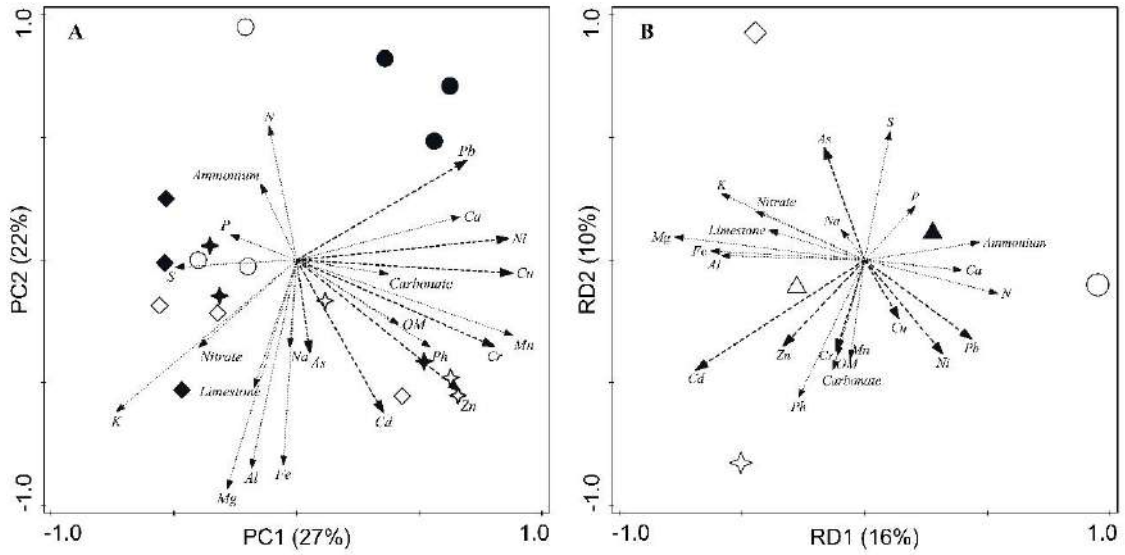

Figure 6.1. (A) Principal component analysis of soil physicochemical properties. Horse (filled symbols) and chicken (empty symbols) manure-derived amendments. Circles: fresh manure; Rhombuses: compost; Stars: bokashi; Dotted arrows: minerals; Dashed arrows: metals; (B) Redundancy analysis displaying the differences observed between experimental factors as explained by soil physicochemical properties. Horse manure-derived amendments: filled triangle; chicken manure-derived amendments: empty triangle; Circle: fresh manure; Rhombus: compost; Star: bokashi.

Table 6.4. Effect of treatments on soil mineral concentrations. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD.

		Al (mg kg^{-1})	P (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Na (mg kg^{-1})	K (mg kg^{-1})	S (mg kg^{-1})
Horse	**Fresh**	10.5 ± 0.9	0.24 ± 0.01	181 ± 12^{a}	2.4 ± 0.0	0.8 ± 0.0	2.6 ± 0.2^{c}	0.40 ± 0.02
	Compost	11.7 ± 0.9	0.24 ± 0.02	135 ± 5^{bc}	3.1 ± 0.2	1.0 ± 0.1	5.1 ± 0.4^{a}	0.51 ± 0.04
	Bokashi	11.8 ± 0.4	0.26 ± 0.01	136 ± 6^{bc}	3.1 ± 0.1	1.0 ± 0.3	4.9 ± 0.8^{ab}	0.45 ± 0.08
Chicken	**Fresh**	11.2 ± 1.9	0.24 ± 0.04	124 ±19^{c}	2.8 ± 0.5	1.3 ± 0.4	4.3 ± 1.1^{ab}	0.51 ± 0.10
	Compost	12.3 ± 0.3	0.25 ± 0.01	142 ± 7^{bc}	3.2 ± 0.0	1.1 ± 0.2	4.9 ± 0.6^{ab}	0.54 ± 0.04
	Bokashi	12.1 ± 0.2	0.25 ± 0.01	147 ± 4^{b}	3.1 ± 0.1	0.8 ± 0.4	3.9 ± 0.2^{b}	0.38 ± 0.08
	Origin (O)	ns	ns	*	ns	ns	ns	ns
	Type (T)	ns	ns	ns	**	ns	**	*
	O x T	ns	ns	**	ns	ns	**	ns

Table 6.5. Effect of treatments on soil metal concentrations. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD.

		Fe (mg kg^{-1})	Mn (µg kg^{-1})	Cu (µg kg^{-1})	Zn (µg kg^{-1})	Cd (µg kg^{-1})	Pb (µg kg^{-1})	Cr (µg kg^{-1})	Ni (µg kg^{-1})	As (µg kg^{-1})
Horse	**Fresh**	8929 ± 419	138 ± 6	34 ± 2	29 ± 1	0.26 ± 0.03^{c}	17 ± 1	11.5 ± 2.5	9.7 ± 0.2^{a}	6.0 ± 0.4
	Compost	9828 ± 463	110 ± 4	25 ± 2	27 ± 2	0.39 ± 0.02^{b}	13 ± 1	9.3 ± 0.3	6.4 ± 0.5^{c}	6.3 ± 0.8
	Bokashi	9664 ± 272	131 ± 28	29 ± 6	30 ± 5	0.48 ± 0.07^{b}	14 ± 1	10.7 ± 3.0	7.9 ± 1.0^{bc}	6.1 ± 0.5
Chicken	**Fresh**	8795 ± 1875	119 ± 15	27 ± 2	31 ± 3	0.43 ± 0.06^{b}	16 ± 3	10.0 ± 1.9	6.9 ± 0.7^{c}	5.0 ± 0.9
	Compost	10194 ± 49	129 ± 28	30 ± 6	25 ± 7	0.49 ± 0.12^{b}	15 ± 2	10.7 ± 1.6	7.5 ± 1.2^{bc}	7.0 ± 1.0
	Bokashi	10337 ± 95	145 ± 21	31 ± 5	36 ± 6	0.66 ± 0.01^{a}	16 ± 1	12.7 ± 0.6	8.5 ± 0.7^{ab}	5.1 ± 1.7
	Origin (O)	ns	ns	ns	ns	**	ns	ns	ns	ns
	Type (T)	*	ns	ns	ns	**	ns	ns	**	ns
	O x T	ns	ns	ns	ns	**	ns	ns	*	ns

6.3.2.2. Microbial parameters

6.3.2.2.1. Activity and biomass

Although the MANOVA performed on soil microbial activity parameters did not show any significant differences ($p = 0.18$) for the factor "origin of the amendment", all these parameters showed higher values when horse manure-derived amendments were used (values were statistically higher only for alkaline phosphatase activity and PMN) (Table 6.6). The origin of the amendment did not have any significant effect on soil microbial biomass parameters (SIR, Cmic, bacterial and fungal gene abundance) (Table 6.7).

Similarly, concerning to the type of amendment, the multivariate analysis of soil microbial activity parameters was not significant ($p = 0.053$). In any case, compost-treated soils showed lower values for most of the microbial activity parameters (Table 6.6). Significantly higher values of microbial biomass parameters (MANOVA, $p<0.05$) were found in fresh manure-treated soils: in particular, fresh manure-treated soils showed higher values of total bacteria (77 and 81% higher than compost- and bokashi-treated soils, respectively) and total fungi (191 and 64% higher than compost- and bokashi-treated soils, respectively).

Table 6.6. Effect of treatments on soil microbial activity parameters. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD. PMN: potentially mineralizable N.

		Arylsulphtase mg ρ-nitrophenol kg DW soil $^{-1}$ h^{-1}	**Alkaline Phosphatase** mg ρ-nitrophenol kg DW soil^{-1} h^{-1}	**β-Glucosidase** mg ρ-nitrophenol kg DW soil $^{-1}$ h^{-1}	**Urease** mg N-NH_4^+ kg DW soil $^{-1}$ h^{-1}	**PMN** mg N-NH_4^+ kg DW soil $^{-1}$	**Soil respiration** µg C g DW soil^{-1} h^{-1}
Horse	**Fresh**	31 ± 3	192 ± 12^{ab}	102 ± 4	16 ± 0	32 ± 9	1.9 ± 0.1^{ab}
	Compost	32 ± 1	206 ± 7^{a}	87 ± 7	18 ± 1	22 ± 2	1.2 ± 0.1^{c}
	Bokashi	32 ± 3	195 ± 3^{a}	101 ± 9	16 ± 6	41 ± 3	2.0 ± 0.5^{a}
Chicken	**Fresh**	30 ± 1	200 ± 11^{a}	102 ± 3	18 ± 1	25 ± 3	1.8 ± 0.1^{ab}
	Compost	26 ± 2	174 ± 7^{b}	93 ± 9	17 ± 0	21 ± 5	1.5 ± 0.2^{bc}
	Bokashi	33 ± 2	187 ± 14^{ab}	93 ± 2	11 ± 1	24 ± 6	1.4 ± 0.2^{bc}
	Origin (O)	ns	*	ns	ns	**	ns
	Type (T)	*	ns	*	*	*	*
	O x T	ns	*	ns	ns	ns	*

Table 6.7. Effect of treatments on soil microbial biomass parameters. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD. SIR: substrate induced respiration; Cmic: microbial biomass carbon.

		SIR µg C g^{-1} DW soil h^{-1}	**Cmic** mg C kg^{-1} DW soil	**Bacterial gene abundance** x 10^{10} copies g^{-1} DW soil	**Fungal gene abundance** x 10^{8} copies g^{-1} DW soil
Horse	**Fresh**	15 ± 1	180 ± 12	2.9 ± 0.5	1.8 ± 0.5
	Compost	12 ± 1	203 ± 17	2.2 ± 0.8	0.6 ± 0.2
	Bokashi	14 ± 2	184 ± 45	1.6 ± 1.0	1.4 ± 1.0
Chicken	**Fresh**	14 ± 1	207 ± 24	3.2 ± 1.0	1.6 ± 0.4
	Compost	13 ± 1	170 ± 21	1.3 ± 0.0	0.6 ± 0.2
	Bokashi	15 ± 1	208 ± 43	1.8 ± 0.2	0.7 ± 0.3
	Origin (O)	ns	ns	ns	ns
	Type (T)	*	ns	**	**
	O x T	ns	ns	ns	ns

6.3.2.2.2. Biodiversity

16S rRNA amplicon sequencing for the original soil (as collected from the field), the amendments, and the amended soils resulted in 1,149,720 reads, clustered into 15,799 OTUs at the 3% dissimilarity level, after quality filtering and removal of singletons. OTU richness did not correlate significantly with the number of reads ($p = 0.296$), indicating that our sequencing effort was sufficient to obtain full coverage of the diversity. However, differences in the number of reads among samples were considerable (from 106,468 to 160,702), and so rarefied richness estimates were used to compare α-diversity values among samples (Table 6.8). Finally, Shannon`s diversity and Pielou's evenness estimates did not correlate with the number of reads.

All amendments themselves showed significantly lower α-diversity than the original soil (Table 6.8). Within each origin of amendment, fresh manure showed, in general, lower diversity than compost and bokashi amendments. This phenomenon was not observed for the amended soils: fresh manure-treated soils did not show lower diversity values than compost- or bokashi-treated soils (Table 6.8). However, both (i) horse manure-derived amendments and (ii) soils treated with horse-manure derived amendments showed significantly higher α-diversity values than chicken manure-derived amendments and soils treated with chicken manure-derived amendments, respectively.

Table 6.8. Values of α-diversity in amendments themselves and amended soils. For the amedments, letters show statistically significant differences according to one-way ANOVA and Duncan's MRT, while for the amended soils, letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values (n = 3) ± SD. RR: rarefied richness. H': Shannon index; J': Pielou's eveness.

		Amendments				**Amended soils**		
		RR	**H'**	**J'**		**RR**	**H'**	**J'**
Horse	**Fresh**	996 ± 30^{d}	4.2 ± 0.1^{e}	0.60 ± 0.00^{e}	**Fresh**	4591 ± 142	7.1 ± 0.1	0.81 ± 0.01
	Compost	2601 ± 600^{b}	5.4 ± 0.2^{c}	0.68 ± 0.00^{cd}	**Compost**	4732 ± 136	7.2 ± 0.0	0.82 ± 0.00
	Bokashi	1895 ± 15^{c}	5.7 ± 0.0^{b}	0.74 ± 0.0^{b}	**Bokashi**	4675 ± 71	7.2 ± 0.1	0.82 ± 0.01
Chicken	**Fresh**	783 ± 13^{d}	4.5 ± 0.0^{e}	0.67 ± 0.00^{d}	**Fresh**	4192 ± 161	6.9 ± 0.1	0.80 ± 0.01
	Compost	948 ± 14^{d}	4.7 ± 0.1^{d}	0.68 ± 0.02^{cd}	**Compost**	4332 ± 78	7.0 ± 0.1	0.80 ± 0.01
	Bokashi	868 ± 14^{d}	4.7 ± 0.0^{d}	0.69 ± 0.0^{c}	**Bokashi**	4116 ± 60	6.9 ± 0.1	0.80 ± 0.01
Original soil		*4012 ± 57^{a}*	*6.8 ± 0.0^{a}*	*0.80 ± 0.01^{a}*				
					Origin (O)	<0.001	<0.001	<0.01
					Type (T)	ns	ns	ns
					O x T	ns	ns	ns

The NMDS ordination performed on prokaryotic OTU composition (Figure 6.2) showed that amended soils separated primarily according to the origin of the amendment (horse manure-derived *vs.* chicken manure-derived) (PERMANOVA, $p<0.01$) and, to a lesser extent, the type of amendment (PERMANOVA, $p<0.05$). In relation to the taxonomical classification, 71.5% of the 16S rRNA sequences were classified to family rank, chosen for the analysis of taxonomic distribution. At family level, the composition of prokaryotic communities was relatively similar among treatments, with the 25 most abundant taxa representing between 40-56% of the total abundance (Figure 6.3).

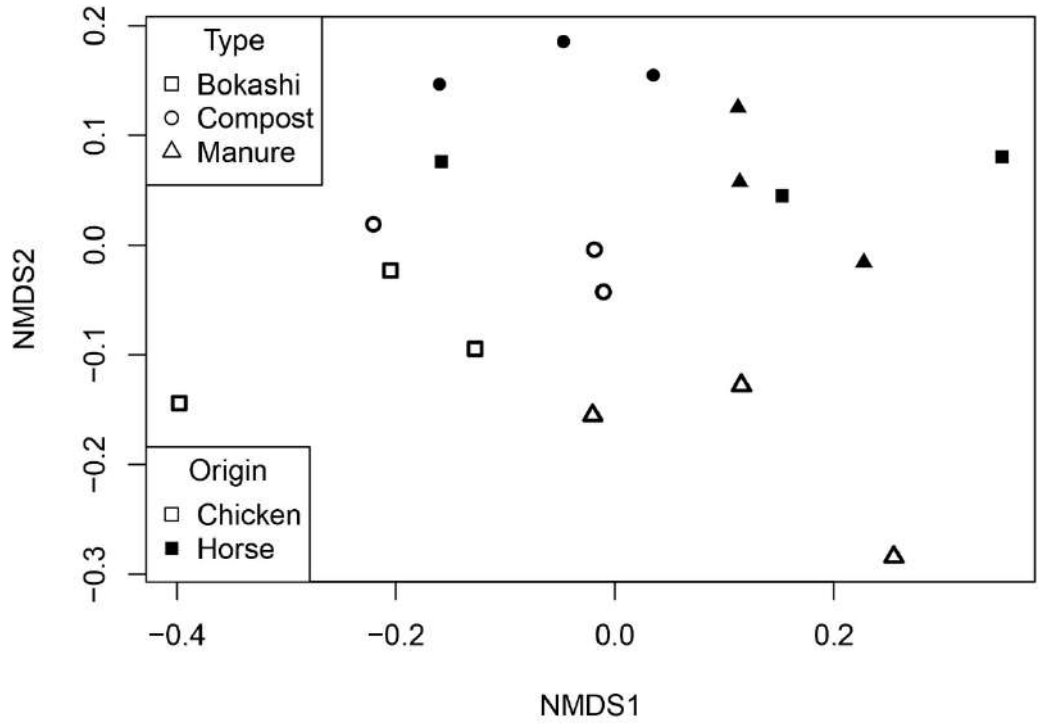

Figure 6.2. Non-metric multidimensional scaling (NMDS) analysis representing patterns of prokaryotic community composition. Bray-Curtis dissimilarities of community composition, based on relative OTU abundances from prokaryotic 16S rRNA amplicon data, are represented as distance in the diagram. Samples are labelled according to legend.

For a more extensive analysis, the abundances of families representing more than 0.1% of the total reads were also studied. Within the amended soils, 18 families (constituting 21% of total abundance) showed significant differences in abundance with respect to amendment origin (Table 6.9). Eight of these were significantly more abundant in chicken manure-amended soils (*i.e., Chitinophagaceae, Blastocatellaceae, Xanthomonadaceae, Oxalobacteraceae, Hymenobacteraceae, Chthoniobacteraceae, Alcaligenaceae* and *PHOS-HE51*), and accounted for 16.3% of the total prokaryotic community (compared to 12.0% for horse manure-amended

soils). The remaining 10 taxa were more abundant in horse manure-amended soils, representing 7.0% of the total prokaryotic community (compared to 3.4 % for chicken manure-amended soils).

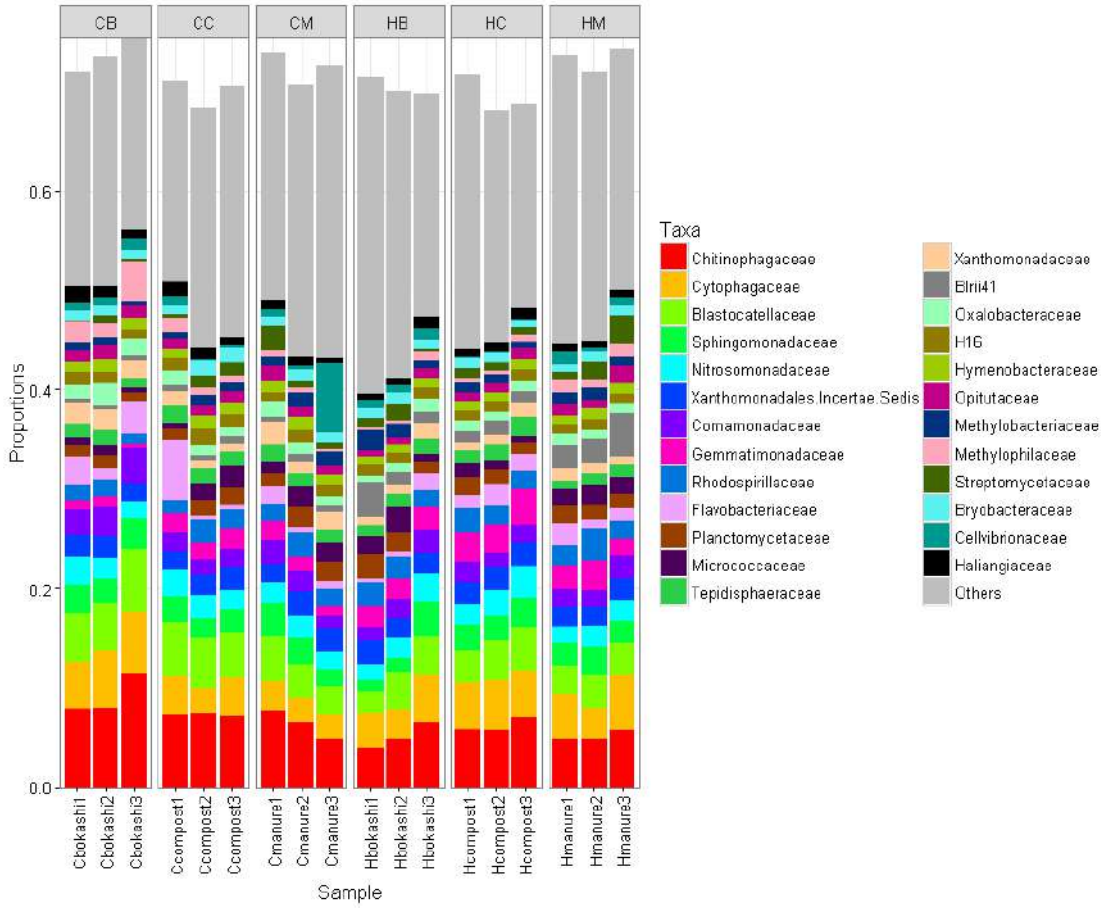

Figure 6.3. Barplot representing the distribution of the 25 most abundant prokaryotic taxa (at family rank) in amended soils, for each individual sample. CB: chicken bokashi-treated soil samples; CC: chicken compost-treated soil samples; CF: chicken manure-treated soil samples; HB: horse bokashi-treated soil samples; HC: horse compost-treated soil samples; HF: horse manure treated soil samples.

With respect to the type of amendment (Table 6.10), 13 taxa showed significant differences in abundance, representing 3.9, 3.1 and 3.8% of the total prokaryotic community at family rank for fresh manure-, compost- and bokashi-amended soils, respectively. Compost-amended soils showed a significantly higher abundance of *Anaerolineaceae*, whereas bokashi application led to a significantly higher abundance of *Haliscomenobacteraceae* (Table 6.10) in amended soils. Fresh manure-amended soils showed a lower abundance of *Fimbriimonadaceae* and *Phycisphaeraceae*, compared to compost- and bokashi-amended soils, but a higher abundance of *Microbacteriaceae*.

Table 6.9. Significant differences in taxa abundance at family rank between horse and chicken manure-derived amendments, based on pooled variances *t*-test. Mean values (n = 9) ± SD. Only those families representing more than 0.1% of the total reads were included in the analysis.

	Relative Abundance		
BACTERIAL TAXA (FAMILY)	**HORSE**	**CHICKEN**	**p value**
Chitinophagaceae	0.055 ± 0.009	0.075 ± 0.017	<0.01
Blastocatellaceae	0.034 ± 0.007	0.047 ± 0.010	<0.01
Gemmatimonadaceae	0.026 ± 0.006	0.014 ± 0.005	<0.001
BIrii41	0.021 ± 0.012	0.005 ± 0.001	<0.01
Xanthomonadaceae	0.011 ± 0.003	0.016 ± 0.005	<0.01
Oxalobacteraceae	0.010 ± 0.002	0.014 ± 0.005	<0.05
Hymenobacteraceae	0.010 ± 0.001	0.012 ± 0.001	<0.01
Micromonosporaceae	0.007 ± 0.002	0.003 ± 0.002	<0.001
Chthoniobacteraceae	0.005 ± 0.001	0.006 ± 0.001	<0.01
Alcaligenaceae	0.004 ± 0.001	0.007 ± 0.002	<0.01
Xanthobacteraceae	0.003 ± 0.001	0.002 ± 0.000	<0.05
Roseiflexaceae	0.003 ± 0.001	0.002 ± 0.001	<0.05
Hyphomonadaceae	0.003 ± 0.000	0.002 ± 0.001	<0.01
Rhodospirillales Incertae Sedis	0.002 ± 0.001	0.002 ± 0.000	<0.01
Nannocystaceae	0.002 ± 0.001	0.001 ± 0.000	<0.05
PHOS-HE51	0.002 ± 0.001	0.003 ± 0.001	<0.01
Burkholderiaceae	0.002 ± 0.001	0.001 ± 0.001	<0.05
Iamiaceae	0.001 ± 0.001	0.001 ± 0.000	<0.05

Table 6.10. Significant differences in taxa abundance at family rank among type of amendment (fresh manure, compost and bokashi) based one-way ANOVA and Duncan's MRT. Mean values (n = 6) ± SD. Only those families representing more than 0.1% of the total reads were included in the analysis.

	Relative Abundance			
BACTERIAL TAXA	**FRESH**	**COMPOST**	**BOKASHI**	**p value**
Hyphomicrobiaceae	0.0121 ± 0.0028 ^{a}	0.0071 ± 0.0017 ^{b}	0.0086 ± 0.0040 ^{ab}	<0.05
Anaerolineaceae	0.0017 ± 0.0003 ^{b}	0.0042 ± 0.0018 ^{a}	0.0016 ± 0.0006 ^{b}	<0.01
Fimbriimonadaceae	0.0020 ± 0.0004 ^{b}	0.0035 ± 0.0006 ^{a}	0.0033 ± 0.0009 ^{a}	<0.01
Bdellovibrionaceae	0.0020 ± 0.0002 ^{b}	0.0023 ± 0.0004 ^{ab}	0.0027 ± 0.0005 ^{a}	<0.05
Phycisphaeraceae	0.0014 ± 0.0003 ^{b}	0.0021 ± 0.0004 ^{a}	0.0020 ± 0.0003 ^{a}	<0.01
Verrucomicrobiaceae	0.0034 ± 0.0010 ^{a}	0.0020 ± 0.0003 ^{b}	0.0026 ± 0.0003 ^{ab}	<0.01
Bacillaceae	0.0039 ± 0.0018 ^{ab}	0.0019 ± 0.0009 ^{b}	0.0077± 0.0050 ^{a}	<0.05
Microbacteriaceae	0.0036 ± 0.0007 ^{a}	0.0018 ± 0.0004 ^{b}	0.0020 ± 0.0013 ^{b}	<0.01
Phaselicystidaceae	0.0012 ± 0.0001 ^{ab}	0.0014 ± 0.0003 ^{a}	0.0010 ± 0.0001 ^{b}	<0.05
Haliscomenobacteraceae	0.0009 ± 0.0003 ^{b}	0.0012 ± 0.0003 ^{b}	0.0020 ± 0.0005 ^{a}	<0.001
Rhodobacteraceae	0.0020 ± 0.0006 ^{a}	0.0012 ± 0.0003 ^{ab}	0.0012 ± 0.0005 ^{b}	<0.05
P3OB-42	0.0008 ± 0.0001 ^{b}	0.0012 ± 0.0003 ^{a}	0.0012 ± 0.0002 ^{ab}	<0.05
Phyllobacteriaceae	0.0039 ± 0.0015 ^{a}	0.0012 ± 0.0003 ^{b}	0.0025 ± 0.0021 ^{ab}	<0.05

Regarding the amendments, fresh manure, compost and bokashi amendments shared 54% of the taxa (184 taxa) at family rank, individually explaining 2.9, 5.9 and 18.8% of the total prokaryotic community, respectively (Figure 6.4). Fresh manure shared 60% (206 taxa) and 55% (189 taxa) with compost and bokashi, respectively, while compost and bokashi shared 64% (218 taxa) between them. Compost had a significantly higher prokaryotic diversity at family rank (304 taxa for compost, compared to 243 and 221 for bokashi and fresh manure, respectively). Regarding the origin of the amendment (horse manure-derived *vs.* chicken manure-derived), 324 and 226 family taxa were found in horse and chicken manure-derived amendments, respectively (62% of the taxa were shared between them).

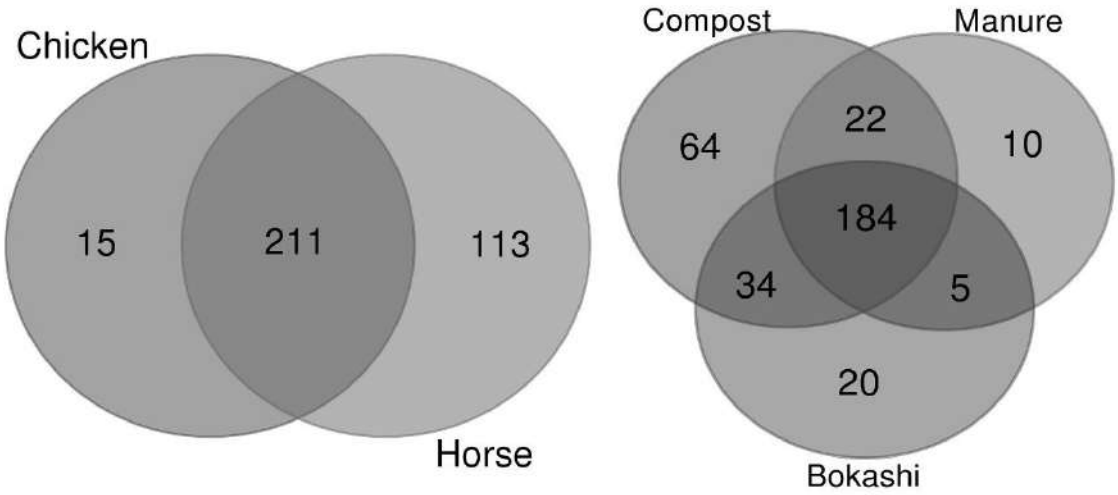

Figure 6.4. Venn diagrams showing number of taxa at family rank for the investigated factors: origin of amendment (horse manure-derived and chicken manure-derived); type of amendment (fresh manure, compost and bokashi).

6.3.2.2.3. Abundance of ARGs and MGE genes

In relation to the relative abundance of ARGs and MGE genes (Figure 6.5, Table 6.S3) in amended soils, the application of fresh chicken manure led to a significant enrichment of all these genes. This effect was also observed in the variation partitioning and redundancy analysis (Figure 6.6; 78.1% of the total variance, pseudo-F = 4.7, $p<0.01$), in which the individual explanatory factors of fresh manure and chicken had a significant impact on the abundance of all studied genes (38.5% of the variation, pseudo-F = 10, $p<0.01$; 21.5% of the variation, pseudo-F = 8.1, $p<0.01$; for fresh manure and chicken explanatory factors, respectively).

Concerning MGE genes, the abundance of the transposase *tnpA* gene was significantly higher in all amended soils, compared to the unamended control soil, suggesting that the application of any of the amendments may potentially enhance HGT. In contrast, apart from the soil amended with fresh chicken manure [which showed a significantly higher abundance (79-fold higher) of the integrase *intI1* gene than the unamended control soil], none of the amended soils showed higher *intI1* abundance than the unamended control soil.

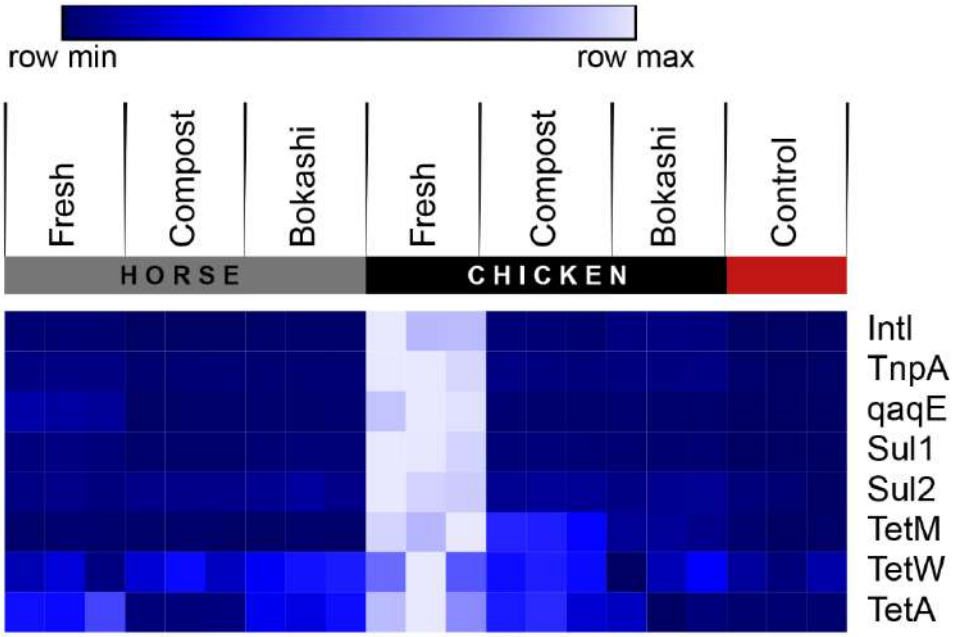

Figure 6.5. Heatmap displaying fold-change (FC) values for each of the studied ARG and MGE in amended samples and unamended control soil. The colour scale reflects the magnitude of the FC value, from blue (lowest) to white (highest).

In relation to ARGs, all amended soils showed significant differences in the abundance of at least one of the studied genes, with respect to the unamended control soil. As abovementioned, the application of fresh chicken manure increased the abundance of all ARGs genes (Figure 6.5, Table 6.S3), compared to all the other treatments. Chicken compost-amended soils showed significantly greater values of sulfonamide (*sul1* and *sul2*, 16- and 2-fold higher, respectively) and tetracycline (*tetM*, *tetW* and *tetA*, 18-, 3- and 5-fold higher, respectively) resistance gene abundances than unamended control soil. Similarly, soils amended with chicken manure-derived bokashi had a higher abundance of *tetM*, *sul1* and *sul2* resistance gene than unamended control soil. The application of fresh horse manure resulted in a significantly higher abundance of *qacEΔ1* (52-fold

higher), *sul1* (21-fold) and *tetA* (6-fold) resistance genes, compared to unamended control soil (Table 6.S3). Soils amended with horse manure-derived bokashi were enriched in *sul1*, *sul2* and *tetA* genes. Finally, soils amended with horse manure-derived compost showed the lowest values of ARGs abundances among all amended soils (*i.e.*, they were only enriched in *sul2* gene).

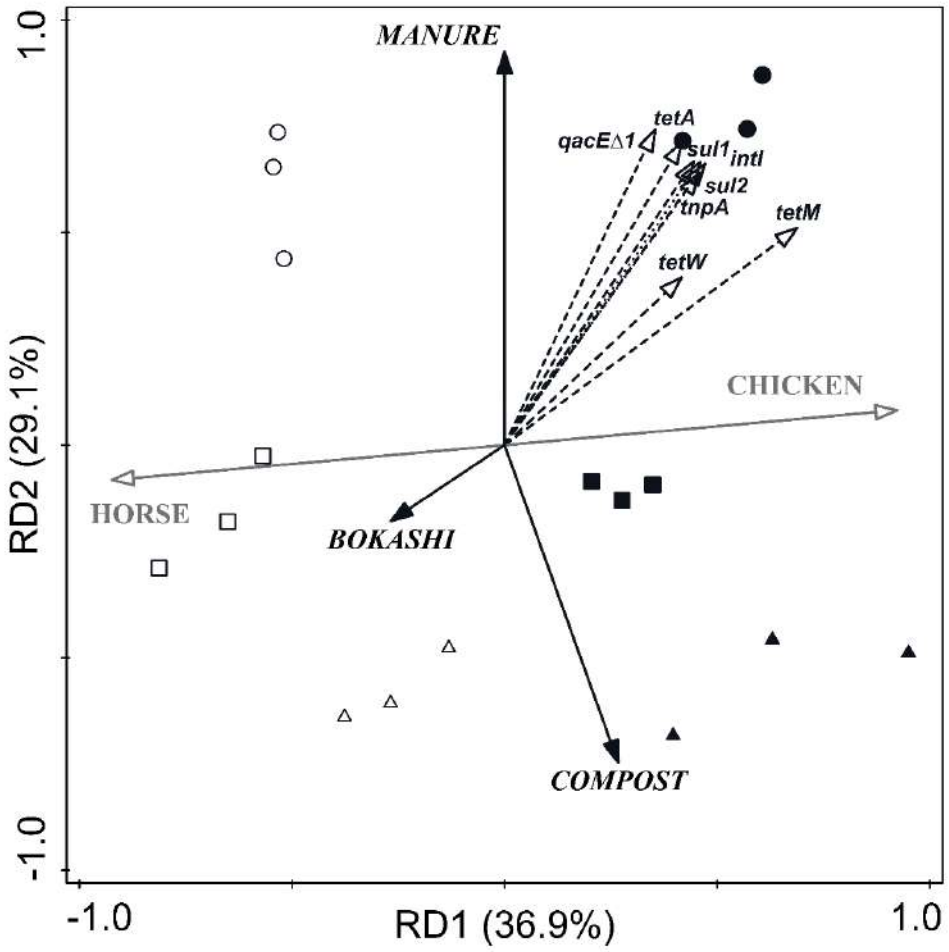

Figure 6.6. Redundancy analysis in which ARGs (dashed vectors) and MGE genes (dotted vectors) are response variables. Experimental factors (solid vectors) are explanatory variables. Empty symbols indicate horse manure-derived amendments; Filled symbols correspond to chicken manure-derived amendments; Circles: fresh manure; Triangles: compost; Squares: bokashi.

The abundances of ARGs and MGE genes were highly correlated (Table 6.11, Figure 6.6). Regarding the correlation between these genes and prokaryotic taxa at family rank (Table 6.S4), *Alcaligenaceae* showed a positive correlation with all ARGs and MGE genes (this family was significantly more abundant in soils amended with chicken manure-derived amendments). *Hyphomonadaceae* was more abundant in horse manure-

amended soils and correlated negatively with all ARGs and MGE genes. *Verrucomicrobiaceae, Rhodobacteraceae and Phyllobacteriaceae* were more abundant in soils amended with fresh manure than in those amended with compost and bokashi, and correlated positively with both ARGs and MGE genes. Finally, *Phycisphaeraceae, Haliscomenobacteraceae and P3OB-42* correlated negatively with ARGs and MGEs genes and were less abundant in fresh manure-amended soils.

Table 6.11. Correlation analysis between ARGs and MGE genes. Pearson correlation coefficient and p values.

		MGE		**ARG**					
		intI	***tnpA***	***qacEΔ1***	***sul1***	***sul2***	***tetM***	***tetW***	***tetA***
MGE	***intl***	-	2.3E-20	8.6E-15	7.4E-20	4.9E-16	7.3E-09	4.0E-05	2.6E-06
	tnpA	0.998	-	1.7E-17	3.5E-27	8.4E-18	7.9E-09	2.3E-05	1.8E-06
ARG	***qacEΔ1***	0.989	0.995	-	6.7E-18	2.9E-14	4.5E-08	4.1E-05	7.2E-07
	sul1	0.998	1.000	0.996	-	1.0E-17	1.1E-08	1.8E-05	1.2E-06
	sul2	0.993	0.996	0.987	1.00	-	7.9E-09	1.1E-05	6.5E-06
	tetM	0.940	0.939	0.924	0.936	0.939	-	2.9E-05	4.4E-06
	tetW	0.814	0.827	0.813	0.832	0.844	0.822	-	2.1E-05
	tetA	0.871	0.877	0.891	0.883	0.854	0.861	0.830	-

6.4. Discussion

6.4.1. Crop yield and nutritional parameters

When used as amendments, livestock residues can improve soil physicochemical and biological properties, while increasing crop yield. Here, all chicken manure-derived amendments led to significantly higher crop yields than horse manure-derived amendments, probably due to their higher content of total N (Table 6.S1). Horse manure has been reported to contain up to 35-70% of the total N content in poultry manure (DEFRA, 2010). A short-cycle crop, such as lettuce, requires fertilization with phytoavailable inorganic N or, alternatively, the application of an easily mineralizable organic amendment, in order to achieve a rapid mineralization of the organic N. During the first growing season, 24-35% of the organic N present in manure-derived compost can be mineralized (Hernández *et al*., 2016). Fresh manure provides more labile OM, which can then be rapidly mineralized. Therefore, it is not surprising that, in our study, fresh manure (particularly, fresh chicken manure) yielded higher crop yield values,

compared to compost and bokashi amendments. In contrast, regarding plant nutritional parameters, no remarkable differences were found among treatments.

6.4.2. Soil physicochemical parameters

At the end of the experiment, no differences in soil OM content were detected in any of the studied treatments. This is not surprising, as changes in soil OM content are usually slow, not expected in such a short-term experiment. In any case, in fresh manure-amended soils, a rapid mineralization of the OM can be expected. Besides, the fresh manure-induced higher crop yield observed here most likely results in an increased amount of root exudates, which could eventually increase the pool of soil organic C (Geisseler and Scow, 2014).

The concentration of Ni was significantly higher in fresh manure-amended soil, compared to compost- and bokashi-amended soil. Nevertheless, none of the metal concentration values detected in our soils exceeded the regulatory limits (VIE-B values) indicated by Law 4/2015 regulating the prevention and correction of soil pollution in the Basque Country (BOE-A-2015-8272).

6.4.3. Soil microbial parameters

Soil microbial parameters are increasingly being used as indicators of soil quality (in particular, to detect the impact of disturbances on soil quality) (Epelde *et al*., 2010), as they provide a quick response and a high sensitivity and ecological relevance (Barrutia *et al*., 2011; Garbisu *et al*., 2011; Pardo *et al*., 2014). Besides, microorganisms play key roles in soil processes (*e.g.*, OM decomposition, nutrient cycling, etc.) and the delivery of essential ecosystem services (Burges *et al*., 2015). Thus, enzyme activities are commonly used as indicators of soil microbial activity (Nannipieri *et al*., 2012). Similarly, other parameters, such as soil respiration and nitrogen mineralization, are often used to detect changes in soil microbial activity (Garaiyurrebaso *et al*., 2017; Lacalle *et al*., 2018).

The application of organic amendments has been frequently reported to enhance soil microbial activity and biomass (Das *et al*., 2017; Dinesh *et al*., 2010; Hernández *et al*., 2016; Reardon *et al*., 2017; Zhen *et al*., 2014). In our study, all microbial activity parameters showed higher values in soils treated with horse manure-derived amendments, compared to soils treated with chicken manure-derived amendments; nonetheless, differences were statistically significant only for alkaline phosphatase activity and PMN

(Table 6.6). These results can be explained by the lower amount of mineral N and P found in horse manure-derived amendments (Table 6.S1), posing the need for organic N and P mineralization. In the absence of the required amount of inorganic nutrients, plants and microorganisms can stimulate OM mineralization by excreting extracellular enzymes (Allison and Vitousek, 2005). The application of compost yielded lower values of several soil microbial activity parameters (see Table 6.6) compared to fresh manure and bokashi, most likely due to the presence of OM in a more stabilized form (Keener *et al*., 2000; Yuan *et al*., 2017). It is a well-known fact that the stability and lability of the OM applied as amendment are critical to explain soil microbial responses (Dijkstra *et al*., 2009).

Concerning soil microbial biomass, fresh manure yielded significantly higher values of both bacterial and fungal cell abundance, compared to compost and bokashi. The utilization of labile organic C sources, such as the ones provided by fresh manure, can stimulate the growth of *r*-strategists, characterized by low substrate use efficiency and high growth rates, often resulting in higher values of microbial biomass and activity, but a lower microbial diversity (Meyer, 1994; Hartmann *et al*., 2015). As the organic C sources become more recalcitrant, a shift towards *K*-strategists is induced, leading to a more efficient use of substrates and a higher diversity (Fierer *et al*., 2007).

Soil microbial diversity is critical for maintaining soil ecosystem functions and services (Delgado-Baquerizo *et al*., 2016). Although in many cases differences were not statistically significant, all α-diversity parameters showed lower values in fresh manure-amended soils. The lack of statistically significant differences between manure-processing types could be due to the fact that both time and plant effects may have minimized differences after 8 weeks of study. Interestingly, horse manure-amended soils showed significantly greater α-diversity values than chicken manure-amended soils. Further, all the amendments used here showed lower microbial diversity values than the original soil. This is not surprising, as the soil ecosystem is well known for its extremely high biodiversity.

It might be argued that the increase in microbial activity and biomass, together with the reduction in microbial diversity, observed in our amended soils, could be just a transient effect. Nonetheless, a regular application of these or similar organic amendments will certainly lead to a long-term shift of the soil microbial community, due to, among other factors, the high levels of OM and nutrients, the presence of heavy metals and organic contaminants, and the presence of antibiotics and ARGs (Das *et al*., 2017; Hartmann *et al*., 2015; Liu *et al*., 2017; Storteboom *et al*., 2007; Tian *et al*., 2015b).

The origin of the amendment was the most relevant factor to explain the composition of soil prokaryotic communities. Das *et al*. (2017) found differences in prokaryotic community composition between soils treated with cattle *vs.* swine compost, and concluded that the main drivers of such differences were soil C and N availability and soil pH values. The type of amendment also had a significant effect on prokaryotic community composition, but to a lesser extent. In this respect, changes in soil physicochemical properties induced by the application of amendments (*e.g.*, quantity and lability of C sources) are known to affect the composition of soil prokaryotic communities. These changes depend, to a great extent, on the stability and lability of the OM applied.

The composition of prokaryotic communities can have a considerable impact on soil function and, in particular, on the soil food web dynamics (Morriën, 2016). At phylum rank, *Proteobacteria*, *Firmicutes* and *Actinobacteria* were more abundant in fresh manure amended soils, compared to compost- and bokashi-amended soils. The first two phyla have been reported as copiotrophs, with higher rates of growth in C- and nutrient-rich environments (Fierer *et al*., 2007). The increased abundance of these phyla in fresh manure-amended soils may be associated to a higher amount of labile C and nutrients. On the other hand, *Acidobacteria*, a taxonomic group reported as oligotrophic (Fierer *et al*., 2007), was more abundant in compost- and bokashi-amended soils, compared to fresh manure-amended soils. Members of this phylum show the ability to grow in the presence of low nutrient concentrations and recalcitrant C substrates, and are usually more abundant in natural ecosystems than in croplands (Pershina *et al*., 2015).

6.4.4. ARGs and MGEs

Livestock manure is often applied to soil in an attempt to improve soil physicochemical and biological properties. However, manure-derived amendments are known to potentially carry antibiotic residues and ARGs (Udikoviv-Kolic *et al*., 2014). Composting has been reported to successfully reduce the amount of antibiotic resistance bacteria and ARGs (Gou *et al.*, 2018). Nonetheless, on the contrary, some authors (Su *et al*., 2014) found an increase in the abundance of ARGs during composting. It is therefore necessary to understand the fate of ARGs during composting before land application (Storteboom *et al*., 2007). Here, we found that, regardless of the treatment, all amended soils had significantly higher abundance of at least one ARG, compared to the unamended control

soil. Significant differences were also detected among treatments, as fresh chicken manure-amended soils showed the highest abundances for all ARGs. Compost- and bokashi-amended soils showed lower values of all ARGs abundances, confirming our hypothesis and in accordance with previous studies (Gou *et al*., 2018; Qian *et al*. 2017; Selvam *et al*., 2012). The high temperatures reached during the preparation of compost (60-70ºC) and bokashi (over 70ºC) are possibly responsible for the reduction in the abundance of ARGs (Selvam *et al*., 2012; Qian *et al*., 2016). Such reduction can also be attributed to the degradation of the antibiotic themselves during composting (Mohring *et al*., 2009; Selvam *et al*., 2012; Storteboom *et al*. 2007; Wang *et al*., 2012) or to changes in their bioavailability (Chessa *et al*., 2016). Yet, compost- and bokashi-amended soils showed higher abundances for some ARGs compared to unamended control soil. This illustrates the complexity of microbial ecology processes during composting (Pruden *et al*., 2013) and our incomplete understanding of how they interact in order to degrade ARGs and residual antibiotics.

Application of manure containing residual antibiotic concentrations can promote the dissemination of ARGs in the soil ecosystem, especially when amendments also contain ARGs that may be transferred to the native soil community. In this study, the abundance of the *tnpA* transposase gene was significantly higher in all amended soils, irrespective of the treatment, compared to unamended control soil. Fresh chicken manure-amended soils were the only ones that showed a significantly higher relative abundance of the class 1 integron integrase *intI1* gene, compared to the unamended control soil. Class 1 integrons can be a source of acquisition and dispersal of ARGs in the environment (Gillings *et al*., 2015). Interestingly, in all amended soils, these two genes (*tnpA, intI1*) were positively correlated with all ARGs (Table 6.11), suggesting a potential risk of dissemination of ARGs via HGT in livestock manure-amended soils, particularly after the application of fresh chicken manure, as previously reported (Zhang *et al*., 2017).

Finally, several taxa at family rank showed positive correlation with all the studied ARGs and MGE genes. Interestingly, *Alcaligenaceae*, *Verrucomicrobiaceae*, *Rhodobacteraceae* and *Phyllobacteriaceae* were significantly more abundant in soils treated with chicken manure-derived amendments and in fresh manure-amended soils. The order *Alcaligenes* has been reported to harbour tetracycline resistant and class 1 integron genes (Agerso and Sanvang, 2005) while members of the family *Rhodobacteraceae* can host a broad range of ARGs (Zhu *et al*., 2017).

6.5. Conclusions

The application of fresh manure resulted in higher values of soil microbial biomass and activity. Fresh chicken manure yielded the highest lettuce crop yield, probably owing to its high content of readily available N. Microbial community composition was altered by the application of manure-derived amendments, with "origin of amendment" being the most determinant factor for such composition. Our findings indicate that special attention must be paid to fresh chicken manure before its application to agricultural soil, since it may increase the abundance of ARGs and class I integrons. Furthermore, two MGE genes (*tnpA, intI1*) were positively correlated with all ARGs, suggesting a risk of dissemination of ARGs via HGT in agricultural soil, as a result of the application of livestock manure-derived amendments. Composting and bokashi-composting processes are highly encouraged to reduce the load of ARGs, as the high temperatures reached during such processes have often shown to be effective at reducing the abundance of ARGs.

6.6. Supplementary information

Table 6.S1. Physicochemical characterization of the original soil (as collected from the field) and the amendments.

		HORSE			CHICKEN		
	Soil	**Fresh**	**Compost**	**Bokashi**	**Fresh**	**Compost**	**Bokashi**
pH	8.5	9.2	9.2	9.9	8.6	8.1	9.0
Organic matter (%)	1.2	81.2	37.0	34.4	81.1	40.2	24.3
C/N	8.6	26.9	24.0	35.9	11.4	10.4	12.1
Total N (%)	0.1	0.6	0.4	0.5	1.7	1.2	1.1
Olsen P (mg kg^{-1} DW)	10	3,744	3,747	2,737	19,423	7,401	4,995
K^+ (mg kg^{-1} DW)	90	15,581	14,435	15,669	25,494	21,630	17,184

Table 6.S2. Primers used for the detection of ARGs and MGE genes.

Gene	Primer	5'→3' sequence	Reference
16S rRNA	16S-F	CGGTGAATACGTTCYCGG	Suzuki *et al*. (2000)
	16S-R	GGWTACCTTGTTACGACTT	
sul1	*sul1*-F	CCGTTGGCCTTCCTGTAAAG	Heuer and Smalla (2007)
	sul1-R	TTGCCGATCGCGTGAAGT	
sul2	*sul2*-F	CGGCTGCGCTTCGATT	Heuer *et al*. (2008)
	sul2-R	CGCGCGCAGAAAGGATT	
tetW	*tetW*-F	GCAGAGCGTGGTTCAGTCT	Smith *et al*. (2004)
	tetW-R	GACACCGTCTGCTTGATGATAAT	
tetM	*tetM-F*	GTGGACAAAGGTACAACGAG	Ng *et al*. (2001)
	tetM-R	CGGTAAAGTTCGTCACACAC	
tetA	*tetA*-F	GGCGGTCTTCTTCATCATGC	Lanz *et al*. (2003)
	tetA-R	CGGCAGGCAGAGCA AGTAGA	
qacEΔ1	*qacEΔ1*-F	ATCGCAATAGTTGGCGAAGT	Sandvang *et al*. (1997)
	qacEΔ1-R	CAAGCTTTTGCCCATGAAGC	
tnpA	*tnpA*-F	CCGATCACGGAAAGCTCAAG	Hu *et al*. (2016)
	tnpA-R	GGCTCGCATGACTTCGAATC	
IntI1	*int1*-F	GCCTTGATGTTACC CGAGAG	Barraud *et al*. (2010)
	int1-R	GATCGGTCGAATGC GTGT	

Table 6.S3. Fold-changes revealing the significant enrichment of ARGs and MGEs relative abundances in amended and unamended control soils. Letters show statistically significant differences by means of one-way ANOVA and Duncan's MRT.

		MGE		ARG					
		intI	***tnpA***	***qacEΔ1***	***sul1***	***sul2***	***tetM***	***tetW***	***tetA***
Horse	**Fresh**	2.7 ± 0.2^{b}	3.4 ± 0.1^{b}	52.2 ± 7.2^{b}	21.3 ± 2.3^{b}	1.4 ± 0.1^{bc}	1.5 ± 0.1^{cd}	1.3 ± 0.4^{bc}	6.3 ± 1.8^{b}
	Compost	1.0 ± 0.0^{b}	2.1 ± 0.2^{c}	4.1 ± 1.0^{c}	7.1 ± 0.3^{cd}	1.6 ± 0.0^{b}	1.1 ± 0.3^{d}	1.8 ± 0.4^{bc}	1.3 ± 0.1^{c}
	Bokashi	1.8 ± 0.1^{b}	2.0 ± 0.1^{c}	9.9 ± 0.7^{c}	14.6 ± 1.5^{bc}	1.8 ± 0.2^{b}	1.0 ± 0.3^{d}	2.4 ± 0.4^{bc}	4.5 ± 0.8^{b}
Chicken	**Fresh**	78.5 ± 5.7^{a}	52.2 ± 1.3^{a}	504 ± 25.7^{a}	459 ± 13.6^{a}	13.9 ± 0.5^{a}	48.2 ± 3.1^{a}	5.2 ± 1.4^{a}	14.2 ± 1.8^{a}
	Compost	3.1 ± 0.5^{b}	3.0 ± 0.4^{b}	10.5 ± 1.8^{c}	16.3 ± 1.7^{bc}	1.8 ± 0.1^{b}	17.7 ± 3.2^{b}	2.6 ± 0.3^{b}	5.4 ± 1.8^{b}
	Bokashi	4.8 ± 0.4^{b}	3.3 ± 0.1^{b}	9.5 ± 1.4^{c}	11.7 ± 1.0^{bc}	1.6 ± 0.1^{b}	4.4 ± 0.6^{c}	1.3 ± 0.8^{bc}	1.7 ± 1.2^{c}
Unamended soil		*1.0 ± 0.1^{b}*	*1.0 ± 0.3^{d}*	*1.0 ± 0.2^{c}*	*1.0 ± 0.1^{d}*	*1.0 ± 0.3^{c}*	*1.1 ± 0.5^{d}*	*1.0 ± 0.2^{c}*	*1.0 ± 0.0^{c}*

Table 6.S4. Pearson's correlations of those prokaryotic taxa (at family rank) representing more than 0.1% of the community and ARGs/MGE genes. ns: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.01$.

TAXA	intI		tnpA		qacEΔ1		sul1		sul2		tetM		tetW		tetA	
Chitinophagaceae	-0,01	ns	-0,03	ns	-0,09	ns	-0,05	ns	-0,03	ns	0,05	ns	-0,01	ns	-0,18	ns
Cytophagaceae	-0,53	*	-0,54	*	-0,56	*	-0,56	*	-0,56	*	-0,62	**	-0,64	**	-0,64	**
Blastocatellaceae	-0,16	ns	-0,18	ns	-0,24	ns	-0,20	ns	-0,18	ns	0,00	ns	-0,10	ns	-0,26	ns
Sphingomonadaceae	0,04	ns	0,02	ns	-0,01	ns	0,02	ns	0,01	ns	-0,05	ns	-0,01	ns	-0,05	ns
Nitrosomonadaceae	-0,14	ns	-0,14	ns	-0,18	ns	-0,14	ns	-0,14	ns	-0,07	ns	-0,05	ns	-0,14	ns
Xanthomonadales Incertae Sedis	0,06	ns	0,09	ns	0,10	ns	0,09	ns	0,08	ns	0,02	ns	0,10	ns	0,01	ns
Comamonadaceae	-0,05	ns	-0,08	ns	-0,11	ns	-0,09	ns	-0,09	ns	-0,14	ns	-0,15	ns	-0,19	ns
Gemmatimonadaceae	-0,32	ns	-0,31	ns	-0,29	ns	-0,29	ns	-0,30	ns	-0,38	ns	-0,19	ns	-0,26	ns
Rhodospirillaceae	-0,01	ns	0,01	ns	0,06	ns	0,03	ns	0,01	ns	-0,07	ns	0,12	ns	0,15	ns
Flavobacteriaceae	-0,18	ns	-0,20	ns	-0,22	ns	-0,21	ns	-0,21	ns	-0,08	ns	-0,23	ns	-0,19	ns
Planctomycetaceae	0,23	ns	0,26	ns	0,29	ns	0,27	ns	0,27	ns	0,19	ns	0,32	ns	0,29	ns
Micrococcaceae	0,23	ns	0,25	ns	0,29	ns	0,26	ns	0,25	ns	0,22	ns	0,31	ns	0,39	ns
Tepidisphaeraceae	0,06	ns	0,04	ns	-0,02	ns	0,04	ns	0,06	ns	0,14	ns	0,11	ns	-0,04	ns
Xanthomonadaceae	0,43	ns	0,40	ns	0,36	ns	0,39	ns	0,40	ns	0,38	ns	0,12	ns	0,17	ns
BIrii41	-0,30	ns	-0,29	ns	-0,23	ns	-0,28	ns	-0,30	ns	-0,42	ns	-0,36	ns	-0,02	ns
Oxalobacteraceae	0,13	ns	0,11	ns	0,07	ns	0,09	ns	0,10	ns	0,11	ns	0,01	ns	-0,06	ns
H16	-0,12	ns	-0,11	ns	-0,13	ns	-0,11	ns	-0,11	ns	0,15	ns	0,09	ns	0,06	ns
Hymenobacteraceae	0,44	ns	0,43	ns	0,41	ns	0,42	ns	0,42	ns	0,49	*	0,34	ns	0,31	ns
Opitutaceae	0,10	ns	0,08	ns	0,06	ns	0,07	ns	0,05	ns	0,07	ns	-0,18	ns	0,16	ns
Methylobacteriaceae	0,30	ns	0,32	ns	0,36	ns	0,33	ns	0,33	ns	0,24	ns	0,35	ns	0,41	ns
Methylophilaceae	-0,26	ns	-0,27	ns	-0,29	ns	-0,29	ns	-0,29	ns	-0,25	ns	-0,34	ns	-0,31	ns
Streptomycetaceae	0,17	ns	0,16	ns	0,16	ns	0,17	ns	0,16	ns	0,10	ns	0,01	ns	0,32	ns
Bryobacteraceae	0,03	ns	0,03	ns	0,02	ns	0,04	ns	0,05	ns	0,22	ns	0,29	ns	0,16	ns
Cellvibrionaceae	0,49	*	0,49	*	0,52	*	0,48	*	0,48	*	0,50	*	0,23	ns	0,37	ns
Haliangiaceae	-0,11	ns	-0,12	ns	-0,15	ns	-0,13	ns	-0,12	ns	0,01	ns	-0,06	ns	-0,09	ns
Rubrobacteriaceae	0,18	ns	0,20	ns	0,24	ns	0,22	ns	0,21	ns	0,16	ns	0,25	ns	0,33	ns
Hyphomicrobiaceae	0,30	ns	0,32	ns	0,38	ns	0,33	ns	0,31	ns	0,18	ns	0,14	ns	0,37	ns
Nocardioidaceae	0,27	ns	0,28	ns	0,33	ns	0,29	ns	0,29	ns	0,24	ns	0,22	ns	0,40	ns
Alcaligenaceae	0,79	***	0,78	***	0,75	***	0,77	***	0,79	***	0,84	**	0,69	**	0,61	**
Caulobacteraceae	0,50	*	0,48	*	0,44	ns	0,47	ns	0,48	*	0,44	ns	0,28	ns	0,25	ns

Chthoniobacteraceae	0,18	ns	0,16	ns	0,10	ns	0,15	ns	0,17	ns	0,36	ns	0,22	ns	0,13	ns
Micromonosporaceae	-0,14	ns	-0,13	ns	-0,08	ns	-0,11	ns	-0,11	ns	-0,26	ns	-0,12	ns	-0,04	ns
Geodermatophilaceae	0,33	ns	0,35	ns	0,41	ns	0,37	ns	0,35	ns	0,24	ns	0,36	ns	0,44	ns
Pseudonocardiaceae	-0,04	ns	-0,04	ns	-0,01	ns	-0,02	ns	-0,02	ns	-0,08	ns	0,05	ns	0,06	ns
env.OPS 17	-0,25	ns	-0,26	ns	-0,31	ns	-0,27	ns	-0,26	ns	-0,26	ns	-0,25	ns	-0,39	ns
Sphingobacteriaceae	0,86	***	0,84	***	0,82	***	0,84	***	0,84	***	0,76	***	0,59	*	0,63	**
Sandaracinaceae	0,05	ns	0,06	ns	0,05	ns	0,05	ns	0,04	ns	0,06	ns	0,16	ns	0,05	ns
Bacillaceae	-0,20	ns	-0,21	ns	-0,19	ns	-0,20	ns	-0,20	ns	-0,29	ns	-0,19	ns	-0,21	ns
Caldilineaceae	0,36	ns	0,38	ns	0,41	ns	0,39	ns	0,39	ns	0,37	ns	0,49	*	0,42	ns
Solirubrobacteraceae	0,33	ns	0,34	ns	0,39	ns	0,36	ns	0,35	ns	0,28	ns	0,34	ns	0,46	ns
Gaiellaceae	0,17	ns	0,18	ns	0,23	ns	0,20	ns	0,19	ns	0,17	ns	0,19	ns	0,36	ns
Pseudomonadaceae	0,24	ns	0,22	ns	0,20	ns	0,23	ns	0,24	ns	0,14	ns	0,22	ns	0,12	ns
Nitrospiraceae	0,25	ns	0,25	ns	0,24	ns	0,26	ns	0,27	ns	0,45	ns	0,43	ns	0,33	ns
Polyangiaceae	0,32	ns	0,34	ns	0,37	ns	0,35	ns	0,35	ns	0,35	ns	0,46	ns	0,33	ns
Bradyrhizobiaceae	0,31	ns	0,32	ns	0,36	ns	0,34	ns	0,32	ns	0,24	ns	0,27	ns	0,42	ns
Erysipelotrichaceae	-0,25	ns	-0,24	ns	-0,22	ns	-0,23	ns	-0,23	ns	-0,20	ns	-0,15	ns	-0,26	ns
Oceanospirillaceae	0,11	ns	0,12	ns	0,18	ns	0,12	ns	0,11	ns	0,04	ns	-0,08	ns	0,10	ns
Fimbriimonadaceae	-0,49	*	-0,51	*	-0,56	*	-0,52	*	-0,50	*	-0,39	ns	-0,42	ns	-0,57	*
Xanthobacteraceae	-0,27	ns	-0,26	ns	-0,22	ns	-0,24	ns	-0,25	ns	-0,34	ns	-0,11	ns	0,00	ns
0319-6G20	-0,25	ns	-0,23	ns	-0,24	ns	-0,24	ns	-0,25	ns	-0,15	ns	-0,09	ns	-0,20	ns
Erythrobacteraceae	-0,10	ns	-0,10	ns	-0,09	ns	-0,10	ns	-0,10	ns	-0,22	ns	-0,19	ns	-0,26	ns
Verrucomicrobiaceae	0,84	***	0,81	***	0,81	***	0,82	***	0,82	***	0,70	**	0,55	*	0,75	***
Roseiflexaceae	-0,01	ns	0,02	ns	0,07	ns	0,03	ns	0,02	ns	-0,07	ns	0,08	ns	0,05	ns
Longimicrobiaceae	-0,25	ns	-0,26	ns	-0,27	ns	-0,25	ns	-0,24	ns	-0,25	ns	-0,17	ns	-0,23	ns
Phyllobacteriaceae	0,66	**	0,67	**	0,70	**	0,68	**	0,68	**	0,57	*	0,58	*	0,67	**
Anaerolineaceae	-0,32	ns	-0,31	ns	-0,32	ns	-0,31	ns	-0,30	ns	-0,18	ns	-0,10	ns	-0,26	ns
Microbacteriaceae	0,41	ns	0,42	ns	0,47	*	0,43	ns	0,42	ns	0,32	ns	0,22	ns	0,53	*
Elev-16S-1332	0,16	ns	0,17	ns	0,21	ns	0,19	ns	0,18	ns	0,11	ns	0,18	ns	0,33	ns
PHOS-HE51	-0,16	ns	-0,18	ns	-0,24	ns	-0,20	ns	-0,18	ns	-0,03	ns	-0,19	ns	-0,27	ns
Bdellovibrionaceae	-0,40	ns	-0,41	ns	-0,44	ns	-0,42	ns	-0,41	ns	-0,40	ns	-0,30	ns	-0,43	ns
Solibacteraceae	-0,37	ns	-0,37	ns	-0,38	ns	-0,36	ns	-0,37	ns	-0,23	ns	-0,22	ns	-0,14	ns
Hyphomonadaceae	-0,71	***	-0,71	***	-0,69	**	-0,70	**	-0,71	***	-0,84	***	-0,70	**	-0,66	**
Geobacteraceae	0,15	ns	0,16	ns	0,17	ns	0,16	ns	0,14	ns	0,27	ns	0,29	ns	0,28	ns
Fibrobacteraceae	0,33	ns	0,28	ns	0,25	ns	0,28	ns	0,29	ns	0,20	ns	0,10	ns	0,12	ns
Ca. Entotheonella fam. incertae sedis	0,12	ns	0,14	ns	0,17	ns	0,15	ns	0,15	ns	0,16	ns	0,18	ns	0,24	ns
Crocinitomicaceae	-0,27	ns	-0,28	ns	-0,28	ns	-0,29	ns	-0,30	ns	-0,23	ns	-0,39	ns	-0,28	ns

288-2	0,31	ns	0,33	ns	0,39	ns	0,35	ns	0,34	ns	0,25	ns	0,36	ns	0,45	ns
NS11-12 marine group	-0,33	ns	-0,34	ns	-0,39	ns	-0,36	ns	-0,35	ns	-0,35	ns	-0,34	ns	-0,53	*
Mycobacteriaceae	0,13	ns	0,14	ns	0,20	ns	0,16	ns	0,14	ns	0,05	ns	0,12	ns	0,32	ns
OPB56	-0,32	ns	-0,34	ns	-0,38	ns	-0,35	ns	-0,34	ns	-0,23	ns	-0,27	ns	-0,37	ns
Glycomycetaceae	-0,17	ns	-0,17	ns	-0,16	ns	-0,16	ns	-0,16	ns	-0,25	ns	-0,14	ns	-0,02	ns
Coxiellaceae	-0,35	ns	-0,35	ns	-0,38	ns	-0,37	ns	-0,35	ns	-0,32	ns	-0,21	ns	-0,49	*
Rhodospirillales Incertae Sedis	-0,16	ns	-0,14	ns	-0,10	ns	-0,12	ns	-0,13	ns	-0,25	ns	0,04	ns	0,00	ns
Rhodocyclaceae	-0,34	ns	-0,33	ns	-0,36	ns	-0,34	ns	-0,34	ns	-0,22	ns	-0,08	ns	-0,26	ns
Rhizobiaceae	0,84	***	0,84	***	0,86	***	0,84	***	0,85	***	0,80	***	0,65	**	0,67	**
Phycisphaeraceae	-0,59	*	-0,60	**	-0,63	**	-0,60	**	-0,58	*	-0,59	**	-0,47	*	-0,69	**
0319-6M6	0,14	ns	0,15	ns	0,20	ns	0,17	ns	0,16	ns	0,10	ns	0,22	ns	0,33	ns
Promicromonosporaceae	-0,10	ns	-0,11	ns	-0,10	ns	-0,09	ns	-0,09	ns	-0,22	ns	-0,09	ns	-0,04	ns
G55	0,10	ns	0,12	ns	0,17	ns	0,13	ns	0,12	ns	0,10	ns	0,18	ns	0,30	ns
Paenibacillaceae	0,21	ns	0,23	ns	0,28	ns	0,24	ns	0,23	ns	0,18	ns	0,22	ns	0,32	ns
ABS-19	-0,32	ns	-0,33	ns	-0,35	ns	-0,33	ns	-0,31	ns	-0,25	ns	-0,29	ns	-0,27	ns
Rhodobacteraceae	0,67	**	0,69	**	0,73	***	0,71	**	0,69	**	0,63	**	0,70	**	0,71	**
Nannocystaceae	-0,25	ns	-0,23	ns	-0,16	ns	-0,23	ns	-0,26	ns	-0,35	ns	-0,34	ns	-0,01	ns
Lentimicrobiaceae	-0,20	ns	-0,18	ns	-0,14	ns	-0,18	ns	-0,19	ns	-0,23	ns	-0,06	ns	-0,01	ns
Haliscomenobacteraceae	-0,52	*	-0,54	*	-0,56	*	-0,55	*	-0,54	*	-0,51	*	-0,51	*	-0,51	*
Chromatiaceae	-0,10	ns	-0,10	ns	-0,10	ns	-0,09	ns	-0,09	ns	-0,14	ns	-0,10	ns	-0,22	ns
Burkholderiaceae	-0,46	ns	-0,45	ns	-0,45	ns	-0,46	ns	-0,46	ns	-0,60	**	-0,46	ns	-0,58	*
Brevibacteriaceae	0,75	***	0,75	***	0,78	***	0,75	***	0,75	***	0,75	***	0,53	*	0,60	**
Nocardiopsaceae	-0,17	ns	-0,17	ns	-0,16	ns	-0,16	ns	-0,15	ns	-0,25	ns	-0,06	ns	-0,11	ns
Phaselicystidaceae	0,06	ns	0,06	ns	0,06	ns	0,07	ns	0,07	ns	0,06	ns	0,07	ns	-0,01	ns
Clostridiaceae 1	0,25	ns	0,27	ns	0,30	ns	0,27	ns	0,25	ns	0,30	ns	0,37	ns	0,43	ns
OM1 clade	0,02	ns	0,05	ns	0,09	ns	0,06	ns	0,05	ns	0,00	ns	0,10	ns	0,06	ns
Archangiaceae	-0,19	ns	-0,18	ns	-0,16	ns	-0,18	ns	-0,19	ns	-0,08	ns	-0,09	ns	0,07	ns
Rhizobiales Incertae Sedis	-0,10	ns	-0,08	ns	-0,03	ns	-0,07	ns	-0,09	ns	-0,20	ns	-0,15	ns	0,00	ns
Iamiaceae	-0,04	ns	-0,03	ns	0,02	ns	-0,01	ns	-0,02	ns	-0,10	ns	0,02	ns	0,20	ns
Parviterribacteraceae	0,38	ns	0,40	ns	0,44	ns	0,42	ns	0,41	ns	0,38	ns	0,48	*	0,53	*
Acetobacteraceae	0,18	ns	0,19	ns	0,24	ns	0,21	ns	0,19	ns	0,14	ns	0,27	ns	0,32	ns
P3OB-42	-0,58	*	-0,58	*	-0,60	**	-0,59	*	-0,58	*	-0,49	*	-0,50	*	-0,53	*
MNG7	0,08	ns	0,10	ns	0,11	ns	0,10	ns	0,09	ns	0,03	ns	0,22	ns	0,16	ns

7| APPLICATION OF SEWAGE SLUDGE TO AGRICULTURAL SOIL INCREASES THE ABUNDANCE OF ANTIBIOTIC RESISTANCE GENES WITHOUT ALTERING THE COMPOSITION OF PROKARYOTIC COMMUNITIES

7. APPLICATION OF SEWAGE SLUDGE TO AGRICULTURAL SOIL INCREASES THE ABUNDANCE OF ANTIBIOTIC RESISTANCE GENES WITHOUT ALTERING THE COMPOSITION OF PROKARYOTIC COMMUNITIES

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Abstract

The application of sewage sludge as soil amendment is a common agricultural practice. However, wastewater treatment plants, sewage sludge and sewage sludge-amended soils have been reported as hotspots for the appearance and dissemination of antibiotic resistance, driven, among other factors, by selection pressure exerted by co-exposure to antibiotics and heavy metals. To address this threat to environmental and human health, soil samples from a long-term (24 years) field experiment, carried out to study the impact of thermally dried and anaerobically digested sewage sludge (at different doses and frequencies of application) on agricultural soil quality, were investigated for the presence of genes encoding antibiotic resistance (ARGs) and mobile genetic elements (MGEs). Sewage sludge-induced changes in specific soil physicochemical and microbial properties, as indicators of soil quality, were also investigated. The application of sewage sludge increased the total concentration of copper and zinc in amended soils, but without affecting the bioavailability of these metals, possibly due to the high values of soil pH and organic matter content. Soil microbal quality, as reflected by the value of the Soil Quality Index, was higher in sewage sludge-amended soils. Similarly, the application of sewage sludge increased soil microbial activity and biomass, as well as the abundance of ARGs and MGE genes, posing a risk of dissemination of antibiotic resistance. In contrast, the composition of soil prokaryotic communities was not significantly altered by the application of sewage sludge. We found correlation between soil Cu and Zn concentrations and the abundance of ARGs and MGE genes. It was concluded that sewage sludge-derived amendments must be properly treated and managed if they are to be applied to agricultural soil.

7.1. Introduction

The use of sewage sludge (SS) as fertilizer is a common agricultural practice aimed at supplying valuable nutrients (*e.g.*, nitrogen-N and phosphorus-P) and organic matter (OM) to agricultural soil, which, on the other hand, allows the reutilization of a by-product of wastewater treatment plants. The application of SS to agricultural soil has shown to enhance its physicochemical and biological properties (and, hence, soil quality), while providing plants with essential nutrients (Singh *et al*., 2011; Latare *et al*., 2014; Lloret *et al*., 2016). Soil microbial parameters which provide information on the activity, biomass and diversity of soil microbial communities are often used as biological indicators of the impact of disturbances (*e.g.*, agricultural practices, contamination) on soil quality (Epelde *et al*., 2010), due to their sensitivity, fast response and ecological relevance (Barrutia *et al*., 2011; Garbisu *et al*., 2011; Pardo *et al*., 2014).

Nevertheless, the application of SS to agricultural soil can pose a risk to the environment, as sewage sludge can contain a variety of toxic contaminants (Roig *et al*., 2012; Petrie *et al*., 2014). Thus, the European Commission implemented the EU Directive 86/278/EEC on sewage sludge, which establishes regulatory guidelines to prevent potential damages to the environment and human health, and settles limit values for the content of heavy metals (European Commission, 1986). There has been much concern about the potential risks for the environment and human health associated to the presence of heavy metals in sewage sludge used as agricultural amendment (Page *et al*., 1987; McBride *et al*., 1997; Wang *et al*., 2003). In particular, toxic heavy metals can negatively impact soil microbial communities and, in consequence, soil functionality (Epelde *et al*, 2010; Burges *et al*, 2015). Regrettably, in the past, much less attention has been paid to other contaminants present in sewage sludge. However, nowadays, previously overlooked contaminants and emerging contaminants are becoming a matter of special concern. In particular, the presence of pharmaceutically active compounds in sewage sludge is currently a topic of much interest and debate (Martín *et al*., 2015). Among pharmaceuticals, special attention has been paid to antibiotics, due to their huge medical relevance worldwide and to the fact that they are poorly metabolized in the human and animal body, resulting in their presence in relevant amounts in wastewaters (Looft *et al*., 2012; Michael *et al*., 2013). Since antibiotics are not eliminated during wastewater treatment, they can reach agricultural soil when sewage sludge is applied as fertilizer. Antibiotic degradation in soil is mainly driven by enzymatic transformations carried out

by microorganisms (McGrath *et al*., 1998). Microbial degradation of antibiotics depends on their bioavailability which, in some cases, is limited due to sorption to the soil matrix and influenced by abiotic factors such as soil pH and temperature. Hence, the assessment of antibiotic degradation rates (and, concomitantly, half-lives) in soil is a difficult task due to, among other factors, the well-known complexity and heterogeneity of the soil matrix (Thiele-Bruhn, 2003). In a review paper on the use of antibiotics in agriculture, Kumar *et al*. (2005) reported half-lives ranging from a few days to 300 days, depending on soil type and properties, as well as on the chemical properties of the antibiotics themselves. Pan and Chu (2016) conducted an experiment to determine the degradation rates of 5 different antibiotics (tetracycline, sulfamethazine, norfloxacin, erythromycin and chloramphenicol) in agricultural soil, and observed half-lives from 2.9 to 43.3 days in non-sterilized soil and from 40.8 to 86.6 days in sterilized soil. Nevertheless, half-lives in soil can be as long as years for some antibiotics (Boxall, 2008). In addition, it must be taken into consideration that their degradation products and secondary metabolites may still exert antimicrobial properties. Then, not surprisingly, wastewater treatment plants have been reported as hotspots for antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) (Pruden *et al*., 2013; Rizzo *et al*., 2013). The dissemination of antibiotic resistance is currently a matter of international concern, with potential devastating consequences for human health (Rizzo *et al*., 2013; Spellberg *et al*., 2013). Antibiotic resistance can be spread among bacteria through horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs), such as plasmids, integrons and transposons (Gaze *et al*., 2011; Jechalke *et al*., 2014).

Antibiotic resistance has frequently been associated with metal resistance (Baker-Austin *et al*., 2006). In fact, the molecular mechanisms underpinning resistance to both antibiotics and heavy metals are often similar (Baker-Austin *et al*., 2006), and largely refer to co-resistance (when different genes encoding for metal and antibiotic resistance are allocated in the same genetic determinant) or cross-resistance (when the same gene provides resistance to both antibiotics and metals) mechanisms (Chapman, 2003).

The aim of this study was to assess the long-term impact of sewage sludge application on: (i) physicochemical and microbial indicators of soil quality, (ii) soil microbial (prokaryotic and eukaryotic) composition, (iii) the abundance of ARGs, and (iv) the risk of their dissemination by HGT. To this aim, soils were collected from an experimental field where plots had been amended for 24 consecutive years with thermally dried and anaerobically digested SS at different rates and frequencies, providing a unique

opportunity to assess the long-term impact of SS application on soil quality, soil microbial communities and, in particular, the presence and abundance of ARGs and MGE genes. We hypothesized that the application of SS will improve soil physicochemical characteristics and stimulate soil microbial activity and biomass, while modifying the composition of microbial communities. In addition, we hypothesized that SS application will pose a risk for the dissemination of ARGs via HGT. Finally, we hypothesized that antibiotic resistance will correlate with total metal concentrations in soil.

7.2. Materials and methods

7.2.1. Site description and experimental design

The long-term (24 years) experimental field is located in the Chartered Community of Navarre, an autonomous community and province in northern Spain. The climate in this region is humid-temperate-Mediterranean (mean annual rainfall = 760 mm; mean temperature = 12.4ºC). This long-term experimental site is dedicated to the study of the impact of the application of SS, using 3-year crop rotations (cereal / cereal / non-cereal), on agricultural soil quality. Neither irrigation nor weed control are used. The experimental soil is a Calcaric Cambisol (FAO-UNESCO, 1997), with a clay-loamy texture, well drained and without salinity problems. Experimental plots (35 m^2; 6 replicates per treatment) are arranged following a factorial design, with combinations of two rates (40 and 80 t ha^{-1}) and three frequencies (every 1, 2 and 4 years) of sewage sludge (thermally dried and anaerobically digested) application, along with an unamended (unfertilized) control. Every year, after crop harvest, the same dehydrated sewage sludge, without any previous storage, has been applied to the land surface and then immediately incorporated to the soil by disc plowing to a depth of 30 cm. Treatments are summarized in Table 7.1.

Table 7.1. Treatment scheme. SS = thermally dried and anaerobically digested sewage sludge.

Treatment	Rate	Frequency	Total amount added (t SS ha^{-1})
40-1	40 t ha^{-1}	Every year	960
40-2	40 t ha^{-1}	Every 2 years	480
40-4	40 t ha^{-1}	Every 4 years	240
80-1	80 t ha^{-1}	Every year	1920
80-2	80 t ha^{-1}	Every 2 years	960
80-4	80 t ha^{-1}	Every 4 years	480
Control	Unamended	Unamended	0

7.2.2. Physicochemical characterization

Composite soil samples (*i.e.*, 6 cores randomly taken per plot; 0-30 cm soil depth) were collected from each plot. After collection, soil samples were immediately transferred to the laboratory in polyethylene bags protected from sunlight. Samples were air-dried at 30ºC and then sieved to <2 mm. Physicochemical analyses of sewage sludge and soil samples were performed according to standard methods (MAPA, 1994). Data are shown in Tables 7.2 and 7.3. Total concentrations of heavy metals were determined via inductively coupled plasma-optical emission spectrometry (ICP-OES). For the estimation of metal availability, (i) $CaCl_2$-extractable (0.01 M), (ii) $NaNO_3$-extractable (0.1 M), and (iii) low molecular weight organic acid (LMWOA) solution-extractable metal fractions in soil were determined as described by Houba *et al*. (2000), Gupta and Aten (1993) and Feng *et al*. (2005), respectively, and then quantified by ICP-OES. Extractable fractions were, in all cases, below the quantification limit (Table 7.S1).

7.2.3. Soil microbial properties

For the determination of soil microbial parameters, soil samples were sieved to <2 mm and stored fresh at 4ºC (not more than one month) until analysis. Samples for molecular analyses were stored at -20ºC. β-glucosidase, arylsulphatase and alkaline phosphatase enzyme activities were determined according to Dick *et al*. (1996) and Taylor *et al*. (2002). β-glucosaminidase and arginine deaminase enzyme activities were measured following Parham and Deng (2000) and Kandeler (1996), respectively. Potentially mineralizable nitrogen (PMN) was measured as described by Powers (1980). Soil respiration was determined following ISO 16072 Norm (2002). Finally, microbial biomass carbon (Cmic) was determined following Vance *et al*. (1987).

For the molecular analyses, DNA extraction was carried out from three aliquots, each of them corresponding to 0.25 g of dry weight (DW) soil from each sample, using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA). Prior to the extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk *et al*., 1997). The amount of DNA was quantified on a ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE). The abundance of 16S rRNA gene fragments for total bacteria and 18S rRNA gene fragments for total fungi was measured by qPCR, following the reaction mixtures, PCR conditions and primers described in Epelde *et al*. (2014).

In order to study the impact of SS application on soil microbial community structure and composition, amplicon libraries were made using a dual indexing approach with sequence-specific primers (Lanzén *et al*., 2016) targeting the V4 region of the 16S (prokaryotes) and 18S (eukaryotes) rRNA genes: 519F (CAGCMGCCGCGGTAA) adapted from Øvreås *et al*. (1997) and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso *et al*. (2012) for 16S rRNA amplification; and 1183F (AATTTGACTCAACRCGGG) and 1443R (GRGCATCACAGACCTG) (Hadziavdic *et al*., 2014; Ray *et al*., 2016) for 18S rRNA amplification. Sequencing was carried out with an Illumina MiSeq V2 platform and pair-ended 2x250 nt at Tecnalia, Spain. Read paired ends were merged, quality filtered (*i.e.*, primer trimming, removal of singletons and chimeric sequences) and clustered into operational taxonomic units (OTUs) as described by Lanzén *et al*. (2016). CREST was used for taxonomical assignments (Lanzén *et al*., 2012).

The presence and abundance of ARGs and MGE genes in soil samples was measured by high-throughput RT-qPCR, using the nanofluidic qPCR BioMark^{TM} HD system, with 48.48 and 96.96 Dynamic Array Integrated Fluidic Circuits (IFCs) (Fluidigm Corporation). A total of 96 validated primer sets (Hu *et al*., 2016) were used: 85 primer sets targeting ARGs, 10 primers sets targeting MGE genes (2 genes encoding integrases, 8 genes encoding transposases) and one reference gene (16S rRNA gene) (Table 7.S2). All samples were pre-amplified (Specific Target Amplication-STA reactions) with a pool of primers (50 nM final concentration for each primer pair; 16 PCR cycles) and, subsequently, treated with exonuclease I. 1:10 dilutions of STA reactions were loaded onto 48.48 or 96.96 Dynamic Array IFCs, following the Fluidigm's Fast Gene Expression Analysis Using EvaGreen Protocol. SsoFastTM EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, Redmond, WA) was used for amplification (with a final primer concentration, both forward and reverse, of 500 nM). The cycling program consisted of 1 min at 95ºC, followed by 30 cycles at 95ºC for 5 seconds and 60ºC for 20 seconds, followed by a melting curve. Four technical replicates were included for each sample. Measurements were conducted in the Gene Expression Unit of the Genomics Facility of SGIker – University of the Basque Country, Spain. Raw data were analyzed with the Fluidigm Real-Time PCR Analysis Software (v.3.1.3) with linear baseline correction and manual threshold settings, in order to obtain threshold cycle (Ct) values. After Ct correction with primer efficiencies, a detection limit Ct value of 27 was chosen since we obtained a highest Ct value of 26.91. The quantification of a specific gene (ARG

or MGE gene) was considered positive when three out of the four technical replicates were above the detection limit. The relative abundance of each gene was expressed as fold change (FC) between the target gene (ARG, MGE gene) and the matched reference 16S rRNA gene, relative to the mean value obtained in unamended control soils for every amended and unamended soil sample, following Livak and Schmittgen (2001):

$$\Delta C_T = C_{T\ (\text{target gene})} - C_{T\ (16S\ \text{rRNA gene})}$$

$$\Delta\Delta CT = \Delta C_{T\ (\text{amended soil})} - \Delta C_{T\ (\text{unamended control mean value})}$$

$$FC = 2^{-\Delta\Delta C_T}$$

where C_T is the q-PCR threshold cycle.

7.2.4. Statistical analysis

We evaluated the impact of SS application on agricultural soil properties, compared to the unamended control soil. Besides, among the different SS-amended soils, we compared the impact and interaction of the rate and frequency of SS application: *Rate* was a fixed factor with two levels: (i) application of 40 t ha^{-1} and (ii) application of 80 t ha^{-1}; *Frequency* was a fixed factor with three levels: (i) every year, (ii) every 2 years and (iii) every 4 years. Statistical significance of SS-amended *vs.* unamended soils for heavy metal concentrations, physicochemical and microbial parameters, and FC values (for ARGs and MGE genes) was determined by pooled variances *t*-test (for equal variances) or Welch's *t*-test (for unequal variances). Differences among experimental factors and their interaction were tested by means of two-way ANOVA and Duncan's multiple range test (when interaction effect was significant) using package *agricolae* of R software (version 3.3.2).

For the assessment of the impact of SS application on soil microbial quality, compared to the unamended control soil, values of all the soil microbial parameters determined here (*i.e.*, microbial indicators of soil quality) were used for the calculation of the Soil Quality Index (SQI) described by Mijangos *et al*. (2010):

$$SQI = 10^{\log m + \frac{\sum_{i=1}^{n}(\log n_i - \log m)}{n}}$$

where *m* is the reference value (set to 100% for the mean value of each parameter in unamended control soil) and *n* corresponds to the measured values for each parameter as percentage of the reference value. The microbial parameters considered for this calculation were: enzyme activities (β-glucosidase, β-glucosaminidase, arylsulphatase, alkaline phosphatase and arginine deaminase), potentially mineralizable N (PMN), soil respiration, microbial biomass carbon (Cmic), bacterial and fungal gene abundance, and α-diversity (RR: rarefied richness, H': Shannon´s index, J': Pielou´s evenness) from 16S and 18S rRNA amplicon sequencing data.

Determination of α-diversity indices, multivariate statistics and visualization of 16S and 18S rRNA amplicon sequencing data were performed with R package *vegan* (Oksanen *et al*., 2013). For both genes, *decostand* function was used to transform OTU distributions into relative abundances. Subsequent calculations of Bray-Curtis dissimilarity matrices for comparisons on OTU community composition were performed as described by Lanzén *et al*. (2016). Matrices were further used to perform non-metric multidimensional scaling (NMDS) with function *metaMDS*. The impact of SS application on soil microbial (prokaryotic and eukaryotic) community composition was assessed with permutational analyses of variance (PERMANOVA), using function *adonis*. Kendall's tau correlations were performed between: (i) microbial taxa at each taxonomic level (*i.e.*, phylum, class, order, family, genus) and the total amount of SS applied to the soil after 24 years; and (ii) total metal concentrations in soil and the abundances of those ARGs and MGE genes significantly enriched in SS-amended soils *vs*. unamended control soil.

In order to assess the significance of the effect of the experimental factors (application of SS at different rates *vs.* no application), as explanatory variables, on the variation in ARG and MGE gene abundance profiles, redundancy analysis (RDA) were performed using Canoco 5 software.

7.3. Results

7.3.1. Soil physicochemical parameters

The application of SS resulted in a very slight reduction of soil pH, compared to unamended control soil (Table 7.2). Values of OM, Olsen P, total K, total N and mineral N were, in general, higher in SS-amended soils; however, differences between SS-

amended *vs*. unamended soil were statistically significant only for OM and Olsen P. Not many statistical differences were detected among SS-amended soils; in any case, soils amended with the highest amount of SS (treatment 80-1 = 80 t SS ha^{-1}, every year) exhibited significantly higher values of mineral N (Table 7.2).

Table 7.2. Impact of sewage sludge (SS) application on soil physicochemical properties. C: unamended control; SS-C: SS-amended *vs*. unamended control, differences based on pooled variances *t*-test or Welch´s *t*-test: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. R: application rate; F: application frequency; RxF: interaction among factors. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT. Mean values (n = 6) ± SD.

	pH 1:2.5	OM (%)	Olsen P (mg kg^{-1})	K^+ (mg kg^{-1})	N total (%)	N mineral (mg $N\text{-}NO_3^-+NH_4^+$ kg^{-1})
40-1	8.5±0.1^{a}	5.0±0.2	268.1±18.2^{ab}	155.7±28.7^{ab}	0.21±0.04	7.7±3.6^{b}
40-2	8.5±0.1^{a}	5.0±0.3	263.7±30.8^{ab}	185.5±37.3^{a}	0.20±0.02	3.1±2.1^{b}
40-4	8.5±0.1^{a}	5.1±0.4	265.3±36.2^{ab}	174.8±26.7^{ab}	0.21±.02	7.4±5.4^{b}
80-1	8.4±0.1^{b}	5.3±0.3	309.1±40.0^{a}	178.5±26.7^{ab}	0.25±0.05	21.0±8.7^{a}
80-2	8.5±0.1^{a}	5.0±0.4	261.0±60.4^{ab}	146.5±17.5^{b}	0.21±0.04	9.4±7.3^{b}
80-4	8.6±0.0^{a}	4.8±0.1	221.±21.3.4^{b}	165.5±19.3^{ab}	0.19±0.04	3.6±2.8^{b}
C	8.6±0.1	4.7±0.1	73.9±14.0	146.3±19.8	0.18±0.03	3.4 ±2.4
SS-C	**	*	***	ns	ns	ns
R	ns	ns	ns	ns	ns	**
F	**	ns	*	ns	ns	***
RxF	**	ns	*	*	ns	**

Regarding heavy metal concentrations, SS-amended soils showed significantly higher values of total Cu and Zn, compared to unamended control soil (Table 7.3). No significant differences were observed between SS-amended *vs.* unamended soil for the other heavy metals tested here (*i.e.*, Cd, Pb, Cr, Ni and As). Among the different SS-amended soils, Zn was the only metal showing significant differences between treatments: in fact, higher Zn values were observed in soils amended with the highest amount of SS (treatment 80-1), compared to all the other amended soils. Metal concentrations in the thermally dried and anaerobically digested sewage sludge used in this study did not exceed the limit values established by the European Commission. As abovementioned, values of extractable metals (0.01 M $CaCl_2$-extractable, 0.1 M $NaNO_3$-extractable and LMWOA-extractable) were below the quantification limit (Table 7.S1).

Table 7.3. Impact of sewage sludge (SS) application on total concentrations of heavy metals in soil. C: unamended control; SS-C: SS-amended *vs.* unamended control, differences based on pooled variances *t*-test or Welch´s *t*-test: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. R: application rate; F: application frequency; RxF: interaction among factors. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT. Mean values (n = 6) ± SD. 86/278/EEC: limit values established by the European Commission (mg kg^{-1} SS).

	Cu mg kg^{-1}	**Zn mg kg^{-1}**	**Cd mg kg^{-1}**	**Pb mg kg^{-1}**	**Cr mg kg^{-1}**	**Ni mg kg^{-1}**	**As mg kg^{-1}**
40-1	30.9±5.4	104.4±5.8^{bc}	1.2±0.1	27.5±2.6	27.6±3.1	31.3±1.9	11.7±1.8
40-2	31.7±4.2	103.8±6.4^{bc}	1.1±0.2	27.8±2.6	31.5±15.6	31.5±2.1	12.5±2.2
40-4	31.4±4.2	107.4±6.3^{b}	1.2±0.1	28.3±2.7	32.4±14.1	31.3±1.9	11.9±1.6
80-1	35.2±3.7	118.4±3.4^{a}	1.2±0.2	29.3±2.8	40.4±20.7	31.8±1.6	12.1±1.3
80-2	28.4±5.9	97.8±12.7^{c}	1.0±0.1	25.8±2.0	29.3±15.3	30.4±1.3	11.3±1.9
80-4	31.1±3.8	97.1±6.2^{c}	1.2±0.2	27.6±3.8	35.7±18.0	31.2±2.3	12.8±1.8
C	26.1±5.0	88.2±4.7	1.1±0.2	25.7±4.4	29.5±15.2	30.6±2.0	12.1±1.0
Sewage sludge	196	936	<3.0	47	73	40	-
SS-C	*	***	ns	ns	ns	ns	ns
R	ns	ns	ns	ns	ns	ns	ns
F	ns	**	ns	ns	ns	ns	ns
RxF	ns	***	ns	ns	ns	ns	ns
86/278/EEC	*1000-1750*	*2500-4000*	*20-40*	*750-1200*	*1000-1500*	*300-400*	-

7.3.2. Soil microbial parameters

In relation to soil microbial activity, all measured parameters showed significantly higher values in SS-amended soils, compared to unamended control soil, with the exception of arginine deaminase activity (in this case, the difference was not statistically significant) (Table 7.4). Among the different SS-amended soils, values of both β-glucosidase and β-glucosaminidase activity were significantly higher in soils amended with the highest amount of SS (treatment 80-1).

Regarding soil microbial biomass, values of total bacteria (number of 16S rRNA gene copies) were significantly higher in SS-amended *vs.* unamended soil (Table 7.4). Furthermore, 18S rRNA gene copy abundance (total fungi) was, in general, higher (14% higher) in SS-amended soils, compared to unamended control soil, but differences were not statistically significant. The experimental factors (*i.e.*, rate and frequency of application) did not lead to significant differences in microbial biomass among SS-amended soils (Table 7.4).

Concerning microbial diversity, after singleton removal and quality filtering, amplicon sequencing generated 3,718,701 prokaryotic (16S rRNA) reads which were then clustered into 9,414 OTUs, and 5,070,574 eukaryotic (18S rRNA) reads clustered into 3,247 OTUs. The number of reads correlated significantly with total OTU richness for both 16S and 18S data ($p<0.001$, for both), indicating that our sequencing effort was insufficient to obtain a full coverage of soil microbial diversity. In consequence, rarefied richness estimates were used to measure α-diversity. Alpha-diversity parameters (RR: rarefied richness, H': Shannon's index, and J': Pielou's eveness) are shown in Table 7.5. All these parameters showed lower values in SS-amended soils, compared to unamended control soil, but in most cases differences were not statistically significant. In fact, statistical differences were only observed for prokaryotic rarefied richness, where unamended control soil yielded significantly higher values.

Table 7.4. Impact of sewage sludge (SS) application on soil microbial activity and biomass. C: unamended control; SS-C: SS-amended *vs.* unamended control, differences based on pooled variances *t*-test or Welch´s *t*-test: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. R: application rate; F: application frequency; RxF: interaction among factors. Letters show significant interaction among factors based on two-way ANOVA and Duncan's MRT. Mean values (n = 6) ± SD. PMN: potentially mineralizable nitrogen; Cmic: microbial biomass carbon.

	Activity							**Biomass**		
	Phosphatase mg NP kg^{-1} DW h^{-1}	**Arilsulphatase** mg NP kg^{-1} DW h^{-1}	**β-Glucosidase** mg NP kg^{-1} DW h^{-1}	**β-Glucosaminidase** mg NP kg^{-1} DW h^{-1}	**Arginine deaminase** mg N-NH_4^+ kg^{-1} DW	**Respiration** mg C kg^{-1} DW h^{-1}	**PMN** mg N-NH_4^+ kg^{-1} DW	**Cmic** mg C kg^{-1} DW	**Bacteria** x 10^{10}copies g^{-1} DW	**Fungi** x 10^{9}copies g^{-1} DW
40-1	375.4±12.2	120.0±8.8	208.2±12.7^{c}	33.5±5.3^{b}	10.1±1.2	3.0±0.4	18.8±3.2	575.6±64.4	3.97±0.35	0.97±0.23
40-2	377.2±10.6	109.5±18.8	217.8±21.4^{bc}	37.7±4.5^{ab}	9.8±0.6	3.1±0.5	17.7±2.6	576.8±54.9	4.17±1.23	1.12±0.14
40-4	374.5±13.4	116.2±8.9	232.8±27.3^{ab}	38.8±6.4^{ab}	10.2±0.8	3.2±0.4	19.8±2.7	625.4±23.7	4.18±0.65	1.09±0.36
80-1	391.8±9.2	117.6±5.1	244.2±14.3^{a}	43.4±2.2^{a}	10.3±0.8	3.2±0.6	20.8±4.4	617.1±54.4	4.97±1.07	1.17±0.36
80-2	371.1±18.8	116.3±9.3	218.7±21.5^{bc}	37.8±7.8^{ab}	10.1±0.6	2.9±0.5	17.6±3.4	634.2±51.6	4.38±0.67	1.07±0.28
80-4	385.9±19.6	119.8±9.7	210.7±7.1^{bc}	34.3±2.0^{b}	10.5±0.6	2.8±0.3	19.1±0.9	613.0±84.9	3.80±0.68	0.90±0.26
C	311.5±19.0	104.0±6.5	170.0±12.6	26.2±3.5	9.5±0.8	2.2±0.2	12.2±1.6	611.9±89.3	3.52±0.35	0.92±0.35
SS-C	***	**	***	***	ns	***	***	ns	*	ns
R	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
F	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
RxF	ns	ns	**	**	ns	ns	ns	ns	ns	ns

Table 7.5. Impact of sewage sludge (SS) application on soil prokaryotic (16S rRNA) and eukaryotic (18S rRNA) α-diversity. C: unamended control; SS-C: SS-amended *vs.* unamended control, differences based on pooled variances *t*-test or Welch´s *t*-test: *: p<0.05; **: p<0.01; ***: p<0.001. R: application rate; F: application frequency; RxF: interaction among factors. Interaction among factors is tested by two-way ANOVA. Mean values (n = 6) ± SD. RR: rarefied richness; H': Shannon´s index; J': Pielou´s index.

	16S rRNA			18S rRNA		
	RR	**H'**	**J'**	**RR**	**H'**	**J'**
40-1	3683±368	6.80±0.33	0.81±0.04	1294±46	4.28±0.18	0.59±0.02
40-2	3658±88	6.83±0.10	0.82±0.01	1288±46	4.20±0.08	0.58±0.01
40-4	3647±134	6.83±0.10	0.82±0.01	1310±31	4.28±0.15	0.59±0.02
80-1	3639±130	6.81±0.17	0.81±0.02	1312±20	4.25±0.06	0.59±0.01
80-2	3669±59	6.79±0.08	0.81±0.01	1288±53	4.29±0.16	0.59±0.02
80-4	3713±138	6.87±0.12	0.82±0.01	1348±39	4.39±0.09	0.60±0.01
C	3832±151	6.86±0.10	0.82±0.01	1321±61	4.34±0.29	0.60±0.04
SS-C	*	ns	ns	ns	ns	ns
F	ns	ns	ns	ns	ns	ns
R	ns	ns	ns	ns	ns	ns
FxR	ns	ns	ns	ns	ns	ns

Finally, values of the SQI, an integrative indicator of all studied microbial parameters, were significantly (p<0.001) higher in SS-amended soils, compared to unamended control soil: SQI = 112.5 and 99.4 for SS-amended and unamended soils, respectively.

Regarding prokaryotic community composition, the NMDS (based on Bray-Curtis dissimilarities in terms of OTU composition) did not separate SS-amended and unamended soils (Fig. 7.1A). Besides, the PERMANOVA analysis, carried out to study the effect of SS application on prokaryotic community composition, was not significant (*i.e.*, no significant difference between SS-amended and unamended control soil, in terms of prokaryotic community composition). By contrast, the ordination analysis for eukaryotic OTU composition did separate SS-amended and unamended control soil (Fig. 7.1B), indicating an effect of SS application on soil eukaryotic community composition. This was further confirmed by the PERMANOVA analysis, carried out to study the effect of SS application on eukaryotic community composition (eukaryotic OTU composition in SS-amended *vs.* unamended control soil), which was significant for both studied factors: presence of amendment (F = 2.705, p = 0.001) and total amount of SS added after 24 years (F = 3.65, p = 0.001).

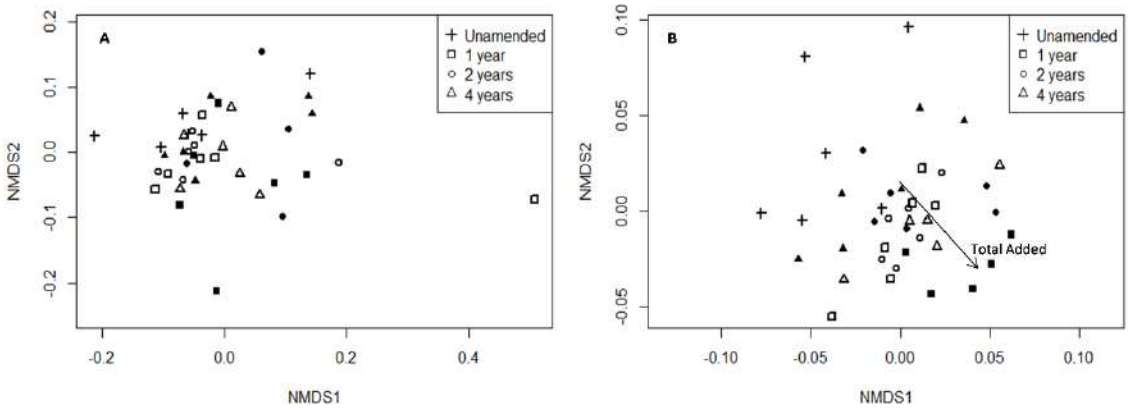


Figure 7.1. Non-metric multidimensional scaling (NMDS) analysis representing patterns of soil (A) prokaryotic and (B) eukaryotic OTU composition. Bray-Curtis dissimilarities of community composition, based on relative OTU abundances from 16S and 18S rRNA amplicons, are represented as distance in the diagram. Samples are labelled according to legend. Empty figures: 40 t of SS ha^{-1}; filled figures: 80 t SS ha^{-1}; Added: total amount of SS applied after 24 years.

Then, 83.3% of the 16S rRNA reads were taxonomically classified to order rank (in comparison, only 54% of the 18S rRNA reads could be classified to order rank; in turn, 87% of the 18S rRNA reads were classified to class rank). In terms of evenness, no clear differences were observed between the studied soils for the 30 more abundant prokaryotic orders (Fig. 7.S1 and Table 7.S3). The same result was observed for the 9 more dominant fungal classes (Fig. 7.S2), together constituting >95% of the fungal abundance. Nevertheless, some rare (less abundant) prokaryotic and eukaryotic taxa abundances displayed significant correlations with the total amount of SS applied after 24 years of study (Table 7.6): within the prokaryotic taxa, the abundance of 8 bacterial genera (*Perlucidibaca, Lysobacter, Arenimonas, Flavihumibacter, Parasegetibacter, Clostridium, Aeromonas*, *Gordonia*) was significantly enriched at higher levels of SS application; similarly, the abundance of two eukaryotic families (*Eccrinaceae, Myxochloridaceae*) and one genus (*Prototheca*) was higher at higher levels of SS application. Nevertheless, some eukaryotic taxa correlated negatively with the total amount of SS applied after 24 years: the abundance of the class *Xanthophyceae*, the order *Chaetothyriales* and the family *Herpotrichiellaceae* was lower at higher levels of SS application.

Table 7.6. Prokaryotic and eukaryotic taxa which significatively correlated with the total amount of sewage sludge applied after 24 years. Taxa correlating negatively are displayed in italics.

Prokaryotic	**Rank**	**p**	**tau**
Clostridia	Class	2.0E-02	0.41
Alteromonadales	Order	1.4E-04	0.55
PYR10d3 Gammaproteobacteria	Order	1.0E-02	0.49
Aeromonadales	Order	2.0E-02	0.43
Clostridiales	Order	2.9E-02	0.42
Oceanospirillales	Order	4.9E-02	0.40
Alteromonadaceae	Family	1.7E-04	0.55
oc58 Oceanospirillales	Family	6.8E-04	0.53
Peptostreptococcaceae (Clostridiales)	Family	2.8E-03	0.49
Aeromonadaceae	Family	3.1E-02	0.43
Clostridiaceae	Family	3.5E-02	0.43
Moraxellaceae	Family	3.9E-02	0.42
Xanthomonadaceae	Family	4.4E-02	0.42
Perlucidibaca	Genus	1.7E-04	0.56
Lysobacter	Genus	1.6E-02	0.45
Arenimonas	Genus	2.7E-02	0.44
Flavihumibacter	Genus	2.9E-02	0.44
Parasegetibacter	Genus	4.0E-02	0.43
Clostridium (Clostridiaceae)	Genus	4.4E-02	0.43
Aeromonas	Genus	4.5E-02	0.43
Gordonia	Genus	4.9E-02	0.43

Eukaryotic	**Rank**	**p**	**tau**
Eccrinales	Phylum	2.8E-03	0.46
Amoebozoa	Phylum	4.0E-02	0.38
Tubulinea	Class	1.3E-02	0.43
Xanthophyceae	*Phylum*	*2.5E-02*	*-0.40*
Chaetothyriales	*Order*	*1.2E-02*	*-0.45*
Microascales	Order	2.9E-05	0.59
Monhysterida	Order	2.0E-02	0.44
Eccrinaceae	Family	6.6E-03	0.47
Herpotrichiellaceae	*Family*	*1.1E-02*	*-0.46*
Myxochloridaceae	Family	3.1E-02	0.43
Prototheca	Genus	3.1E-02	0.45

Table 7.7. Fold change (FC) values of MGE genes and ARGs that showed significant differences between SS-amended and unamended control soil, based on pooled variances *t*-test or Welch´s *t*-test: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. Mean values ± SD.

	Gene	SS-amended	Unamended	p
MGE	tnpA-07	15.1 ± 9.3	4.0 ± 2.1	*
ARG	aac(6')-Ib-01	6.0 ± 5.4	2.1 ± 3.0	*
	aadE	2.3 ± 0.9	1.1 ± 0.5	**
	tet(32)	3.3 ± 1.9	1.0 ± 0.3	***
	tetPA	3.1 ± 1.5	1.1 ± 0.4	**
	tetT	8.7 ± 9.1	1.0 ± 0.0	***
	vanB-01	3.0 ± 1.7	1.1 ± 0.4	*

In relation to ARGs and MGE genes, out of the 95 studied genes, 86 were amplified in the studied soil samples (SS-amended and unamended control soil), while 74 genes were amplified in the sewage sludge itself (*i.e.*, in the thermally dried and anaerobically digested SS used here). The redundancy analysis (Fig. 7.S3), which explained 97% of the total variation, showed a significant (pseudo-F = 143, p = 0.002) separation between soil samples and SS amendment, based on the abundance values of ARGs and MGE genes. The 25 genes that better explained such separation showed higher abundance values in the SS amendment (Fig. 7.S3). In SS-treated soils, 7 genes showed a significantly higher abundance, compared to unamended control soil (Table 7.7): one of these genes encodes for a MGE (a transposase, tnpA-07), while the other 6 encode ARGs conferring resistance to aminoglycosides [aac(6´)-Ib-01, aadE], tetracyclines [tet(32), tetPA, tetT] and vancomycin [vanB-01]. The treatment with the lowest rate and frequency of SS application (treatment 40-4 = 40 t ha^{-1}, every 4 years) showed a significantly higher abundance of one MGE gene (tnpA-07, encoding a transposase) and 3 ARGs (aadE, aminoglycoside resistance gene; tetPA, tetracycline resistance gene; vanB-01, vancomycin resistance gene), compared to the unamended control soil. The RDA based on gene abundances (as response variables) displayed a significant (pseudo-F = 143, p = 0.002) separation of SS-amended and unamended soil samples (Fig. 7.2), indicating that ARG and MGE profiles were significantly affected by the addition of SS.

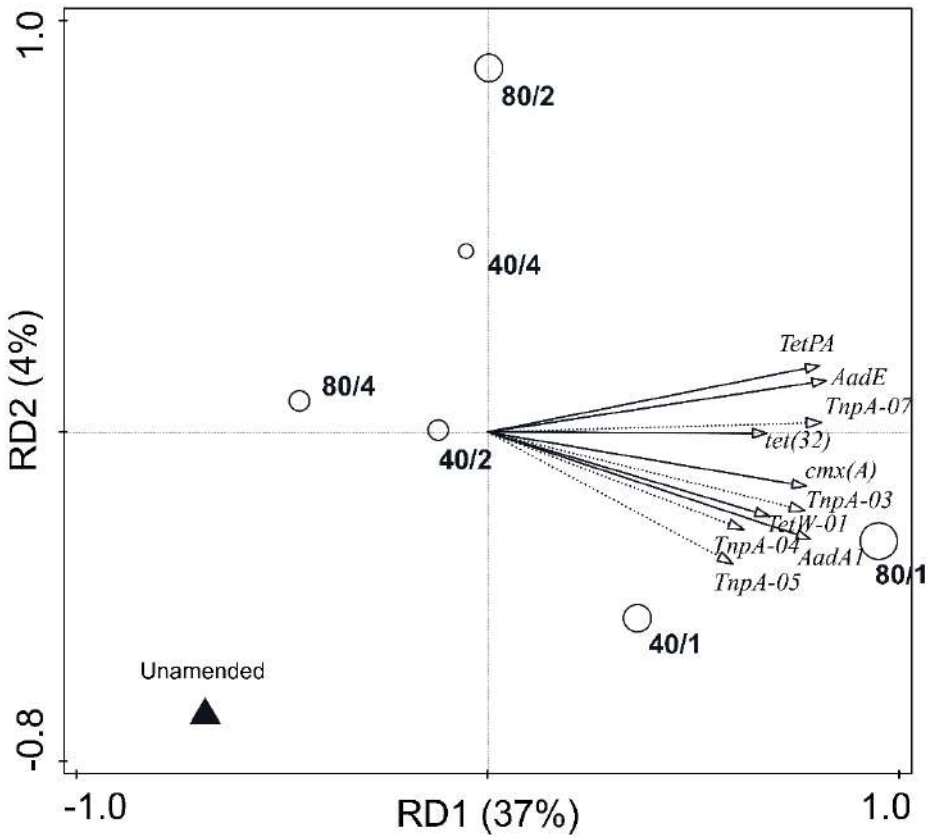


Figure 7.2. Redundancy analysis displaying (as response variables) the ARGs and MGE genes with the best fit for explaining the differences observed between SS-amended and unamended control soil. First and second axes account for the 37 and 4% of the explained variation, respectively. Size of circles represents the total amount of SS applied after 24 years. Solid lines represent ARGs; Dotted lines represent MGE genes.

Finally, interestingly, we found correlation between soil Cu and Zn total concentration and the abundance of ARGs and MGE genes (Table 7.8). In fact, Zn total concentration in soil correlated with the abundance of 5 out of the 7 genes that showed a higher abundance in SS-amended *vs*. unamended control soil.

Table 7.8. Kendall's tau correlations between total metal concentrations in soil and the abundance of those ARGs and MGE genes that showed a higher abundance in SS-amended *vs*. unamended control soil. ns: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

	MGE	**ARG**					
	tnpA-07	**aac(6')-Ib-01**	**aadE**	**tet(32)**	**tetPA**	**tetT**	**vanB-01**
Cu	*	ns	*	ns	*	ns	ns
Zn	***	ns	***	*	***	*	ns
Cd	**	ns	*	ns	*	ns	ns
Pb	*	ns	*	ns	ns	ns	ns
Cr	ns	ns	ns	ns	ns	ns	ns
Ni	*	ns	*	ns	ns	ns	ns
As	ns	ns	ns	ns	ns	ns	ns

7.4. Discussion

7.4.1. Soil physicochemical parameters

The application of SS to agricultural soil is a common practice worldwide, given its potential agronomic benefits (derived from its capacity to supply valuable nutrients and OM to the soil) (Gaskin *et al*., 2003, Mantovi *et al*., 2005; Singh *et al*., 2011) and environmental gains derived from (i) the concomitant reduction in the need for energetically-expensive synthetic fertilizers and (ii) the reutilization of a by-product. Enhanced values of soil OM have been widely reported after SS application (Wang *et al*., 2008; Lloret *et al*., 2016). Here, the long-term application of SS led to significantly higher values of OM in SS-amended soils, compared to unamended control soil. Besides, the long-term application of SS resulted in a slight, but statistically significant, reduction in soil pH, which could be attributed to nitrification of the ammonium contained in sewage sludge (Cytryn *et al*., 2012) and/or to the production of organic acids during OM decomposition (Angin *et al*., 2012).

On the other hand, increased values of essential nutrients (*e.g.*, N and P) are commonly reported after the application of SS to agricultural soil (Parat *et al*., 2005; Wang *et al*., 2008; Latare *et al*., 2014). In this study, the application of SS resulted in significantly higher values of Olsen P. Given its tendency to attach to the solid fraction during wastewater treatment processes, high quantities of P are frequently found in SS (Alvarenga *et al*., 2015). In contrast, no significant differences were observed between SS-amended and unamended control soil for the content of K^+, total N and mineral N. Sewage sludge is known to often contain high quantities of organic N which can then be mineralized by soil microorganisms following its application to agricultural soil (Parat *et al*., 2005). In our study, soil samples were taken after crop harvest, and then it is possible that a great part of the soil inorganic N, resulting from OM mineralization, might have been taken up and assimilated by the plants. Nevertheless, the treatment with the highest rate and frequency of SS application (treatment 80-1 = 80 t ha^{-1}, every year) showed significantly greater mineral N values in soil than all the other treatments (Table 7.2), which could be due to a high rate of N mineralization under those conditions, resulting in values of soil mineral N higher than those required by the plants. While for K^+, this nutrient can form soluble compounds in the liquid fraction during wastewater treatment processes (Alvarenga *et al*., 2015), thus limiting its presence in SS.

Nonetheless, the application of SS to agricultural soil entails a variety of environmental risks, derived from the presence of toxic contaminants (*e.g.*, heavy metals, organic contaminants) in SS-derived amendments (Mattana *et al*., 2014). The problem of metal contamination associated to the use of SS as agricultural fertilizer has been widely reported (Gaskin *et al*., 2003; Antolín *et al*., 2005; Mantovi *et al*., 2005; Lloret *et al*., 2016). Here, after 24 years of SS application, amended soils exhibited significantly higher concentrations of Cu and Zn. Total metal concentrations in the thermally dried and anaerobically digested SS were much lower than the limit values established by the EU Directive 86/278/EEC (European Commission, 1986) (Table 7.3). Nevertheless, regarding contaminant ecotoxicity, metal bioavailable concentrations are more environmentally relevant than total metal concentrations (Alkorta *et al*., 2006). In this sense, all extractable metal fractions measured here to estimate metal bioavailability were below the quantification limit, probably due to the high values of OM content and, above all, soil pH. The presence of OM can reduce soil metal bioavailability due to the formation of metallo-organic complexes and macro-aggregates that are chemically stable (Mohapatra *et al*., 2016). On the other hand, a high pH is known to reduce the bioavailability of most metals in soil (Soler-Rovira *et al*., 2010). In our study, SS application led to a slight, but statistically significant, decrease in soil pH, which could *a priori* lead to higher metal bioavailability; nevertheless, the high soil pH of our study site (around 8.5) severely restricts metal bioavailability. In any case, the higher total concentrations of Cu and Zn found in SS-amended soils must be considered a potential risk in the long-term, since changes in environmental conditions could, in the future, lead to an increase in metal mobility, solubility and bioavailability (McBride *et al*., 1995; Parat *et al*., 2007).

7.4.2. Soil microbial parameters

Soil microorganisms play an essential role in important soil processes (Bastida *et al*., 2008; Fernández *et al*., 2009). Owing to their sensitivity, rapid response, integrative character and ecological relevance, microbial parameters have acquired much importance for the assessment of the impact of disturbances, including agricultural practices and contamination, on soil functioning (Mijangos *et al*., 2006; Muñoz-Leoz *et al*., 2012; Burges *et al*., 2015). In this respect, the application of organic amendments has frequently been associated with an increase in soil microbial activity and biomass (Roig *et al*., 2012;

Reardon *et al*., 2016). Here, SS application resulted in a stimulation of soil microbial activity, as reflected by the higher values of soil enzyme activities. This is probably due to the supply of OM and, concomitantly, substrates susceptible for enzymatic hydrolysis (Singh *et al*., 2011). This increase in soil microbial activity after SS application has been broadly reported (Kizilkaya and Bayrakli, 2005; Carbonell *et al*., 2009; Roig *et al*., 2012; Mattana *et al*., 2014).

Regarding soil microbial biomass, the application of SS led to a small but significant increase in total bacteria, which may be explained by the increase in available C sources in the absence of N limitation (the C:N ratio of the SS was 5.5). Antolín *et al*. (2005) and Xue and Huang (2013) also found an enhanced soil microbial biomass following SS application.

No significant variations were observed among the different SS-amended soils (regardless of the rate and frequency of application) in terms of soil microbial activity and biomass. In fact, the only significant difference observed among SS-amended soils corresponded to β-glucosidase and β-glucosaminidase enzyme activities, whose values were significantly higher in soils amended with the highest amount of SS (Treatment 80-1). These enzyme activities are linked to C-cycle and higher values are probably explained by the higher amount of OM applied to those soils.

However, in disagreement with our results, the presence of heavy metals in SS sludge has been reported to reduce soil microbial activity (*i.e.*, by inhibiting enzyme activity) (Paz-Ferreiro *et al*., 2012; Roig *et al*., 2012) and biomass (Fernandez *et al*., 2009). In contrast, 6 years after its last application, Parat *et al*. (2005) reported higher microbial biomass values in SS-amended soil in spite of the high concentration of heavy metals. These contradictory results may possibly be due to the complex nature of the interactions between microorganisms, heavy metals, sewage sludge and the soil matrix. Roig *et al*. (2012) indicated that SS stability may have a stronger influence on its ecotoxicity than the potentially toxic elements present in it. In any event, the increased values of total Cu and Zn in soil, as a consequence of SS application, did not seem to significantly affect the status of soil microorganisms, as reflected by the values of the different microbial indicators of soil quality determined here. In fact, the application of SS resulted in higher values of the SQI, compared to unamended control soil.

Concerning microbial diversity, in general, soil microbial diversity was not significantly affected by SS application (Table 7.5). However, SS-amended soils exhibited a small but significantly lower rarefied richness of prokaryotes, compared to

unamended control soil. This effect has also been reported by other authors (Lloret *et al*., 2016), and may be linked to a selection filter provided by the introduction of a considerable amount of specific substrates provided by the amendment and/or to the adverse impact of heavy metals (Martin-Laurent *et al*., 2001). In contrast to our findings, Chen *et al*. (2016) reported an increase in soil bacterial diversity following long-term SS application. Similarly, under controlled laboratory conditions, Mattana *et al*. (2014) reported that the application of SS-based amendments increased soil prokaryotic diversity after 28 days of incubation.

The use of organic amendments can induce changes in soil microbial community composition (Marschner *et al*., 2003), that may then lead to changes in soil functionality (Fierer *et al*., 2012) and, in particular, the soil food web (Morriën, 2016). Here, we investigated changes in soil prokaryotic and eukaryotic communities after 24 years of SS application through metabarcoding of 16S and 18S rRNA amplicons. Our results indicate that the application of SS did not significantly change the composition of soil prokaryotic OTUs, as reflected by the NMDS analysis (Fig. 7.1A). This fact could be explained by the resistance and resilience of soil prokaryotic communities (Allison and Martiny, 2008). Nevertheless, the composition of eukaryotic OTUs did display differences between SS-amended and unamended control soil (Fig. 7.1B).

Furthermore, several prokaryotic and eurkaryotic taxa (ranging from class to genus level and from phylum to genus, respectively) exhibited significant correlation with the total amount of SS applied to the soil after 24 years. Regarding prokaryotic taxa, the relative abundance of 8 genera was significantly correlated with the total amount of SS applied after 24 years (Table 7.6). These taxa could also be responsible for the increase in bacterial biomass observed in SS-amended plots. Among them, *Lysobacter* was the most abundant, followed by *Clostridium*. The genus *Lysobacter*, which belongs to *Gammaproteobacteria*, has been linked to the degradation of a variety of phenolic compounds found in SS, such as bisphenol A (Yang *et al*., 2014) and nonylphenol (Zheng *et al*., 2018), so its higher abundance may be associated with a higher presence of phenolics compounds in soils amended with higher amounts of SS. The genus *Clostridium* belongs to the class *Clostridia*, the only prokaryotic taxon at class rank that showed a significant correlation with the total amount of SS applied after 24 years (Table 7.6). This genus is commonly found in municipal wastes and can be promoted by anaerobic digestion processes (Goberna *et al*., 2009), as it can oxidize acetate during the anaerobic decomposition of OM (Karakashev *et al*., 2006). Rahube *et al*. (2014) reported

an increased abundance of *Clostridium perfringens* (a wastewater pathogen), owing to SS application to soil. On the other hand, the genus that exhibited the best correlation with the total amount of SS applied after 24 years was *Perlucidibaca*, which contains just one specie, *Perlucidibaca piscinae* (Song *et al*., 2008), and is commonly found in drinking water wells (Navarro-Moya *et al*., 2013). However little is known about its functionality in the environment (Vandermaesen *et al*., 2017).

Concerning eukaryotic communities, *Prototheca* was the only genus that showed a positive correlation with the total amount of SS applied after 24 years. *Prototheca* is an oportunistic, pathogenic green algae that can act as human pathogen causing protothecosis (Velez-Mejiaa and Velez-Londoño, 2017). Species belonging to this genus have been reported to degrade polycyclic aromatic hydrocarbons (Ueno *et al*., 2008). Therefore, the reason for its increased abundance may be the higher amount of these organic contaminants in soils amended with higher loads of SS. By contrast, the family *Herpotrichiellaceae*, which belongs to the order *Chaetothyriales*, correlated negatively with the total amount of SS applied after 24 years. Some species of this family have been suggested for the biocontrol of soil-borne pathogens (Narisawa *et al*., 2000). Besides, some members of *Chaetothyriales* have the ability to degradate volatile aromatic hydrocarbons such as toluene (Badali *et al*., 2011).

Finally, in relation to the problem of antibiotic resistance, SS has been reported as a reservoir of ARB and ARGs (Rizzo *et al*., 2013). Stabilization through anaerobic digestion or the addition of lime may significantly reduce the load of ARB in SS (Munir *et al*., 2011). Nevertheless, such stabilization can also lead to sorption of antibiotics onto SS particles, making them less bioavailable (Li *et al*., 2013). Su *et al*. (2015) detected 156 different ARGs (encoding resistance to virtually all known antibiotic groups) and MGE genes in composted SS. Here, we observed a higher abundance of ARGs in sewage sludge itself, compared to soils amended or unamended with SS. Nevertheless, out of the 95 genes studied here, 86 were detected in soil samples, while only 74 were detected in the sewage sludge. This latter circumstance may be due to the well-known fact that soil bacteria are an important reservoir of ARGs (Lang *et al*., 2010; Forsberg *et al*., 2012). In any case, the application of SS may: (i) increase the abundance of certain ARGs already present in the soil and/or (ii) add new ones which were not previously present.

Increased abundances of ARGs and MGE genes have been frequently detected in soils after the application of SS (Rahube *et al*., 2014; Chen *et al*., 2016; Xie *et al*., 2016). Here, the effect of SS application did have a significant effect on the abundance of those

genes (Fig. 7.2); in fact, SS-treated soils exhibited a significant enrichment of 7 genes (*i.e.*, genes encoding resistance to aminoglycoside, tetracycline and vancomycin, and a gene encoding a transposase), compared to the unamended control. The increased abundance of these genes in amended soil could be due to HGT from populations present in the SS itself or due to the stimulation of soil microbial biomass and activity. Even those soils amended with the lowest total amount of SS after 24 years (treatment 40-4: 40 t ha^{-1}, every 4 years) showed significantly higher abundances for 3 ARGs and 1 MGE gene, compared to unamended control soil. However, it must be stated that the treatment with the lowest rate and frequency of SS application investigated here doubles the level of SS treatment usually applied in our region (*i.e.*, 20 t ha^{-1}, every 4 years). This was done in an attempt to accentuate the potential effects of SS application on soil quality. Interestingly, these increased gene abundances occurred in the absence of changes in prokaryotic OTU composition. MGEs can favour the dissemination of ARGs among bacterial populations through HGT, without altering the taxonomical composition. Furthermore, MGEs play a key role in the evolution of co-resistance, as they can simultaneously carry antibiotic and metal resistance determinants (Allen *et al*., 2010). Then, the presence of heavy metals in SS may lead to an increase in antibiotic resistance due to co-selection (Baker-Austin *et al*., 2006). Antibiotic resistance gene abundances have been reported to correlate with metal concentrations in soil (Knapp *et al*., 2011; Hu *et al*., 2016). In this respect, we found correlation between soil Cu and Zn total concentration and the abundance of ARGs and MGE genes (Table 7.8). The presence of metals (particularly, Cu and Zn) in amended soils may have triggered co-selection processes between metal and antibiotic resistance, leading to the higher abundance of some ARGs detected here (Knapp *et al*., 2017). This link has been attributed to the fact that some structural and functional resistance systems are shared for both metals and antibiotics (Baker-Austin *et al*., 2006). Moreover, the positive correlation found here between Cu and Zn concentration and the abundance of a transposase-encoding MGE gene, highlights the risk of dissemination of the increased ARGs. To this regard, it has been reported that the Zn finger of TnpA protein (a protein associated to mobilizable transposon Tn4555, an important antibiotic resistance element encoding a broad spectrum β-lactamase) has an important role in transposition and can mediate protein/protein interactions with integrase or other Tn4555 proteins to facilitate insertion into the preferred sites (Bacic *et al*., 2007).

7.5. Conclusions

Our study reveals that the long-term application of thermally dried and anaerobically digested SS to agricultural soil improves its physicochemical and microbial properties, thus ameliorating soil quality. Nonetheless, SS application led to Cu and Zn accumulation in soil, but without enhancing their bioavailability. The composition of soil prokaryotic communities was not significantly altered by SS application. Nevertheless, the application of SS resulted in changes in the abundance of some rare taxa, which correlated significantly with the total amount of SS applied after 24 years. Besides, sewage sludge application increased the abundance of some ARGs and MGE genes, posing a risk of dissemination of antibiotic resistance. We found correlation between soil Cu and Zn concentration and antibiotic resistance, suggesting mechanisms of co-selection for heavy metals and antibiotics. It was concluded that SS must be properly treated and managed prior to its application to agricultural soil, in order to minimize the introduction of contaminants and, in particular, the risk of dissemination of antibiotic resistance.

7.6. Supplementary information

Table 7.S1. Impact of sewage sludge (SS) application on $CaCl_2$-extractable (0.01 M), (ii) $NaNO_3$-extractable (0.1 M), and (iii) low molecular weight organic acid (LMWOA) solution-extractable metal fractions in soil. C: unamended control.

	$CaCl_2$ 0.01M						
	Cu mg kg^{-1}	**Zn mg kg^{-1}**	**Cd mg kg^{-1}**	**Pb mg kg^{-1}**	**Cr mg kg^{-1}**	**Ni mg kg^{-1}**	**As mg kg^{-1}**
40-1	0.52±0.05	0.40±0.21	0.03±0.02	0.52±0.27	0.17±0.04	0.12±0.09	1.73±0.88
40-2	0.54±0.09	0.29±0.08	0.03±0.01	0.41±0.20	0.13±0.03	0.16±0.15	1.39±0.51
40-4	0.60±0.05	0.35±0.23	0.03±0.01	0.42±0.35	0.17±0.04	0.17±0.09	1.55±0.59
80-1	0.62±0.06	0.28±0.06	0.03±0.02	0.48±0.16	0.17±0.03	0.17±0.12	1.43±0.46
80-2	0.51±0.04	0.22±0.03	0.02±0.01	0.56±0.27	0.13±0.03	0.18±0.08	1.04±0.52
80-4	0.46±0.05	0.27±0.19	0.03±0.01	0.40±0.24	0.14±0.03	0.12±0.07	0.93±0.39
C	0.43±0.04	0.17±0.05	0.03±0.01	0.44±0.27	0.17±0.05	0.16±0.09	0.98±0.57
	$NaNO_3$ 0.1M						
	Cu mg kg^{-1}	**Zn mg kg^{-1}**	**Cd mg kg^{-1}**	**Pb mg kg^{-1}**	**Cr mg kg^{-1}**	**Ni mg kg^{-1}**	**As mg kg^{-1}**
40-1	0.22±0.13	0.11±0.05	0.01±0.01	0.12±0.05	0.03±0.01	0.08±0.02	0.19±0.08
40-2	0.17±0.05	0.27±0.39	0.01±0.00	0.09±0.07	0.04±0.01	0.07±0.03	0.16±0.17
40-4	0.23±0.02	0.13±0.06	0.01±0.01	0.17±0.10	0.03±0.02	0.09±0.03	0.17±0.15
80-1	0.34±0.11	0.15±0.04	0.01±0.00	0.14±0.07	0.03±0.01	0.10±0.04	0.23±0.13
80-2	0.30±0.34	0.18±0.22	0.02±0.03	0.22±0.32	0.04±0.04	0.10±0.10	0.42±0.44
80-4	0.14±0.04	0.09±0.03	0.01±0.01	0.15±0.07	0.03±0.01	0.08±0.02	0.14±0.10
C	0.05±0.03	0.40±0.75	0.01±0.00	0.18±0.09	0.03±0.02	0.06±0.02	0.27±0.21
	LMWOA						
	Cu mg kg^{-1}	**Zn mg kg^{-1}**	**Cd mg kg^{-1}**	**Pb mg kg^{-1}**	**Cr mg kg^{-1}**	**Ni mg kg^{-1}**	**As mg kg^{-1}**
40-1	0.47±0.12	0.79±0.05	0.06±0.01	0.40±0.10	0.31±0.07	0.65±0.12	0.58±0.45
40-2	0.44±0.12	0.87±0.13	0.04±0.02	0.38±0.27	0.29±0.02	0.60±0.12	0.34±0.31
40-4	0.64±0.19	0.91±0.12	0.05±0.03	0.42±0.18	0.33±0.02	0.62±0.09	0.63±0.53
80-1	0.84±0.16	1.23±0.12	0.04±0.01	0.50±0.11	0.33±0.05	0.71±0.13	1.27±1.07
80-2	0.49±0.22	0.78±0.24	0.03±0.01	0.41±0.19	0.29±0.03	0.64±0.16	0.71±0.46
80-4	0.29±0.10	0.59±0.12	0.05±0.01	0.38±0.23	0.29±0.07	0.55±0.09	0.40±0.36
C	0.14±0.12	0.45±0.12	0.04±0.02	0.62±0.18	0.26±0.05	0.57±0.13	1.21±0.53

Table 7.S2. MGE genes and ARGs targeted in this study and their classification by target drug and mechanism of resistance. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes; MLSB: Macrolide-Lincosamide-Streptogramin B resistance.

Gene	Forward Primer	Reverse Primer	Classification	Resistance mechanism
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	16S rRNA	NA
intI	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	MGEs/Integrase	integrase
intI1	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	MGEs/Integrase	integrase
tnpA-03	AATTGATGCGGACGGCTTAA	TCACCAAACTGTTTATGGAGTCGTT	IS6 Group	transposase
IS613	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT	IS613	transposase
tnpA-01	CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT	IS21 Group	transposase
tnpA-04	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC	IS6 Group	transposase
tnpA-07	GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG	ISEcp1B	transposase
tnpA-05	GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT	IS6 Group	transposase
Tp614	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT	Tp614	transposase
tnpA-02	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	IS4 Group	transposase
catB3	GCACTCGATGCCTTCCAAAA	AGAGCCGATCCAAACGTCAT	FCA	deactivate
catB8	CACTCGACGCCTTCCAAAG	CCGAGCCTATCCAGACATCATT	FCA	deactivate
cmlA1-02	AGGAAGCATCGGAACGTTGA	ACAGACCGAGCACGACTGTTG	FCA	efflux
cmx(A)	GCGATCGCCATCCTCTGT	TCGACACGGAGCCTTGGT	FCA	efflux
catA1	GGGTGAGTTTCACCAGTTTTGATT	CACCTTGTCGCCTTGCGTATA	FCA	deactivate
aacC2	ACGGCATTCTCGATTGCTTT	CCGAGCTTCACGTAAGCATTT	Aminoglycoside	deactivate
aacC4	CGGCGTGGGACACGAT	AGGGAACCTTTGCCATCAACT	Aminoglycoside	deactivate
aacC	CGTCACTTATTCGATGCCCTTAC	GTCGGGCGCGGCATA	Aminoglycoside	deactivate
aac(6')-Ib-01	GTTTGAGAGGCAAGGTACCGTAA	GAATGCCTGGCGTGTTTGA	Aminoglycoside	deactivate
aadA1	AGCTAAGCGCGAACTGCAAT	TGGCTCGAAGATACCTGCAA	Aminoglycoside	deactivate
aadE	TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT	Aminoglycoside	deactivate
aph6ia	CCCATCCCATGTGTAAGGAAA	GCCACCGCTTCTGCTGTAC	Aminoglycoside	deactivate
aph(2')-Id-02	TGAGCAGTATCATAAGTTGAGTGAAAAG	GACAGAACAATCAATCTCTATGGAATG	Aminoglycoside	deactivate
strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside	deactivate
strA	CCGGTGGCATTTGAGAAAAA	GTGGCTCAACCTGCGAAAAG	Aminoglycoside	deactivate
ampC-01	TGGCGTATCGGGTCAATGT	CTCCACGGGCCAGTTGAG	Beta Lactamase	deactivate
ampC-02	GCAGCACGCCCCGTAA	TGTACCCATGATGCGCGTACT	Beta Lactamase	deactivate
bla1	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA	Beta Lactamase	deactivate
bla-ACC-1	CACACAGCTGATGGCTTATCTAAAA	AATAAACGCGATGGGTTCCA	Beta Lactamase	deactivate
blaCMY2-01	AAAGCCTCAT GGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA	Beta Lactamase	deactivate
blaCTX-M-04	CTTGGCGTTGCGCTGAT	CGTTCATCGGCACGGTAGA	Beta Lactamase	deactivate
blaCTX-M-05	GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT	Beta Lactamase	deactivate
blaGES	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG	Beta Lactamase	deactivate

bla-L1	CACCGGGTTACCAGCTGAAG	GCGAAGCTGCGCTTGTAGTC	Beta Lactamase	deactivate
blaOXY	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT	Beta Lactamase	deactivate
blaPAO	CGCCGTACAACCGGTGAT	GAAGTAATGCGGTTCTCCTTTCA	Beta Lactamase	deactivate
mecA	GGTTACGGACAAGGTGAAATACTGAT	TGTCTTTTAATAAGTGAGGTGCGTTAATA	Beta Lactamase	protection
Pbp5	GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT	Beta Lactamase	protection
penA	AGACGGTAACGTATAACTTTTTGAAAGA	GCGTGTAGCCGGCAATG	Beta Lactamase	protection
carB	GGAGTGAGGCTGACCGTAGAAG	ATCGGCGAAACGCACAAA	MLSB	efflux
ereA	CCTGTGGTACGGAGAATTCATGT	ACCGCATTCGCTTTGCTT	MLSB	deactivate
erm(36)	GGCGGACCGACTTGCAT	TCTGCGTTGACGACGGTTAC	MLSB	protection
ermA	TTGAGAAGGGATTTGCGAAAAG	ATATCCATCTCCACCATTAATAGTAAACC	MLSB	protection
lmrA-01	TCGACGTGACCGTAGTGAACA	CGTGACTACCCAGGTGAGTTGA	MLSB	efflux
lnuA-01	TGACGCTCAACACACTCAAAAA	TTCATGCTTAAGTTCCATACGTGAA	MLSB	deactivate
matA/mel	TAGTAGGCAAGCTCGGTGTTGA	CCTGTGCTATTTTAAGCCTTGTTTCT	MLSB	efflux
mdtA	CCTAACGGGCGTGACTTCA	TTCACCTGTTTCAAGGGTCAAA	MLSB	efflux
oleC	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG	MLSB	efflux
pikR1	TCGACATGCGTGACGAGATT	CCGCGAATTAGGCCAGAA	MLSB	protection
vatC-01	CGGAAATTGGGAACGATGTT	GCAATAATAGCCCCGTTTCCTA	MLSB	deactivate
vgbB-01	CAGCCGGATTCTGGTCCTT	TACGATCTCCATTCAATTGGGTAAA	MLSB	efflux
vgbB-02	ATACGAGCTGCCTAATAAAGGATCTT	TGTGAACCACAGGGCATTATCA	MLSB	deactivate
sul2	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT	Sulfonamide	protection
sul1	CAGCGCTATGCGCTCAAG	ATCCCGCTGCGCTGAGT	Sulfonamide	protection
dfrA12	CCTCTACCGAACCGTCACACA	GCGACAGCGTTGAAACAACTAC	Sulfonamide	deactivate
folA	CGAGCAGTTCCTGCCAAAG	CCCAGTCATCCGGTTCATAATC	Sulfonamide	deactivate
tet(32)	CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA	Tetracycline	protection
tet(34)	CTTAGCGCAAACAGCAATCAGT	CGGTGATACAGCGCGTAAACT	Tetracycline	unknown
tetA-01	GCTGTTTGTTCTGCCGGAAA	GGTTAAGTTCCTTGAACGCAAACT	Tetracycline	efflux
tetB-01	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA	Tetracycline	efflux
tetC-01	CATATCGCAATACATGCGAAAAA	AAAGCCGCGGTAAATAGCAA	Tetracycline	efflux
tetD-01	TGCCGCGTTTGATTACACA	CACCAGTGATCCCGGAGATAA	Tetracycline	efflux
tetE	TTGGCGCTGTATGCAATGAT	CGACGACCTATGCGATCTGA	Tetracycline	efflux
tetG-01	TCAACCATTGCCGATTCGA	TGGCCCGGCAATCATG	Tetracycline	efflux
tetH	TTTGGGTCATCTTACCAGCATTAA	TTGCGCATTATCATCGACAGA	Tetracycline	efflux
tetJ	GGGTGCCGCATTAGATTACCT	TCGTCCAATGTAGAGCATCCATA	Tetracycline	efflux
tetK	CAGCAGTCATTGGAAAATTATCTGATTATA	CCTTGTACTAACCTACCAAAAATCAAAATA	Tetracycline	efflux
tetL-01	AGCCCGATTTATTCAAGGAATTG	CAAATGCTTTCCCCCTGTTCT	Tetracycline	efflux
tetM-01	CATCATAGACACGCCAGGACATAT	CGCCATCTTTTGCAGAAATCA	Tetracycline	protection
tetO-01	ATGTGGATACTACAACGCATGAGATT	TGCCTCCACATGATATTTTTCCT	Tetracycline	protection
tetPA	AGTTGCAGATGTGTATAGTCGTAAACTATCTATT	TGCTACAAGTACGAAAACAAAACTAGAA	Tetracycline	efflux
tetPB-01	ACACCTGGACACGCTGATTTT	ACCGTCTAGAACGCGGAATG	Tetracycline	protection

tetQ	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT	Tetracycline	protection
tetR-02	CGCGATAGACGCCTTCGA	TCCTGACAACGAGCCTCCTT	Tetracycline	efflux
tetS	TTAAGGACAAACTTTCTGACGACATC	TGTCTCCCATTGTTCTGGTTCA	Tetracycline	protection
tetU-01	GTGGCAAAGCAACGGATTG	TGCGGGCTTGCAAAACTATC	Tetracycline	unknown
tetT	CCATATAGAGGTTCCACCAAATCC	TGACCCTATTGGTAGTGGTTCTATTG	Tetracycline	protection
tetX	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT	Tetracycline	unknown
tetW-01	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC	Tetracycline	protection
tetV	GCGGGAACGACGATGTATATC	CCGCTATCTCACGACCATGAT	Tetracycline	efflux
vanA	AAAAGGCTCTGAAAACGCAGTTAT	CGGCCGTTATCTTGTAAAAACAT	Vancomycin	protection
vanB-01	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT	Vancomycin	protection
vanC-01	ACAGGGATTGGCTATGAACCAT	TGACTGGCGATGATTTGACTATG	Vancomycin	protection
vanG	ATTTGAATTGGCAGGTATACAGGTTA	TGATTTGTCTTTGTCCATACATAATGC	Vancomycin	protection
vanHB	GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT	Vancomycin	protection
vanRA-01	CCCTTACTCCCACCGAGTTTT	TTCGTCGCCCCATATCTCAT	Vancomycin	protection
vanSA	CGCGTCATGCTTTCAAAATTC	TCCGCAGAAAGCTCAATTTGTT	Vancomycin	protection
vanTE	GTGGTGCCAAGGAAGTTGCT	CGTAGCCACCGCAAAAAAAT	Vancomycin	protection
vanXA	CGCTAAATATGCCACTTGGGATA	TCAAAAGCGATTCAGCCAACT	Vancomycin	protection
vanXB	AGGCACAAAATCGAAGATGCTT	GGGTATGGCTCATCAATCAACTT	Vancomycin	protection
acrA-01	CAACGATCGGACGGGTTTC	TGGCGATGCCACCGTACT	Multidrug	efflux
acrA-02	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC	Multidrug	efflux
adeA	CAGTTCGAGCGCCTATTTCTG	CGCCCTGACCGACCAAT	Multidrug	efflux
ceoA	ATCAACACGGACCAGGACAAG	GGAAAGTCCGCTCACGATGA	Multidrug	efflux
cmeA	GCAGCAAAGAAGAAGCACCAA	AGCAGGGTAAGTAAAACTAAGTGGTAAATCT	Multidrug	efflux

Table 7.S3. Impact of sewage sludge (SS) application on the relative abundance (%) of the 30 most abundant prokaryotic taxa at order rank: C: unamended control; SS-C: SS-amended *vs.* unamended control, differences based on pooled variances *t*-test or Welch´s *t*-test: ns, not significant; *: p<0.05; **: p<0.01; ***: p<0.001. R: application rate; F: application frequency; RxF: interaction among factors. Interaction among factors is tested by two-way ANOVA. Mean values (n = 6) ± SD.

	40-1	**40-2**	**40-4**	**80-1**	**80-2**	**80-4**	**C**	**SS-C**	**R**	**F**	**RxF**
Acidobacteria group 6 (DA023)	12.7±2.2	13.8±1.3	12.8±1.0	12.3±1.5	13.0±1.5	13.5±0.8	13.6±0.9	ns	ns	ns	ns
Xanthomonadales	7.1±2.0	6.3±0.6	6.5±0.6	7.1±0.7	7.0±0.8	6.3±0.8	6.3±1.1	ns	ns	ns	ns
Burkholderiales	5.2±1.8	4.2±0.6	5.0±1.9	5.0±1.7	4.9±0.8	4.6±0.8	4.1±0.6	ns	ns	ns	ns
Sphingobacteriales	4.4±1.4	4.0±1.2	4.1±0.6	4.4±0.7	5.8±2.0	4.9±1.6	4.4±1.4	ns	ns	ns	ns
Rhizobiales	4.2±0.4	4.3±0.5	4.5±0.2	4.4±0.6	4.2±0.2	4.6±0.4	4.4±0.6	ns	ns	ns	ns
Acidobacteria group 4	2.9±0.6	3.2±0.5	3.2±0.4	2.9±0.5	3.0±0.3	3.2±0.7	3.3±0.3	ns	ns	ns	ns
AKIW543	3.0±0.7	3.4±0.5	3.2±0.4	3.0±0.5	2.7±0.6	3.1±0.6	3.0±0.5	ns	ns	ns	ns
Myxococcales	2.9±0.5	2.9±0.3	3.1±0.1	2.9±0.2	2.9±0.5	3.2±0.3	3.0±0.2	ns	ns	ns	ns
Rhodospirillales	3.1±1.3	2.9±0.7	3.0±0.6	2.7±0.5	2.3±0.5	2.5±0.9	2.9±0.7	ns	ns	ns	ns
Cytophagales	2.6±0.3	2.6±0.8	2.8±0.2	2.8±0.4	3.1±0.8	3.1±0.8	2.6±0.8	ns	ns	ns	ns
Solirubrobacterales	2.3±1.1	2.9±0.7	2.6±0.4	2.4±0.5	2.1±0.9	2.4±0.8	2.5±0.7	ns	ns	ns	ns
Planctomycetales	2.4±0.4	2.5±0.3	2.5±0.4	2.5±0.3	2.4±0.5	2.5±0.2	2.5±0.2	ns	ns	ns	ns
Sphingomonadales	2.3±0.3	2.3±0.3	2.4±0.4	2.2±0.4	2.6±0.3	2.5±0.3	2.6±0.3	ns	ns	ns	ns
WD2101 soil group	2.2±0.4	2.3±0.3	2.3±0.4	2.1±0.3	2.2±0.2	2.3±0.2	2.3±0.1	ns	ns	ns	ns
Nitrosomonadales	2.0±0.2	1.9±0.2	2.0±0.2	1.9±0.1	2.1±0.4	2.0±0.1	1.9±0.1	ns	ns	ns	ns
Micrococcales	1.6±0.4	1.8±0.3	1.7±0.3	1.6±0.3	1.5±0.3	1.7±0.4	1.4±0.2	ns	ns	ns	ns
Chthoniobacterales	1.2±0.1	1.3±0.1	1.3±0.1	1.2±0.2	1.4±0.1	1.4±0.3	1.4±0.1	ns	ns	ns	ns
Flavobacteriales	2.0±3.0	1.4±1.5	1.1±0.5	1.4±0.7	1.4±0.8	0.9±0.6	1.1±1.2	ns	ns	ns	ns
Gemmatimonadales	1.4±0.7	1.1±0.5	0.9±0.6	1.1±0.6	0.7±0.5	0.9±0.7	1.3±0.6	ns	ns	ns	ns
Nitrospinaceae order incertae sedis	1.1±0-3	1.1±0.2	1.1±0.1	1.0±0.1	0.9±0.1	1.0±0.2	1.1±0.2	ns	*	ns	ns
Propionibacteriales	1.0±0.2	1.1±0.1	1.1±0.2	1.0±0.2	0.9±0.2	1.0±0.1	1.0±0.1	ns	ns	ns	ns
Acidimicrobiales	0.9±0.3	1.1±0.2	1.0±0.2	1.0±0.2	0.8±0.2	0.9±0.3	0.9±0.2	ns	ns	ns	ns
Frankiales	0.9±0.2	1.0±0.2	0.9±0.1	0.8±0.2	0.8±0.2	0.9±0.2	0.9±0.2	ns	ns	ns	ns
KD4-96	0.8±0.2	0.8±0.1	0.9±0.2	0.8±0.1	0.8±0.2	0.8±0.2	0.8±0.1	ns	ns	ns	ns
Bacillales	0.7±0.2	0.8±0.0	0.8±0.0	0.7±0.2	0.7±0.1	0.8±0.1	0.8±0.1	ns	ns	ns	ns
GR-WP33-30	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	ns	ns	ns	ns
Corynebacteriales	0.5±0.2	0.7±0.1	0.6±0.1	0.8±0.2	0.6±0.2	0.5±0.1	0.5±0.1	ns	ns	ns	ns
Pseudomonadales	0.6±0.3	0.5±0.2	0.5±0.2	0.6±0.3	0.5±0.1	0.6±0.2	1.0±1.4	ns	ns	ns	ns
Rubrobacterales	0.5±0.3	0.7±0.2	0.6±0.2	0.5±0.1	0.4±0.3	0.5±0.3	0.6±0.2	ns	ns	ns	ns
Micromonosporales	0.6±0.3	0.6±0.1	0.6±0.2	0.5±0.2	0.4±0.2	0.5±0.3	0.6±0.2	ns	ns	ns	ns

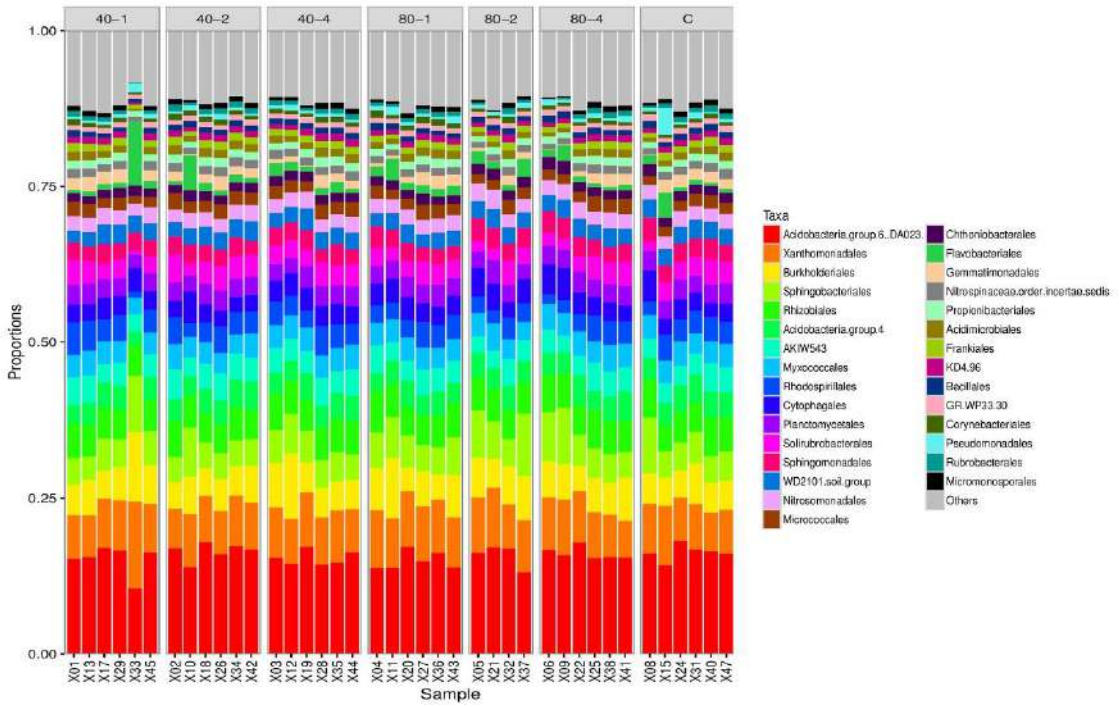


Figure 7.S1. Barplot representing the relative abundance of the 30 most abundant prokaryotic taxa at order rank, for each individual sample. C, unamended control soil.

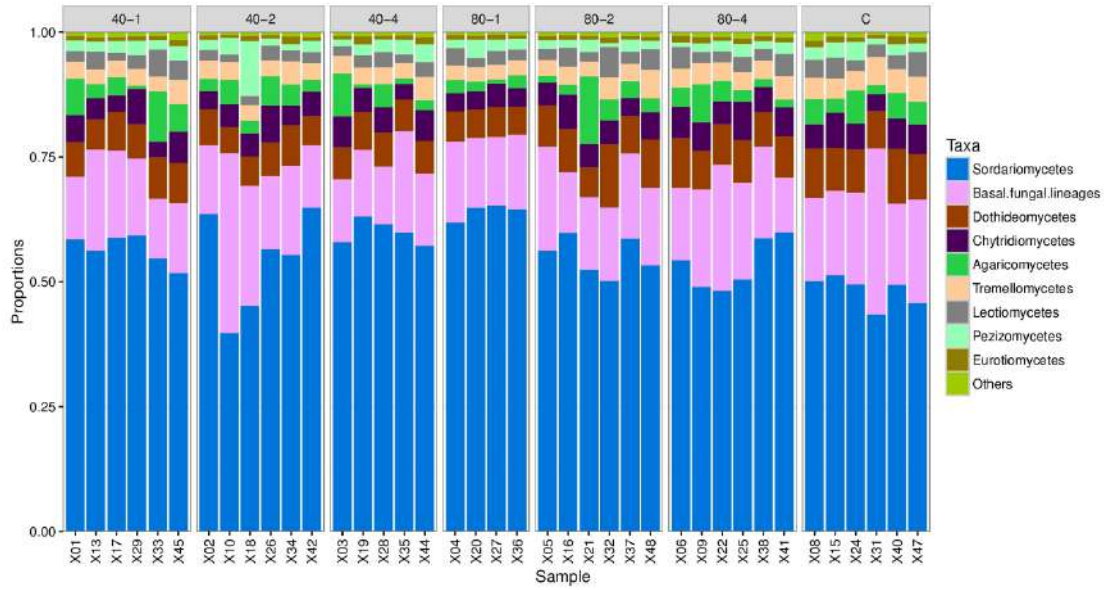


Figure 7.S2. Barplot representing the relative abundance of the 9 most abundant fungal taxa at class rank, for each individual sample. C, unamended control soil.

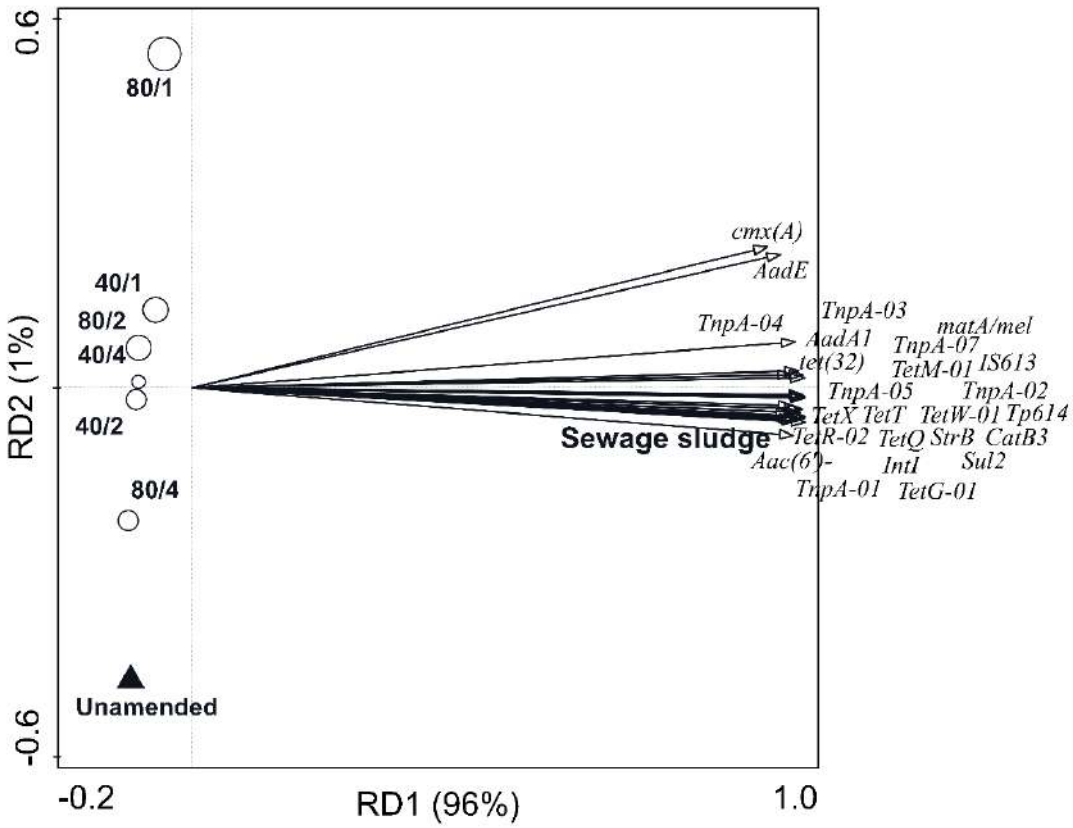


Figure 7.S3. Redundancy analysis displaying (as response variables) the 25 ARGs or MGEs with the best fit in explaining the variation between SS-amended soil, unamended soil and the sewage sludge itself. First and second axes account for the 96 and 1% of the explained variation, respectively. Size of circles represents the total amount of SS applied after 24 years.

7.7. Data on links between structural and functional prokaryotic diversity in long-term sewage sludge amended soil

Urra, J., Alkorta, I., Mijangos, Garbisu, C., 2018, published in ***Data in Brief****,* 20, 1787-1796.

Abstract

The application of sewage sludge to agricultural soil induces co-exposure of prokaryotic populations to antibiotics and heavy metals, thus exerting a selection pressure that may lead to the development of antibiotic resistance. Here, soil samples from a long-term factorial field experiment in which sewage sludge was applied to agricultural soil, at different rates (40 and 80 t ha-1) and frequencies (every 1, 2 and 4 years) of application, were studied to assess: (i) the effect of sewage sludge application on prokaryotic community composition, (ii) the links between prokaryotic community composition and antibiotic resistance profiles, and (iii) the links between antibiotic resistance and metal(oid) concentrations in amended soil. We found no significant impact of sewage sludge on prokaryotic community composition. Some antibiotic resistance genes (ARGs) correlated positively with particular prokaryotic taxa, being Gemmatimonadetes the taxon with the greatest number of positive correlations at phylum level. No positive correlation was found between prokaryotic taxa and genes encoding resistance to sulfonamides and FCA. All metal(oid)s showed positive correlations with, at least, one ARG. Metal(oid) concentrations in soil also showed positive correlations with mobile genetic element genes, particularly with the gene tnpA-07. These data provide useful information on the links between soil prokaryotic composition and resistome profiles, and between antibiotic resistance and metal(oid) concentrations, in agricultural soils amended with sewage sludge.

7.7.1. Specifications Table

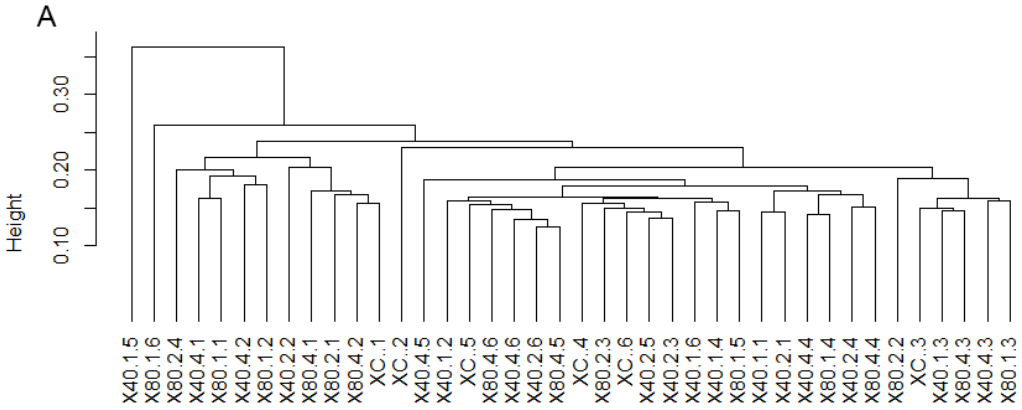
Subject area	*Biology*
More specific subject area	*Soil health, soil quality, soil resistome, prokaryotic functional diversity, prokaryotic structural diversity, heavy metals, metalloids, organic amendments, antibiotic resistance.*
Type of data	*Tables and Figures.*
How data was acquired	*Illumina MiSeq V2Platform; BioMark™ HD System and Dynamic Array Integrated Fluidic Circuits (IFCs); Inductively Coupled Plasma Optical Emission-Spectrometry (ICP-OES, VARIAN).*
Data format	*Analyzed*
Experimental factors	*Sewage sludge was added to agricultural soil following a factorial design with combinations of two rates (40 & 80 t ha^{-1}) and three frequencies (every 1, 2 and 4 years) of application, as well as an unamended control.*
Experimental features	*16S rRNA metabarcoding was carried out following a dual indexing approach in an Illumina MiSeq V2 platform. Relative abundance of ARGs and MGE genes was measured by HT-qPCR. Heavy metal(oid) concentration in soil was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).*
Data source location	*Derio, Spain.*
Data accessibility	*Data are available in the article.*
Related research article	*J. Urra, I. Alkorta, I. Mijangos, L. Epelde, C. Garbisu, Application of sewage sludge to agricultural soil increases the abundance of antibiotic resistance genes without altering the composition of prokaryotic communities. Sci. Total Environ. 647 (2019), 1410-1420.*

7.7.2. Value of the Data

- Data are useful for depicting the links between soil prokaryotic composition and antibiotic resistance profiles after long-term application of sewage sludge.
- Data are useful to show the long-term impact of sewage sludge application on the composition of prokaryotic communities in agricultural soil.
- Our data provide useful information on the links between metal(oid) concentrations in soil and the abundance of antibiotic resistance genes.

7.7.3. Data

We evaluated the effect of sewage sludge application on soil prokaryotic communities and the soil resistome, as reflected by the abundance of antibiotic resistance genes (ARGs) and mobile genetic element (MGE) genes. We investigated the correlations between ARG abundance and metal(oid) concentrations in soil. Hierarchical clustering did not show differences, neither among sewage sludge treatments (Figure 7.3A) nor with respect to the total amount of sewage sludge applied to soil after 24 years (Figure 7.3B).



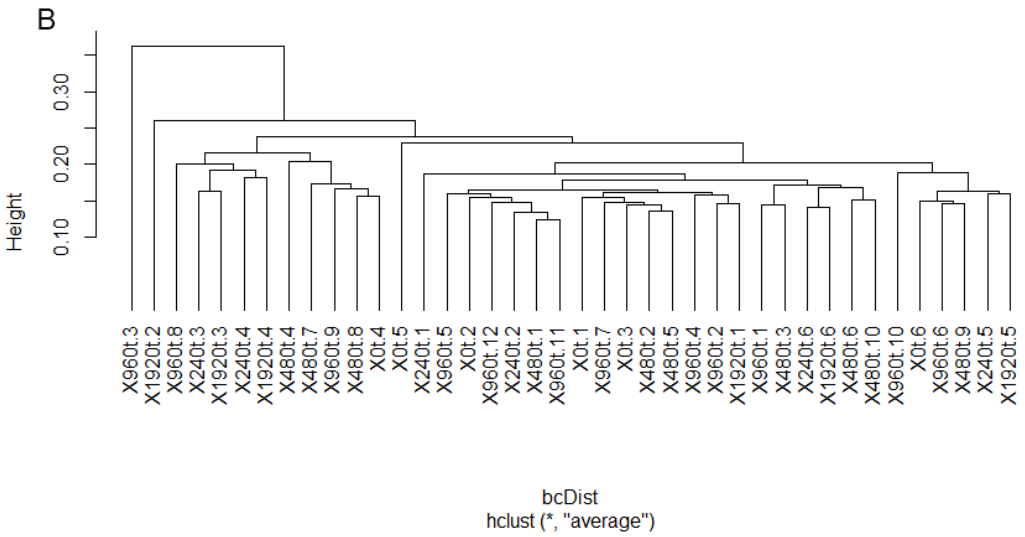


Figure 7.3. Hierarchical clustering of soil samples, based on Bray Curtis dissimilarities of prokaryotic OTUs obtained from 16S rRNA metabarcoding. Samples are arranged according to: (A) sewage sludge treatment; and (B) total amount of sewage sludge applied during the 24-year experiment. Treatments: 40-1: 40 t ha^{-1} every year; 40-2: 40 t ha^{-1} every 2 years; 40-4: 40 t ha^{-1} every 4 years; 80-1: 80 t ha^{-1} every year; 80-2: 80 t ha^{-1} every 2 years; 80-4: 40 t ha^{-1} every 4 years; C: control, unamended. Total amount of sewage sludge applied during the 24-year experiment (in t ha^{-1}): 0, 240, 480, 960 and 1920.

In relation to taxonomical classification, 96.6 and 55.3% of the 16S rRNA sequences were classified to phylum and family rank, respectively. At both levels, taxa distribution was similar among all soil samples and it did not show any significant differences (i) between sewage sludge amended *vs.* unamended soil (Figure 7.4); (ii) among sewage sludge treatments (Figure 7.5); and (iii) with respect to the total amount of sewage sludge added to the soil after 24 years (Figure 7.6).

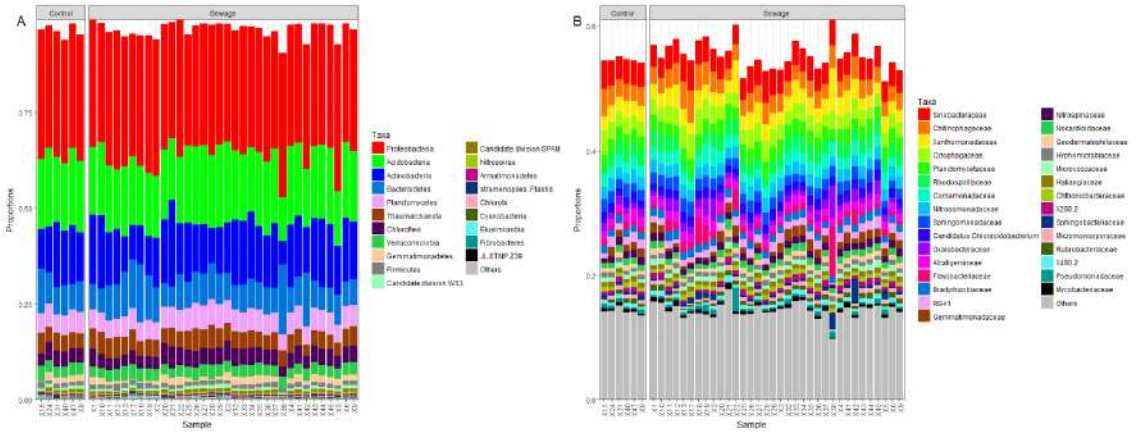


Figure 7.4. Barplots representing the composition of: (A) the 20 most abundant prokaryotic taxa at phylum rank; and (B) the 30 most abundant taxa at family rank, for all sewage-amended and unamended soil samples. Control: unamended samples. Sewage: sewage sludge amended samples.

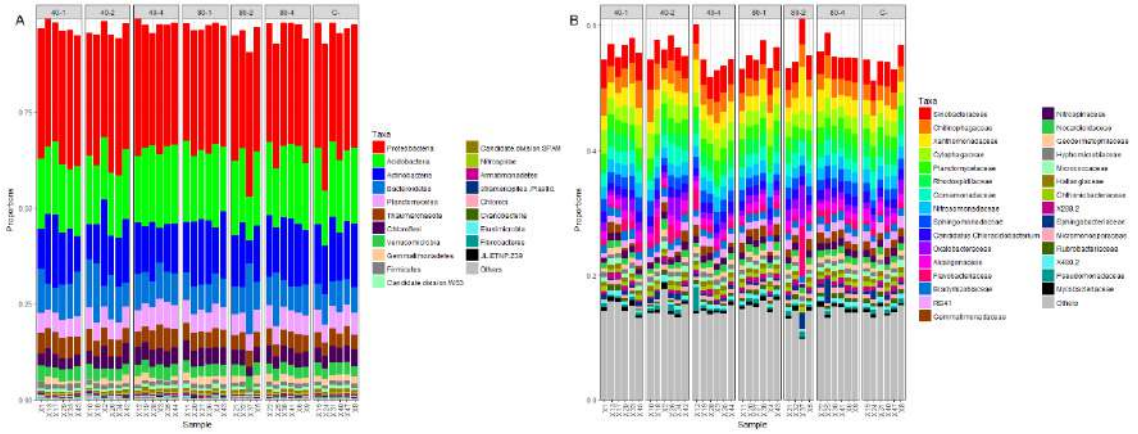


Figure 7.5. Effect of treatments on the composition of: (A) the 20 most abundant prokaryotic taxa at phylum rank; and (B) the 30 most abundant taxa at family rank. Treatments: 40-1: 40 t ha^{-1} every year; 40-2: 40 t ha^{-1} every 2 years; 40-4: 40 t ha^{-1} every 4 years; 80-1: 80 t ha^{-1} every year; 80-2: 80 t ha^{-1} every 2 years; 80-4: 40 t ha^{-1} every 4 years; C: control, unamended.

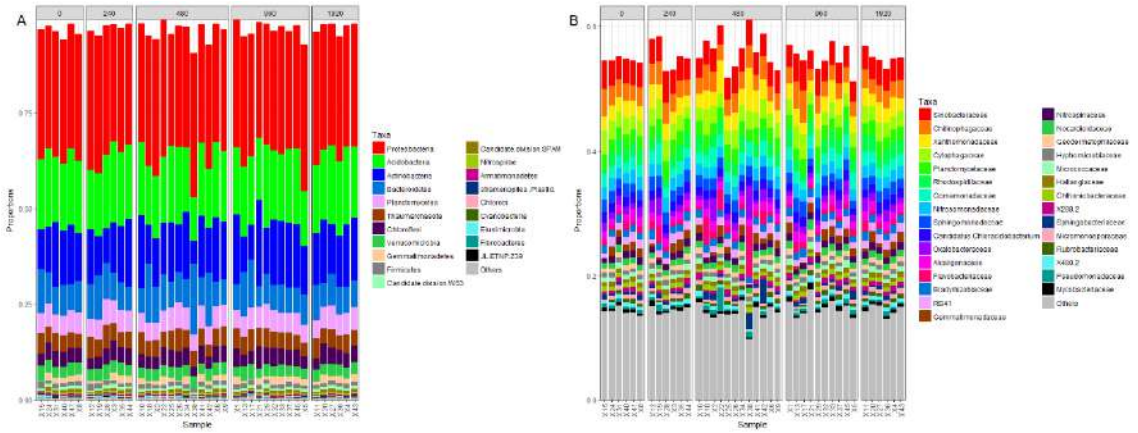


Figure 7.6. Effect of the total amount of sewage sludge applied during the 24-year experiment on the composition of: (A) the 20 most abundant prokaryotic taxa at phylum rank; and (B) the 30 most abundant taxa at family rank. Total amount of sewage sludge applied during the 24-year experiment (in t ha^{-1}): 0, 240, 480, 960 and 1920.

The 10 most abundant prokaryotic taxa at phylum level accounted for 89.4% of the total community. Among them, *Proteobacteria*, the most dominant taxon, showed the greatest number of negative correlations (Table 7.9).

Table 7.9. Kendall's tau correlations between the 10 most abundant prokaryotic phyla. Negative correlation are displayed in italics. ns: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

	Proteobacteria	**Acidobacteria**	**Actinobacteria**	**Bacteroidetes**	**Planctomycetes**	**Chloroflexi**	**Verrucomicrobia**	**Gemmatimonadetes**	**Firmicutes**	**Candidate division WS3**
Proteobacteria		***	**	***	***	***	*	ns	ns	**
Acidobacteria	***-0.49***		ns	**	***	***	ns	ns	ns	***
Actinobacteria	***-0.32***	-		***	ns	***	***	***	ns	ns
Bacteroidetes	**0.43**	***-0.34***	***-0.69***		ns	***	***	***	ns	ns
Planctomycetes	***-0.45***	**0.61**	-	-		***	ns	ns	*	***
Chloroflexi	***-0.43***	**0.37**	**0.37**	***-0.41***	**0.44**		**	***	*	**
Verrucomicrobia	**0.22**	-	***-0.59***	**0.50**	-	***-0.32***		***	ns	ns
Gemmatimonadetes	-	-	**0.49**	***-0.45***	-	**0.51**	***-0.44***		**	ns
Firmicutes	-	-	-	-	***-0.24***	***-0.28***	-	***-0.29***		ns
Candidate division WS3	***-0.31***	**0.55**	-	-	**0.63**	**0.29**	-	-	-	

No correlation was found between prokaryotic taxa and the abundance of ARGs for sulfonamides and FCA (Tables 7.10). Several ARGs belonging to the other antibiotic groups studied here (aminoglycoside, β-lactamase, MLSB, tetracycline, vancomycin, multidrug) correlated with some prokaryotic taxa (Table 7.10).

Table 7.10. Kendall's tau significant correlations between the 10 most abundant prokaryotic phyla and the abundance of ARGs. Negative correlations are displayed in italics. Genes that were not amplified during the HT-qPCR analysis are highlighted in grey. *: p<0.05; **: p<0.01; ***: p<0.001. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB: Macrolide-Lincosamide-Streptogramin B resistance.

		Proteobacteria	Acidobacteria	Actinobacteria	Bacteroidetes	Planctomycetes	Chloroflexi	Verrucomicrobia	Gemmatimonadetes	Firmicutes	Candidate division WS3
FCA	catB3										
	catB8										
	cmlA1-02										
	cmx(A)										
	catA1										
Aminoglycoside	aacC2										
	aacC4			0.24*							*-0.24**
	aacC				*-0.26**			*-0.24**			
	aac(6')-Ib-01										
	aadA1										
	aadE										
	aph6ia										
	aph(2')-Id-01										
	strB										
	strA										
β-Lactamase	ampC-01										
	ampC-02	*-0.30**									*-0.25**
	bla1										
	bla-ACC-1										
	blaCMY2-01										
	blaCTX-M-04										
	blaCTX-M-05										
	blaGES										
	bla-L1			0.33**	*-0.29**			*-0.27**	0.28*		
	blaOXY		0.24*								
	blaPAO										
	mecA										
	Pbp5										
	penA										
MLSB	carB										
	ereA										
	erm(36)										
	ermA										
	lmrA-01										
	lnuA-01	*-0.30**									
	matA/mel										
	mdtA										
	oleC										
	pikR1										
	vatC-01										
	vgbB-01										
	vgbB-02										

Sulfonamide	**sul2**							
	sul1							
	dfrA12							
	folA							
Tetracycline	**tet(32)**							
	tet(34)							
	tetA-01							
	tetB-01				0.28*	0.26*		
	tetC-01							
	tetD-01							
	tetE							
	tetG-01							
	tetH							
	tetJ							
	tetK							
	tetL-01							
	tetM-01							
	tetO-01							*-0.26**
	tetPA						0.25*	
	tetPB-01			0.22*	*-0.24**			
	tetQ							
	tetR-02							
	tetS							
	tetU-01							
	tetT		0.27*					0.25*
	tetX							
	tetW-01							
	tetV							
Vancomycin	**vanA**							
	vanB-01							
	vanC-01							
	vanG							
	vanHB					0.27*	*-0.24**	
	vanRA-01						0.27*	
	vanSA							
	vanTE							
	vanXA							
	vanXB							
Multidrug	**acrA-01**	*-0.25**						
	acrA-02	*-0.25**				0.24*		
	adeA							
	ceoA							
	cmeA							

Concerning MGE genes, *Actinobacteria* and *Gemmatimonadetes* showed some positive correlations (Table 7.11). All metal(oid)s correlated positively with at least one ARG (Table 7.12).

Table 7.11. Kendall's tau significant correlations between the 10 most abundant prokaryotic phyla and the abundance of MGE genes. Negative correlations are displayed in italics. *: p<0.05; **: p<0.01; ***: p<0.001. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB: Macrolide-Lincosamide-Streptogramin B resistance.

		Proteobacteria	Acidobacteria	Actinobacteria	Bacteroidetes	Planctomycetes	Chloroflexi	Verrucomicrobia	Gemmatimonadetes	Firmicutes	Candidate division WS3
Integrase	**intI**							*-0.34***	0.24*		
	intI1										
Transposase	**tnpA-03**										
	IS613										
	tnpA-01										
	tnpA-04										
	tnpA-07										
	tnpA-05										
	Tp614			0.26*							
	tnpA-02			0.25*					0.25*		

Table 7.12. Kendall's tau significant correlations between metal(oid) concentration in soil and abundance of ARGs. Negative correlations are displayed in italics. Genes that were not amplified during the HT-qPCR analysis are highlighted in grey. *: p<0.05; **: p<0.01; ***: p<0.001. . FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB: Macrolide-Lincosamide-Streptogramin B resistance.

		Cu	Zn	Cd	Pb	Cr	Ni	As
FCA	**catB3**							
	catB8		-0.38 *			-0.38 *		
	cmlA1-02							
	cmx(A)		0.57 ***		0.38 *			
	catA1							
Aminoglycoside	**aacC2**							
	aacC4							
	aacC							
	aac(6')-Ib-01							
	aadA1		0.42 *					
	aadE	0.47 **	0.63 ***		0.35 *			
	aph6ia							
	aph(2')-Id-01							
	strB				0.35 *			
	strA							

β-Lactamase	**ampC-01**						
	ampC-02						
	bla1						
	bla-ACC-1						0.44 **
	blaCMY2-01						0.49 **
	blaCTX-M-04				0.35 *		
	blaCTX-M-05						
	blaGES						
	bla-L1	-0.50 **		-0.35 *		-0.42 *	
	blaOXY						
	blaPAO						
	mecA						
	Pbp5						
	penA						
MLSB	**carB**						
	ereA						
	erm(36)				0.49 **		
	ermA						
	lmrA-01						
	lnuA-01						
	matA/mel		0.45 **		0.50 **		
	mdtA						
	oleC						
	pikR1						
	vatC-01				0.34 *		
	vgbB-01						
	vgbB-02	0.35 *		0.41 *		0.40 *	
Sulfonamide	**sul2**						
	sul1						
	dfrA12						0.41 *
	folA						
Tetracycline	**tet(32)**		0.38 *				
	tet(34)				-0.44 **		
	tetA-01	-0.34 *					
	tetB-01						
	tetC-01						
	tetD-01						
	tetE						
	tetG-01		0.39 *				
	tetH				0.41 *		
	tetJ						
	tetK						
	tetL-01				-0.36 *		
	tetM-01				0.50 **		
	tetO-01				0.45 **		
	tetPA	0.42 *	0.58 ***				
	tetPB-01					0.36 *	
	tetQ						
	tetR-02						
	tetS						0.38 *
	tetU-01						
	tetT	0.35 *	0.41 *				
	tetX						
	tetW-01		0.50 **				
	tetV						

Vancomycin	**vanA**						
	vanB-01						
	vanC-01			0.39 *			
	vanG						0.34 *
	vanHB						
	vanRA-01	0.34 *					0.52 **
	vanSA		-0.35 *	-0.37 *			
	vanTE						
	vanXA						
	vanXB			0.34 *			
Multidrug	**acrA-01**						
	acrA-02						
	adeA			0.57 ***			
	ceoA						
	cmeA						

The gene *intI1* exhibited a significant negative correlation with Cu (Table 7.13). Most of the studied metal(oids) were positively correlated with, at least, one transposase encoding gene.

Table 7.13. Kendall's tau significant correlations between metal(oid) concentrations in soil and the abundance of MGE genes. Negative correlations are displayed in italics. *: p<0.05; **: p<0.01; ***: p<0.001. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB: Macrolide-Lincosamide-Streptogramin B resistance.

		Cu	Zn	Cd	Pb	Cr	Ni	As
Integrase	**intI**							
	intI1	*-0,34 **						
Transposase	**tnpA-03**		0,39 *					
	IS613							*-0,36 **
	tnpA-01							
	tnpA-04		0,36 *					
	tnpA-07	0,45 **	0,65 ***	0,39 *			0,34 *	
	tnpA-05				0,38 *			
	Tp614							
	tnpA-02							

7.7.4. Experimental Design, Materials, and Methods

Experimental plots (35 m^2; 6 replicates per treatment) were located in Navarre, Spain. A factorial design, with combinations of two rates (40 and 80 t ha^{-1}) and three frequencies (every 1, 2 and 4 years) of sewage sludge (thermally dried and anaerobically digested) application, was followed in this field experiment. An unamended control was also included. Sewage sludge was annually (for 24 consecutive years) incorporated to the soil

by disc plowing to a depth of 30 cm. The cropping system consists of 3-year crop rotations (cereal / cereal / non-cereal) with no irrigation or weed control (Urra *et al*., 2019c). Composite soil samples (*i.e.*, 0-30 cm soil depth; 6 cores randomly taken per plot) were collected from each plot. The soil is a Calcaric Cambisol, with a clay-loamy texture, an alkaline pH (around 8.5) and an organic matter content of *ca.* 5%.

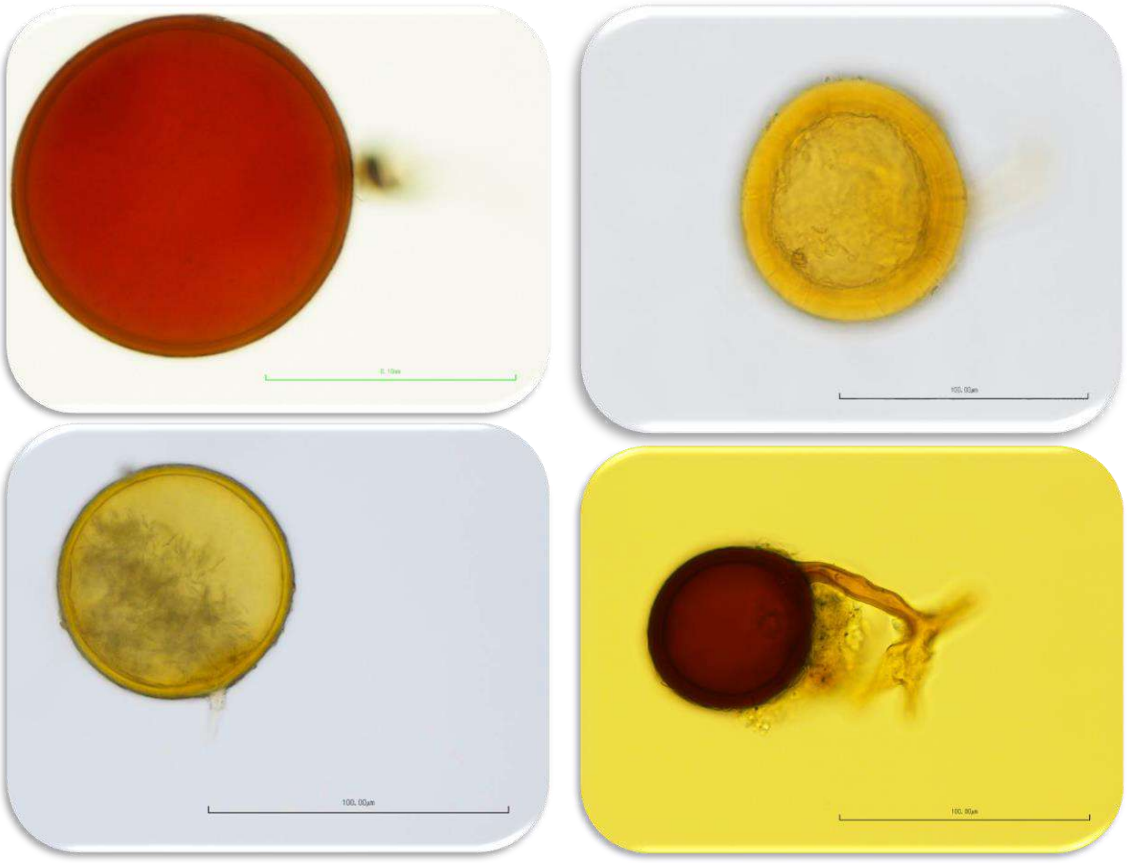
Soils were dried at room temperature and then sieved (<2 mm). Total concentrations of metal(oid)s (*i.e.* Cu, Zn, Cd, Pb, Cr, Ni, As) in soil samples were determined via inductively coupled plasma-optical emission spectrometry (ICP-OES). Samples for molecular analyses were stored at -20ºC. DNA was extracted from soil samples (3 soil aliquots per sample, 0.25 g DW soil each) using the Power Soil DNA Isolation Kit (MO Bio Laboratories, CA). Prior to DNA extraction, samples were washed twice in 120 mM K_2PO_4 (pH 8.0). Amplicon libraries were prepared with a dual indexing approach using sequence-specific primers targeting the V4 hypervariable region of the 16S rRNA gene according to the reaction mixtures, PCR conditions and primers described in Urra *et al*. (2018). Sequencing was carried out in an Illumina MiSeq V2 platform and pair-ended 2x250 nt. Merging of the read paired ends, quality filtering and clustering into operational taxonomic units (OTUs) was following Lanzén *et al*. (2016). Taxonomical assignments were carried out using CREST and SilvaMod v128 (Lanzén *et al*., 2012).

High-throughput RT-qPCR (HT-qPCR), with the nanofluidic qPCR BioMark™ HD system and 48.48 and 96.96 Dynamic Array Integrated Fluidic Circuits (IFCs) (Fluidigm Corporation), was used for the detection and quantification of ARGs and MGE genes in soil samples. 96 validated primer sets (Hu *et al*., 2016) targeting 85 ARGs, 10 MGEs and one reference structural gene (16S rRNA gene) were used. All samples were pre-amplified and treated with exonuclease I and then loaded onto the IFCs, following the Fluidigm's Fast Gene Expression Analysis Using EvaGreen Protocol. SsoFastTM EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, Redmond, WA) was used for amplification. The cycling program consisted of 1 min at 95ºC, followed by 30 cycles at 95ºC for 5 seconds and 60ºC for 20 seconds, followed by a melting curve. Data were then analyzed with the Fluidigm Real-Time PCR Analysis Software (v.3.1.3) with linear baseline correction and manual threshold settings, in order to obtain threshold cycle (Ct) values. Four technical replicates were included for each sample, and quantification of a specific gene was considered positive when 3 out of the 4 replicates were above the detection limit, which was established according to the lowest positive amplification

value recorded in our experiment. Each ARG and MGE gene was normalized according to the matched structural reference gene (16S rRNA), in order to obtain a relative abundance value.

Metabarcoding data visualization and hierarchical clustering were performed with R package *vegan* (Oksanen *et al*., 2015). Hierarchical clustering of prokaryotic OTUs was performed based on Bray-Curtis dissimilarity matrices to determine differences in soil prokaryotic composition between sewage sludge amended and unamended soil. In order to investigate the links between (i) the most dominant prokaryotic taxa, (ii) soil prokaryotic community composition and antibiotic resistance profiles, and (iii) total metal(oid) concentrations in soil and abundance of ARGs and MGE genes, Kendall's tau correlations were performed.

8| INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGI INCREASES LETTUCE YIELD WITHOUT ALTERING NATURAL SOIL COMMUNITIES



8. INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGI INCREASES LETTUCE YIELD WITHOUT ALTERING NATURAL SOIL COMMUNITIES

Epelde, J., Urra, J., Anza, M., Gamboa, J., Garbisu, C., under revision in ***Archives of Agronomy and Soil Science****.*

Abstract

Arbuscular mycorrhizal fungi (AMF) can be an environmentally-friendly alternative or complement to chemical fertilizers in agriculture. Initially, through ITS metabarcoding, we characterized the composition of fungal communities from three organic orchard soils, as well as from three adjacent non-cultivated soils. Organic orchard management had a negative impact on AMF community composition, leading to reduced alpha diversity and relative abundance of AMF taxa, compared to non-cultivated soil. In this situation, using trap plants, we multiplied the AMF communities from the abovementioned orchard and non-cultivated soils. Afterwards, a microcosm experiment was carried out to study the effect of inoculation with these AMF communities on (i) lettuce yield and nutritional quality (two consecutive crops), and (ii) fungal communities. During the second crop cycle, AMF inoculations led to higher lettuce yields (by an average of 186%). In contrast, AMF inoculations did not substantially modify either lettuce nutritional quality or the abundance and diversity of fungal or AMF communities. Furthermore, the origin of the AMF inoculum did not have a clear influence on its ulterior effect on lettuce yield. Much research is needed to better predict under which specific conditions and through which mechanisms AMF inoculation can contribute to enhanced crop yield and nutritional quality.

8.1. Introduction

Traditionally, agricultural production has been maximized through the intensive application of mineral fertilizers and pesticides (Robertson and Vitousek 2009), leading to a variety of negative impacts on agroecosystems, such as, for instance, soil contamination with trace elements, pesticides and other harmful substances (Muñoz-Leoz *et al*. 2012, 2013). Furthermore, intensive agricultural practices are known to cause

adverse effects on soil microbial communities and, hence, ecological processes responsible for soil fertility and, in general, soil functioning (Culman *et al*. 2010; Mijangos *et al*. 2006; Postma-Blaauw *et al*. 2010).

In the current scenario and projections of human population growth, we undoubtedly need to urgently increase agricultural productivity and production, but it is important to always emphasize that such increase must be achieved according to sustainability principles. Thus, sustainable agricultural practices are gaining increased interest throughout the world. Among them, the use of biostimulants is being thoroughly tested as an environmentally-friendly alternative or complement to the intensive use of chemical fertilizers and pesticides. Biostimulants have been defined as "any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content" (du Jardin 2015). Microbial biostimulants include mycorrhizal fungi, plant growth-promoting rhizobacteria and bacterial endosymbionts (du Jardin 2015).

The inoculation of Arbuscular Mycorrhizal Fungi (AMF) is of particular interest for organic farmers, as it offers an alternative to inorganic fertilizers due to their capacity to increase nutrient uptake and plant growth (Verbruggen *et al*. 2013). Arbuscular mycorrhizal fungi increase root volume and help plants to take up nutrients (*e.g.*, phosphorus, nitrogen, sulfur) and water. In addition, they protect plants against root pathogens and adverse environmental (biotic and abiotic) conditions (Rillig *et al*. 2016), while actively collaborating in maintaining soil structure (Berruti *et al*. 2016). In return, plants provide the symbiotic fungus with carbon and a protective niche. Besides, according to van der Heijden (1998), the belowground diversity of AMF determines aboveground plant biodiversity, productivity and ecosystem variability.

Many authors (Dai *et al*. 2013; Oehl *et al*. 2010) have reported the impact of land use and management on AMF diversity. The richness and composition of AMF can also vary depending on the specific climatic and edaphic conditions (Tedersoo 2017). In this respect, in a study by Williams *et al*. (2012), plants inoculated with native AMF (those that were originally extracted from the same soil where they were subsequently inoculated) showed a better performance than those inoculated with non-native AMF. Johnson *et al*. (2010) indicated that the edaphic origin of the AMF should be considered when managing for their benefits in agriculture.

The specific questions investigated in this study were: (Q1) Does organic farming affect soil fungal and AMF community structure, compared to non-cultivated soil? (Q2)

Does AMF inoculation affect the yield and nutritional quality of lettuce crops? If so, is such an effect influenced by the origin (native *vs.* non-native) of the inoculum? (Q3) Does AMF inoculation modify the abundance of natural AMF and the composition of natural fungal and AMF communities? To this purpose, we characterized the taxonomic composition of fungal and AMF communities from three organic orchard soils as well as from three adjacent non-cultivated soils through ITS metabarcoding. Afterwards, we multiplied the AMF communities from the orchard and non-cultivated soils using trap plants. Finally, a microcosm experiment was carried out to study the effect of inoculation with these multiplied AMF communities on (i) lettuce yield and nutritional quality (two consecutive crops), and (ii) fungal and AMF communities.

8.2. Materials and methods

8.2.1. Soil sampling and analysis

Soil was sampled from three cultivated vegetable orchards under organic management, as well as from three corresponding adjacent/contiguous non-cultivated semi-natural grassland areas (6 soil samples in total). The three organic orchards were located in the Basque Country (northern Spain): one of them under oceanic climate (Larrabetzu, province of Biscay) and two under Mediterranean climate (Monasterioguren and Salcedo, province of Araba). These soils are periodically fertilized with manure and managed with minimal tillage. At the time of sampling, a variety of vegetables (cabbage, leek, endive) were growing in the organic orchards.

Each sample consisted of a composite of 10 soil cores taken randomly (10 cm depth, 23 mm diameter). Soil samples were immediately taken to the laboratory and sieved to <2 mm. Subsamples were taken for DNA analysis and kept at -20ºC (see below). The remaining soil was air-dried at room temperature until constant weight and kept at 4ºC. Then, the following physicochemical properties were determined: particle size distribution was estimated by laser diffractrometry; soil pH was measured with a pH-meter in a soil suspension with deionized water (1:2.5, w:v); soil organic matter was determined by elemental analysis of carbon after dry combustion (LECO TruSpec CHN-S, LECO Corp., USA) according to ISO 10694 (1995) and subtracting carbonates; total nitrogen content was also determined by elemental analysis following ISO 13878 (1998);

Olsen P was measured following Watanabe and Olsen (1965); and electrical conductivity was measured according to ISO 11265 (1994).

The physicochemical properties of the six different soils initially characterized here are shown in Table 8.1. The soil from the Monasterioguren orchard and from its adjacent non-cultivated area had a lower and higher proportion of sand (coarse + fine) and clay, respectively, compared to Larrabetzu and Salcedo orchard and adjacent soils. Soil pH values were over 8 for Larrabetzu and Salcedo soils (lower values were detected in Monasterioguren soils). Values of organic matter content varied between 1.78% (Larrabetzu orchard soil) and 6.72% (Monasterioguren adjacent non-cultivated soil). Soil nitrogen content was lower in Larrabetzu and Salcedo soils, compared to Monasterioguren soils. By contrast, phosphorus levels were relatively similar in all soils.

Table 8.1. Physicochemical characterization of the studied soils. LO: Larrabetzu orchard; LA: Larrabetzu adjacent non-cultivated; MO: Monasterioguren orchard; MA: Monasterioguren adjacent non-cultivated; SO: Salcedo orchard; SA: Salcedo adjacent non-cultivated

	LO	**LA**	**MO**	**MA**	**SO**	**SA**
Coarse sand (%)	11.98	5.66	3.66	12.55	4.01	10.5
Fine sand (%)	42.33	44.25	10.22	12.71	33.47	29.2
Silt (%)	34.29	37.4	49.2	42.38	38.74	38.68
Clay (%)	11.4	12.69	36.92	32.06	23.78	21.62
Classification	Sandy-loam	Medium loam	Silty-clay-loam	Clay-loam	Medium loam	Medium loam
pH 1:2.5	8.37	8.39	7.7	6.61	8.58	8.76
Organic matter (%)	1.78	5.35	4.39	6.72	2.66	2.15
Nitrogen (%)	0.13	0.19	0.26	0.47	0.16	0.14
Phosphorus (mg kg^{-1})	28.12	21.37	21.64	31.45	31.08	28.46
Conductivity (mS cm^{-1})	0.13	0.15	0.14	0.22	0.2	0.19

8.2.2. Characterization of fungi

DNA extraction from soil and the preparation of fungal amplicon libraries were carried out as described in Lanzén *et al*. (2015). Soil DNA was extracted using PowerSoil DNA Isolation kit (Mo-Bio Laboratories, Carlsbad, CA, USA). The primers ITS1F (CTTGGTCATTTAGAGGAAGTAA; Gardes and Bruns, 1993) and ITS2R (GCTGCGTTCTTCATCGATGC; White *et al*. 1990) were used for amplification of fungi, using a dual-indexed adapter-linked protocol based on D'Amore *et al*. (2016) with five random nucleotides inserted between the linker and forward primer (5NDI), as described by Schirmer *et al*. (2015). In order to minimize PCR drift, in the first PCR,

triplicates were made for each sample and then pooled. Pair-ended sequencing was carried out using an Illumina MiSeq with the V2 kit at Tecnalia Corporation, Spain.

Sequence data processing and taxonomic classification were performed as follows: read-pairs were quality-filtered and overlapped using vsearch (Rognes *et al*. 2016; options fastq_maxdiff = 5). Overlapped sequences were trimmed to remove primers and 5NDI using cutadapt (Martin 2011), discarding non-matching sequences, and thereafter they were quality filtered and trimmed using vsearch (option fastq_maxee = 0.5), discarding shorter sequences. Quality-filtered overlapped sequences were then clustered into OTUs (ultimately error-corrected exact sequence variants) using Swarm v2 (Mahé *et al*. 2015). Swarm OTUs were subjected to reference based and then *de novo* chimera filtering, using UCHIME as implemented in vsearch. Remaining chimera-filtered Swarm OTUs were then further clustered into OTUs based on overall sequence similarity (minimum 97%) using vsearch. OTU abundances were obtained by mapping reads back to the representative OTU sequences, again vsearch (Rognes *et al*. 2016). Taxonomic classification was carried out by aligning representative OTU sequences to the UNITE reference database using blastn (v.2.6.0+ task megablast) and then analysed using CREST LCAClassifier (v3.0.3) with default parameters (Lanzén *et al*. 2012). Unclassified OTUs were excluded from further analysis. Statistical analyses were based on relative taxon abundances derived by CREST. When the analyses were focused on AMF, only the relative taxon abundances of the Glomeromycota division were considered.

8.2.3. AMF multiplication

Trap plants (sorghum-*Sorghum bicolor* L. Moench and alfalfa-*Medicago sativa* L.) were used for the multiplication of AMF from the three orchard soils and the three adjacent non-cultivated soils. To this purpose, sampled soil was mixed (1:4 w/w) with sterile (autoclaved twice – two consecutive days – for 15 minutes at 120ºC) sand. Subsequently, Osmocote Pro 5-6M 17-11-10+2MgO+TE (ICL Specialty Fertilizers) slow release fertilizer was applied (0.5 g kg^{-1} dose) to such mixtures. Plant seeds were surface sterilized with a 2x-diluted sodium hypochlorite solution, and rinsed several times with deionized water. Plants were grown under controlled conditions in a growth chamber: 14/10 h light/dark cycle, 20/16°C day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 μmol photon m^{-2} s^{-1}. Throughout the

experimental period, plants were bottom watered periodically as needed. After 6 months of plant growth, AMF spores were isolated by wet sieving (Gerdemann and Nicolson 1963). AMF colonized roots were stained with 0.05% Trypan blue solution (Phillips and Hayman, 1970) for examination under an optical microscope. We quantified 84-218 spores g^{-1} of soil. According to the method by McGonigle *et al*. (1990), the percentage of AMF root colonization was over 66%. These data confirmed the right multiplication of AMF under our experimental conditions. Finally, rhizosphere samples from trap plants were taken and maintained in plastic bags at 4°C for one month.

8.2.4. Microcosm experiment

A microcosm experiment, under the same controlled conditions described above, was performed to assess the effects of AMF inoculation on lettuce yield and nutritional quality, as well as on soil AMF communities. Seven AMF inoculation treatments, in triplicate, were studied: (1) control: no AMF inoculation; (2) inoculation with AMF multiplied from the Larrabetzu orchard; (3) inoculation with AMF multiplied from a non-cultivated area adjacent to the Larrabetzu orchard; (4) inoculation with AMF multiplied from the Monasterioguren orchard; (5) inoculation with AMF multiplied from a non-cultivated area adjacent to the Monasterioguren orchard; (6) inoculation with AMF multiplied from the Salcedo orchard; (7) inoculation with AMF multiplied from a non-cultivated area adjacent to the Salcedo orchard. For all treatments, lettuce plants were grown in soil collected from the Monasterioguren orchard. Rhizospheric soil, from trap plants containing around 25,000 AMF spores (the amount of soil was variable, between 114 and 301 grams of soil, as it depended on spore abundance; the influence of this variability on the amount of soil for the results obtained was not statistically significant), was added as inoculum to a mixture of non-sterilized Monasterioguren soil (2 kg minus the quantity of soil used for the inoculum) and sand (500 g). The mixtures were placed in 3 L pots and lettuce seeds were directly sown. Two consecutive lettuce crops were grown in the same pots, each lasting for approximately 7 weeks.

At the end of each crop cycle, lettuce shoots were cut and dried at 70ºC for 48 h to then determine dry weight as indicator of plant yield. Then, shoots were milled and digested with a mixture of $HNO_3/HClO_4$ (Zhao *et al*. 1994), prior to the determination of the following elements by inductively coupled plasma atomic emission spectrometry (ICP-AES): aluminum, phosphorus, calcium, magnesium, sodium, potassium, sulfur,

iron, manganese and molybdenum. Total nitrogen content was measured following ISO 13878 (1998).

After each crop cycle, a representative soil sample was taken from each pot, air-dried at room temperature, and sieved to <2 mm. In these soil samples, the protein related to the easily extractable glomalin was determined following Jorge-Araújo *et al*. (2015).

At the end of the microcosm experiment, the diversity of fungi and AMF was studied in root samples from the following selected treatments: (i) control: no AMF inoculation – treatment 1 above; (ii) inoculation with AMF from a non-cultivated area adjacent to the Larrabetzu orchard – treatment 3 above; and (iii) inoculation with AMF from a non-cultivated area adjacent to the Monasterioguren orchard – treatment 5 above. These three treatments were chosen because after the second crop cycle, they showed significantly higher values of lettuce yield, compared to control treatment (see Figure 8.2 below). To this purpose, lettuce roots were thoroughly washed with distilled water and, then, ground in liquid nitrogen using a pestle and mortar. DNA was extracted from ground lettuce roots with NucleoSpin® Plant II kit (Macherey Nagel). Once the DNA was extracted, fungal and AMF diversity was determined as described above.

8.2.5. Statistical analysis

Diversity indices and rarefied richness were calculated using the R package vegan (Oksanen *et al*. 2015). The mycorrhizal growth response (MGR) was calculated as the ratio of the shoot dry weights of mycorrhizal and nonmycorrhizal plants (Eddiwal, 2015). For alpha diversity values and relative taxon abundances, one-way ANOVA analyses and Tukey's HSD-tests were performed to establish significant differences among groups (*i.e.*, among the different soils initially characterized, and among the soils from the microcosm experiment). Venn diagrams were used to determine the shared number of Glomeromycota OTUs among treatments from the microcosm experiment (Oliveros 2007-2015).

8.3. Results

8.3.1. Characterization of fungal and AMF community

Regarding fungal alpha diversity values, orchard soils showed lower values than adjacent non-cultivated soils (*i.e.*, on average ± standard error, rarefied richness = 401 ± 18 and

524 ± 61; Shannon index = 2.9 ± 0.1 and 3.5 ± 0.4; Simpson index = 0.8 ± 0.0 and 0.9 ± 0.0, for orchard and adjacent soils, respectively), although the differences were not statistically significant. Figure 8.1a shows the relative abundances of different fungal phyla. Ascomycota was the most abundant phylum in orchard soils. Zygomycota ($p<0.006$) and Glomeromycota ($p<0.04$) had a significantly lower relative abundance in orchard soils, compared to adjacent non-cultivated soils.

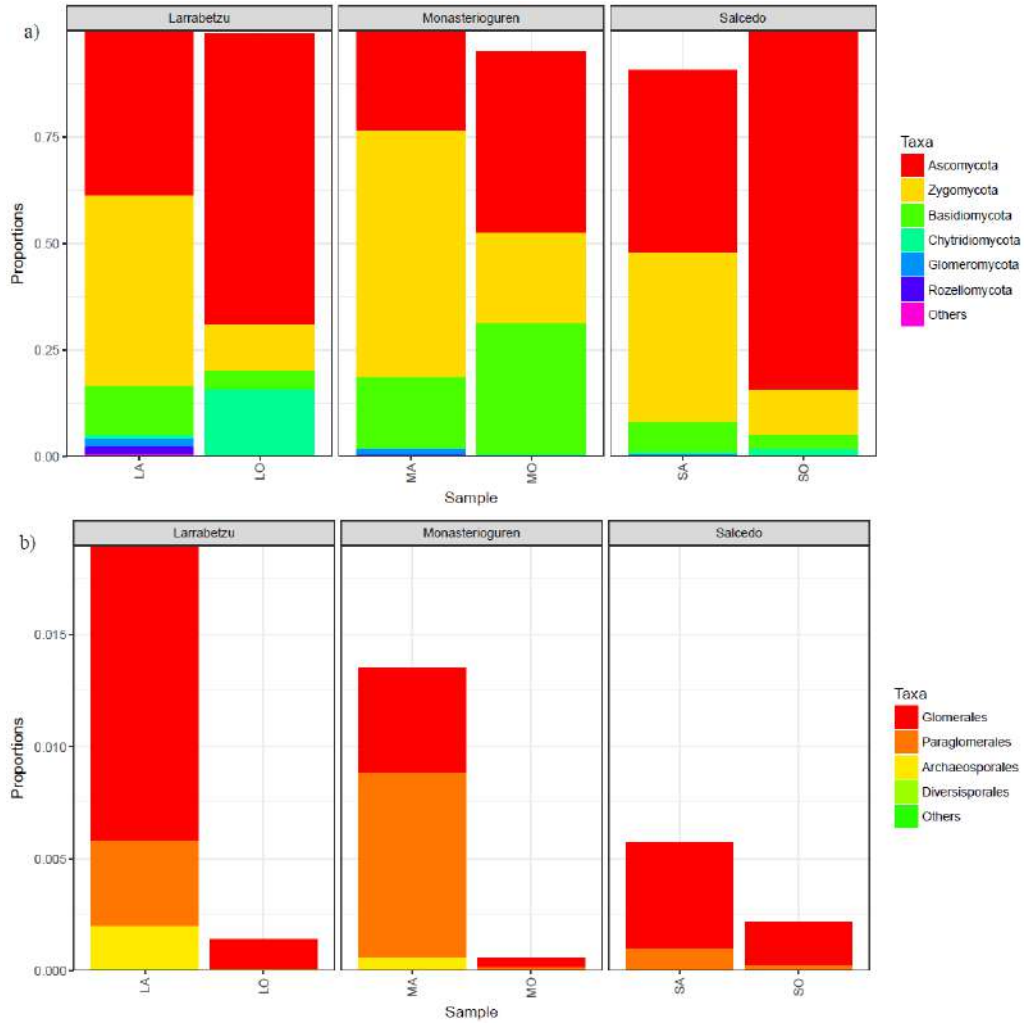


Figure 8.1. Relative abundances with respect to the total sequences obtained of (a) fungal phyla and (b) Glomeromycota orders in the studied soils. LO: Larrabetzu orchard; LA: Larrabetzu adjacent non-cultivated; MO: Monasterioguren orchard; MA: Monasterioguren adjacent non-cultivated; SO: Salcedo orchard; SA: Salcedo adjacent non-cultivated.

Regarding Glomeromycota, lower values of rarefied richness (10 *vs.* 11), Shannon index (2.1 *vs.* 2.6) and Simpson index (0.8 *vs.* 0.9) were observed in orchard soils than in adjacent non-cultivated soils, but again the differences were not statistically significant. As seen in Figure 8.1b, the relative abundance of Glomeromycota orders was lower in

orchard soil, compared to adjacent non-cultivated soils (Glomerales was the most abundant order). At lower taxonomic levels, the relative abundance of the genus *Paraglomus* ($p<0.002$) and the species *Paraglomus laccatum* ($p<0.02$) were significantly lower in orchard soils.

8.3.2. Microcosm experiment

Regarding lettuce yield, no significant differences between treatments were observed after the first crop cycle (Figure 8.2). However, after the second crop cycle, lettuce yield (in terms of shoot dry weight) was significantly lower in control non-inoculated pots, compared to pots inoculated with AMF from the Larrabetzu orchard, from the non-cultivated area adjacent to the Larrabetzu orchard, and from the non-cultivated area adjacent to the Monasterioguren orchard (Figure 8.2); the MGR of these treatments was 2.08, 1.88 and 2.42, respectively.

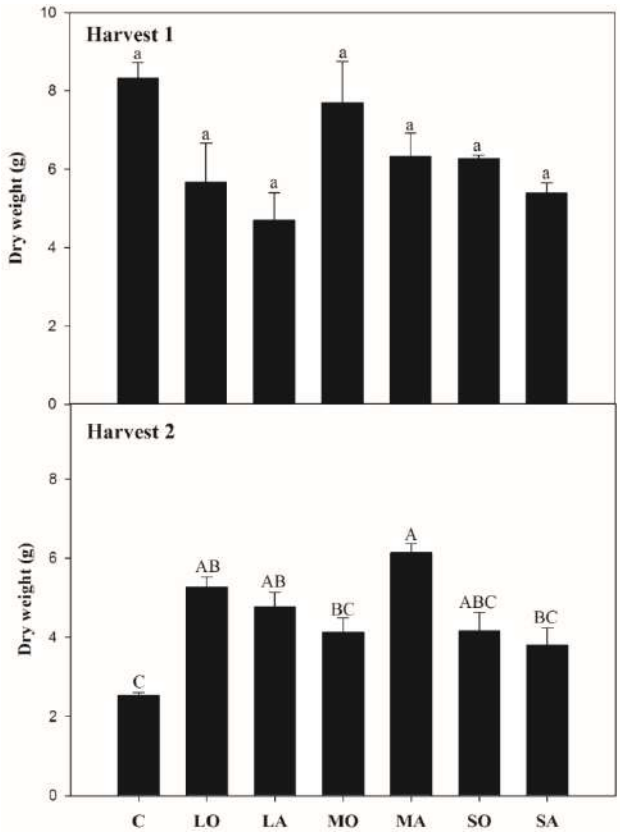


Figure 8.2. Effect of treatments on lettuce yield. Mean values (n = 3) ± standard errors. Bars with different lower case (first harvest) and upper case (second harvest) letters are significantly different (P < 0.05 or lower) according to Tukey's post-hoc test. C: control; LO: Larrabetzu orchard; LA: Larrabetzu adjacent non-cultivated; MO: Monasterioguren orchard; MA: Monasterioguren adjacent non-cultivated; SO: Salcedo orchard; SA: Salcedo adjacent non-cultivated.

As far as nutritional quality was concerned, after the first crop cycle, control non-inoculated lettuces had a significantly higher calcium concentration than those inoculated with AMF from both Monasterioguren soils (Table 8.2). Also, control non-inoculated lettuces had a significantly lower sulfur concentration than those inoculated with AMF from the Salcedo non-cultivated adjacent soil. After the second crop cycle, control non-inoculated lettuces had a significantly lower concentration of magnesium than those inoculated with AMF from the Monasterioguren non-cultivated adjacent soil. In addition, control non-inoculated lettuces had, in general, a significantly higher concentration of sulfur than lettuces grown under the other treatments.

No major differences between control non-inoculated soil and AMF inoculated soil were found regarding protein related to the easily extractable glomalin (Table 8.3). No clear trend was observed among AMF inoculated treatments regarding the effect of origin (Larrabetzu *vs.* Monasterioguren *vs.* Salcedo) or management (orchard *vs.* adjacent non-cultivated).

Table 8.2. Effect of treatments on lettuce nutritional quality. Mean values (n = 3) ± standard errors. Values followed with different lower case (first crop cycle) and upper case (second crop cycle) letters are significantly different (P < 0.05 or lower) according to Tukey's post-hoc test.

TREATMENT	N (%)	Al (g kg^{-1})	P (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Na (g kg^{-1})	K (g kg^{-1})	S (g kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Mo (mg kg^{-1})
AFTER THE FIRST CROP CYCLE											
Control	**2.9 ± 0.1^{a}**	**5.01 ± 1.37^{a}**	**5.2 ± 0.4^{ab}**	**30 ± 2^{b}**	**4.0 ± 0.1^{a}**	**4.1 ± 0.3^{ab}**	**35 ± 2^{ab}**	**3.9 ± 0.3^{ac}**	**2784 ± 834^{a}**	**69 ± 5^{a}**	**0.49 ± 0.03^{a}**
Larrabetzu orchard	3.6 ± 0.1^{a}	2.32 ± 0.71^{a}	5.0 ± 0.2^{ab}	19 ± 1^{ac}	3.5 ± 0.2^{a}	4.0 ± 0.2^{ab}	34 ± 1^{a}	4.0 ± 0.1^{abc}	1635 ± 490^{a}	189 ± 82^{a}	0.51 ± 0.17^{a}
Larrabetzu adjacent	3.5 ± 0.2^{a}	4.01 ± 0.04^{a}	4.4 ± 0.0^{a}	29 ± 4^{ab}	3.4 ± 0.0^{a}	3.4 ± 0.2^{a}	35 ± 0^{ab}	3.5 ± 0.1^{a}	2651 ± 45^{a}	72 ± 3^{a}	0.49 ± 0.03^{a}
Monasterioguren orchard	3.5 ± 0.4^{a}	2.01 ± 0.80^{a}	5.8 ± 0.2^{b}	18 ± 1^{c}	3.9 ± 0.3^{a}	5.6 ± 0.4^{b}	36 ± 2^{ab}	4.5 ± 0.1^{bc}	1302 ± 488^{a}	56 ± 3^{a}	0.67 ± 0.1^{a}
Monasterioguren adjacent	3.6 ± 0.2^{a}	2.37 ± 0.61^{a}	5.1 ± 0.1^{ab}	17 ± 1^{c}	3.8 ± 0.0^{a}	4.8 ± 0.2^{ab}	43 ± 2^{b}	4.7 ± 0.1^{bc}	1223 ± 273^{a}	45 ± 2^{a}	0.85 ± 0.12^{a}
Salcedo orchard	3.7 ± 0.2^{a}	3.15 ± 0.51^{a}	5.7 ± 0.3^{ab}	22 ± 1^{abc}	3.6 ± 0.1^{a}	4.7 ± 0.1^{ab}	32 ± 1^{a}	4.2 ± 0.1^{abc}	2255 ± 404^{a}	70 ± 4^{a}	1.05 ± 0.11^{a}
Salcedo adjacent	4.1 ± 0.2^{a}	2.01 ± 0.41^{a}	5.8 ± 0.1^{b}	20 ± 1^{abc}	3.9 ± 0.2^{a}	5.4 ± 0.4^{b}	36 ± 2^{ab}	4.9 ± 0.1^{b}	1233 ± 270^{a}	51 ± 6^{a}	1.00 ± 0.01^{a}
AFTER THE SECOND CROP CYCLE											
Control	**2.0 ± 0.1^{A}**	**1.80 ± 0.55^{AB}**	**5.8 ± 0.2^{AB}**	**25 ± 5^{AB}**	**4.1 ± 0.2^{A}**	**8.9 ± 0.3^{A}**	**33 ± 1^{A}**	**5.2 ± 0.1^{B}**	**938 ± 283^{AB}**	**62 ± 11^{A}**	**1.12 ± 0.21^{A}**
Larrabetzu orchard	2.4 ± 0.3^{A}	0.68 ± 0.24^{AB}	5.1 ± 0.2^{AB}	16 ± 1^{A}	3.5 ± 0.3^{A}	9.9 ± 2.1^{A}	36 ± 3^{A}	3.8 ± 0.2^{A}	407 ± 123^{AB}	54 ± 21^{A}	0.62 ± 0.15^{A}
Larrabetzu adjacent	2.6 ± 0.3^{A}	1.15 ± 0.34^{AB}	4.8 ± 0.1^{AB}	18 ± 1^{AB}	3.6 ± 0.1^{A}	8.0 ± 1.0^{A}	47 ± 1^{A}	3.6 ± 0.1^{A}	658 ± 124^{AB}	26 ± 2^{A}	0.69 ± 0.00^{A}
Monasterioguren orchard	2.1 ± 0.0^{A}	0.95 ± 0.17^{AB}	5.7 ± 0.4^{AB}	23 ± 3^{AB}	4.9 ± 0.5^{AB}	10.6 ± 1.1^{A}	41 ± 6^{A}	4.4 ± 0.3^{AB}	527 ± 109^{AB}	36 ± 3^{A}	1.22 ± 0.08^{A}
Monasterioguren adjacent	2.5 ± 0.1^{A}	3.05 ± 0.90^{A}	5.2 ± 0.1^{AB}	33 ± 5^{B}	6.1 ± 0.2^{B}	11.2 ± 0.7^{A}	32 ± 0^{A}	3.8 ± 0.1^{A}	1534 ± 390^{A}	54 ± 6^{A}	1.04 ± 0.04^{A}
Salcedo orchard	2.5 ± 0.2^{A}	0.74 ± 0.08^{AB}	6.0 ± 0.5^{B}	17 ± 1^{AB}	3.8 ± 0.3^{A}	10.4 ± 1.5^{A}	50 ± 11^{A}	4.4 ± 0.3^{AB}	385 ± 39^{AB}	28 ± 1^{A}	0.77 ± 0.03^{A}
Salcedo adjacent	3.2 ± 0.5^{A}	0.29 ± 0.09^{B}	4.3 ± 0.2^{A}	16 ± 1^{A}	4.7 ± 0.3^{AB}	13.5 ± 0.8^{A}	46 ± 7^{A}	3.4 ± 0.1^{A}	178 ± 41^{AB}	16 ± 2^{A}	0.80 ± 0.06^{A}

Table 8.3. Effect of treatments on protein related to the easily extractable glomalin. Mean values (n = 3) ± standard errors. Values followed with different lower case (first crop cycle) and upper case (second crop cycle) letters are significantly different (P < 0.05 or lower) according to Tukey's post-hoc test.

TREATMENT	Easily extractable glomalin (mg kg^{-1})
AFTER THE FIRST CROP CYCLE	
Control	**894 ± 24^{ab}**
Larrabetzu orchard	1143 ± 110^{a}
Larrabetzu adjacent	893 ± 31^{ab}
Monasterioguren orchard	904 ± 48^{ab}
Monasterioguren adjacent	850 ± 20^{b}
Salcedo orchard	818 ± 12^{b}
Salcedo adjacent	804 ± 13^{b}
AFTER THE SECOND CROP CYCLE	
Control	**975 ± 15^{AB}**
Larrabetzu orchard	1113 ± 89^{A}
Larrabetzu adjacent	959 ± 9^{AB}
Monasterioguren orchard	984 ± 33^{AB}
Monasterioguren adjacent	978 ± 25^{AB}
Salcedo orchard	855 ± 20^{B}
Salcedo adjacent	879 ± 18^{B}

As abovementioned, after the second lettuce crop cycle, the diversity of root fungi and AMF was studied in three selected treatments: (i) control: no AMF inoculation; (ii) inoculation with AMF from a non-cultivated area adjacent to the Larrabetzu orchard; and (iii) inoculation with AMF from a non-cultivated area adjacent to the Monasterioguren orchard. In this case, no significant differences were observed among treatments in terms of fungal or AMF alpha diversity, obtaining the following mean values for all three treatments for fungi and AMF, respectively: rarefied richness = 88.3 and 5.0, Shannon index = 1.8 and 1.3 and Simpson index = 0.7 and 0.6. In terms of composition, the Venn diagram (Figure 8.3) shows that a high number (44.8 and 66.7% for fungi and AMF, respectively) of the OTUs were shared among the three treatments. Interestingly, AMF inoculum treatments contained >13% of unique fungal OTUs. The most abundant fungal taxa were Olpidiaster, *Rhizophagus* and Glomeraceae (Figure 8.4a), the last two being the most abundant among the AMFs (Figure 8.4b). No statistically significant differences were observed between treatments in terms of relative abundance.

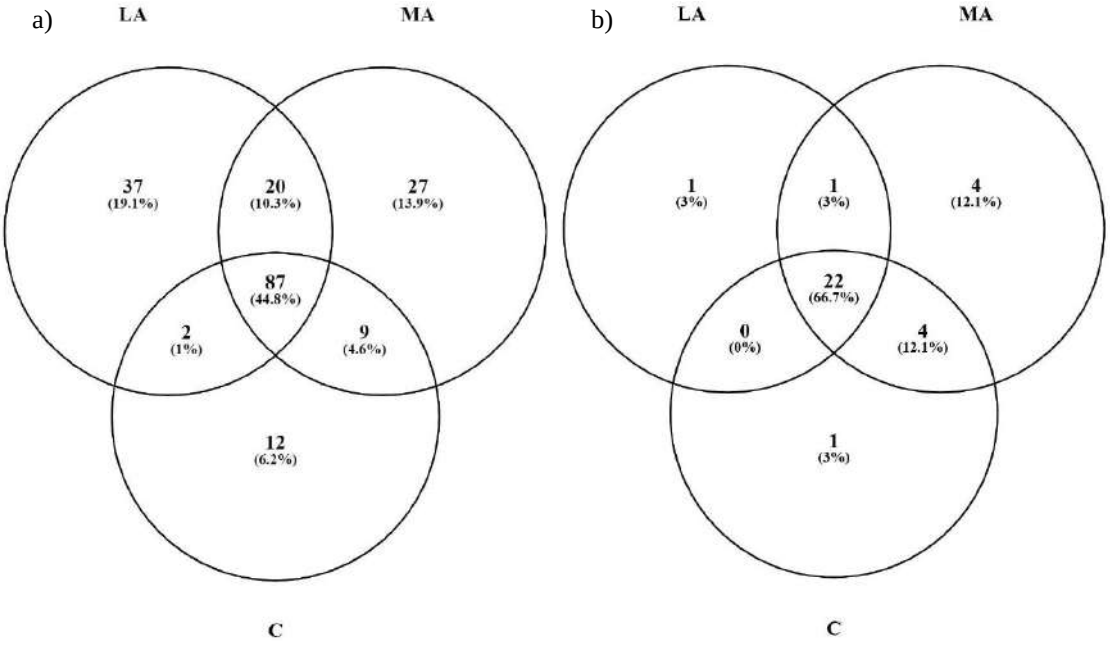
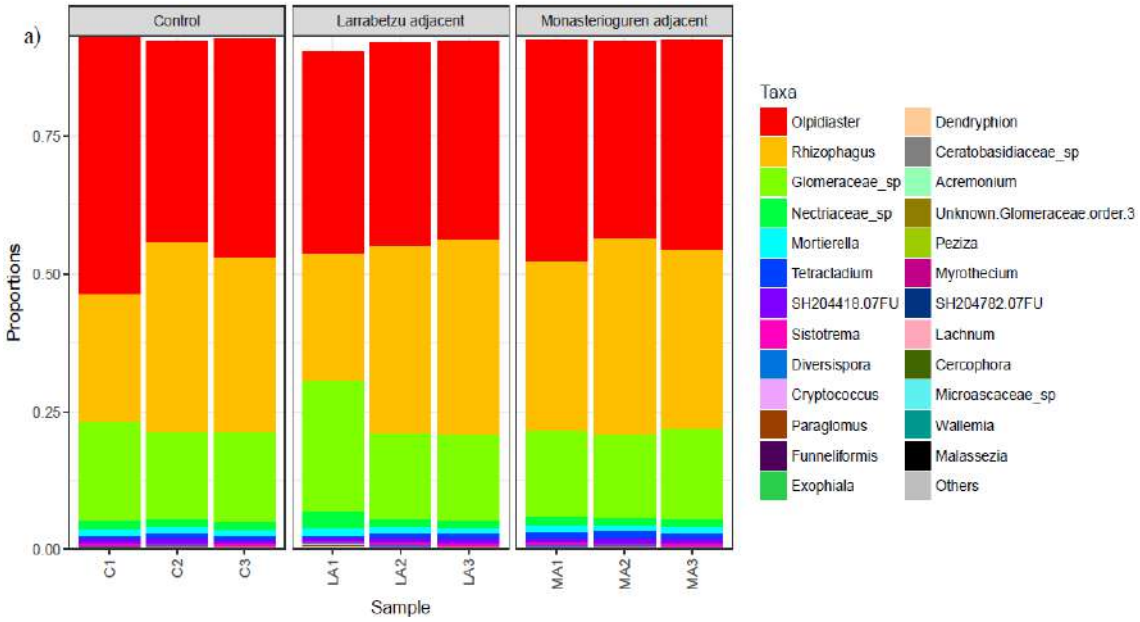


Figure 8.3. Venn diagrams of (a) fungal and (b) Glomeromycota OTUs subjected to different AMF treatments. C: control: no AMF inoculation; LA: inoculation with AMF from a non-cultivated area adjacent to the Larrabetzu orchard; MA: inoculation with AMF from a non-cultivated area adjacent to the Monasterioguren orchard.



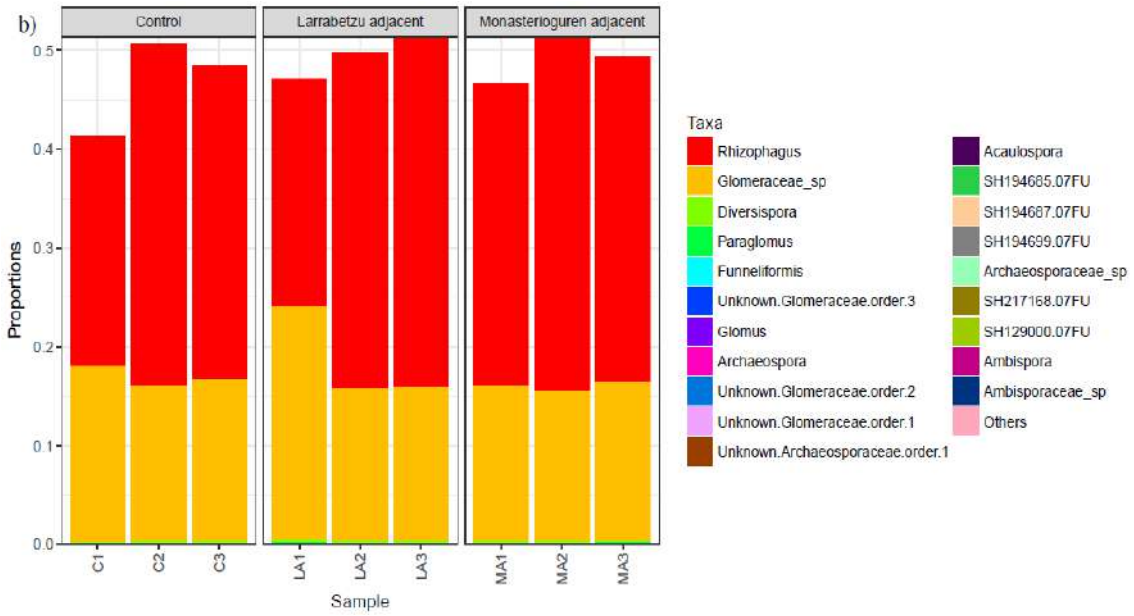


Figure 8.4. Relative abundances of (a) fungi and (b) Glomeromycota at the order level. C: control: no AMF inoculation; LA: inoculation with AMF from a non-cultivated area adjacent to the Larrabetzu orchard; MA: inoculation with AMF from a non-cultivated area adjacent to the Monasterioguren orchard.

8.4. Discussion

8.4.1. (Q1) Does organic farming affect soil fungal and AMF community structure, compared to non-cultivated soil?

In organic orchard soils, Glomeromycota and Zygomycota showed a significantly lower relative abundance, compared to adjacent non-cultivated soils. More specifically, the relative abundance of the genus *Paraglomus* was significantly lower in orchard soils. Previous studies have also reported that more intensive agricultural management appears to impact negatively on *Paraglomus* spp. (Gosling *et al*. 2014). Moreover, fungal alpha diversity values in general and Glomeromycota in particular were lower in organic orchard soils. A priori, a lower diversity might imply a reduction in the well-known benefits provided by AMF, since the probability of having different species providing a diverse array of traits and benefits, as well as the probability of finding the optimal plant-fungi combination for crop yield, is reduced (Verbruggen *et al*. 2013).

High levels of phosphorus and nitrogen in soil are known to have a negative effect on AMF. Similarly, the use of biocides and the application of intensive tillage can be detrimental for AMF populations (Verbruggen and Kiers 2010a). In this respect, conventional agronomic management has been reported (Colombo *et al*. 2014) to have an

adverse impact on AMF diversity. On the contrary, a priori, one would expect organic management to have a lower impact on AMF than conventional management. There are studies that demonstrate the ability of organic farming to sustain a greater AMF diversity and network complexity, relative to conventional farming (Banerjee *et al*. 2019; Manoharan *et al*. 2017). Verbruggen *et al*. (2010b) observed that the number of AMF taxa was highest in grasslands, intermediate in organically-managed fields, and lower in conventionally-managed fields; in addition, AMF communities of organically-managed fields were more similar to those of natural grasslands than those found under conventional management.

Here, in the organic orchards, Glomerales was the most common order within Glomeromycota. Furthermore, in the microcosm experiment with lettuce, most of the identified AMF belonged to *Rhizophagus* and Glomeraceae, two taxa frequently used in commercial inocula (Berruti *et al*. 2016). AMF belonging to these taxa might be more resistant to disturbances caused by cultivation practices, such as those used in the studied organic orchards. In this respect, one genotype of *Funneliformis mosseae* (which belongs to Glomerales) has been found to have a global distribution, possibly related to its optimum adaptation to agricultural conditions (Rosendahl *et al*. 2009).

8.4.2. (Q2) Does AMF inoculation affect the yield and nutritional quality of lettuce crops? If so, is such an effect influenced by the origin (native vs. non-native) of the inoculum?

After the second harvest, a significant positive effect of AMF inoculation on lettuce yield was observed, as also indicated by the MGR values. AMF play a greater role in stimulating plant growth under conditions of reduced nutrient availability. In contrast, no consistent differences in lettuce nutritional quality were detected. Lekberg and Koide (2005) reported that AMF inoculation increased root colonization by an average of 29%, resulting in a significant plant biomass increase of 23%. In a review paper by Berruti *et al*. (2016), it was described that plant yield and nutrition were improved by AMF inoculation in 84 and 92% of the analysed studies, respectively (81 experiments for plant yield and 112 for plant nutritional status).

In accordance to other studies (Pellegrino *et al*. 2011), in our study, the origin (Larrabetzu *vs.* Monasterioguren *vs.* Salcedo) or management (organic orchard site *vs.* non-cultivated site) did not have a clear influence on the effect of AMF inocula. On the contrary, Williams *et al*. (2012) found that plants inoculated with indigenous AMF had a

significantly greater survival than those inoculated with commercial AMF. Besides, Johnson *et al*. (2010) showed that certain grass ecotypes adapt to their local soil fertility and indigenous AMF communities.

8.4.3. (Q3) Does AMF inoculation modify natural AMF abundance and natural fungal and AMF community composition?

In the microcosm experiment, the abundance of AMF was not modified by AMF inoculation. Equally, the composition of AMF communities was not significantly altered by AMF inoculation. Other studies (Rodríguez-Caballero *et al*. 2017) observed that AMF inoculum (in this case, with *Rhizophagus intraradices*) can modify rhizosphere community while improving plant performance. Then, our results indicate that the stimulation in lettuce growth observed in the microcosm experiment was not caused by an increase in AMF abundance, as a result of AMF inoculation. Rather, the stimulation in lettuce growth might have been due to the inoculum performing a beneficial effect on plant growth in a context of reduced nutrient availability; in any case, our data suggest that the responsible taxa for such effect are not relevant in quantitative terms. By contrast, Verbruggen *et al*. (2013) reported that plant responses to AMF inoculation are, to a large extent, driven by increases in AMF abundance rather than by the introduction of new strains. Another explanation for increased lettuce yield could be related to synergistic effects on soil microbial communities induced by the addition of AMF inoculum. In this regard, the soils that received AMF inoculation showed a relatively high abundance of unique fungal OTUs. However, no statistically significant differences were observed among treatments in terms of relative abundance. Nevertheless, an experimental design with greater statistical power could yield different results.

Finally, the AMF communities from the three orchard soils and adjacent non-cultivated soils were different from those present in the lettuce roots from the microcosm experiment, in agreement with Varela-Cervero *et al*. (2015). In fact, 50% of the total fungi identified in the roots from the microcosm experiment corresponded to Glomeromycota, whereas only 8% of the total fungi corresponded to this taxonomical group in the studied sites (in the three orchard soils and their corresponding adjacent non-cultivated soils). However, the diversity indices were significantly higher in the studied sites. In both cases, it is necessary to take into consideration that the relatively short maximum sequence

length returned by Illumina platforms is a limitation for AMF diversity studies, where commonly used amplicon lengths range from 540 to 1500 bp (Öpik and Davison, 2016).

8.5. Conclusions

Organic orchard management had a negative impact on AMF community composition, leading to reduced alpha diversity and relative abundance of AMF taxa, compared to non-cultivated soil. In this situation, one of the alternatives would be to inoculate the soil with AMF, which could be of native or non-native origin. After the second crop cycle, AMF inoculation led to higher lettuce yields. This stimulation of crop yield was not due to an increase neither in AMF abundance, nor due to a major change in AMF or fungal community composition. The origin of the AMF inoculum did not have a clear influence on its effect on lettuce yield. It was concluded that if we are to facilitate the use of AMF as biostimulants, much research is needed to better predict under which specific conditions and through which mechanisms AMF inoculation will contribute to enhanced crop yield and nutritional quality.

9| SÍNTESIS

9. SÍNTESIS

Los suelos están actualmente sometidos a una fuerte y preocupante presión antrópica. La demanda de este recurso para la producción de alimento, fibra, combustible y soporte físico para la promoción de infraestructuras aumenta muy rápidamente, como consecuencia del acelerado crecimiento demográfico mundial que estima en torno a los 9.700 millones de personas para el año 2050 (UN, 2019). Bajo esta estimación, se ha indicado que la producción de alimentos debe aumentar en un 70% para 2050 en aras de satisfacer la demanda global (ELD, 2015). Este aumento en la producción de alimento, hoy en día, se encuentra supeditado a la mejora del rendimiento productivo por unidad de superficie, el cual deriva de la intensificación agrícola. Ejemplo de ello es el incremento, a nivel global, de la producción de cereales que se vio cuadruplicado entre los años 1961 y 2016, periodo en el que la superficie cultivable a escala global se incrementó del 10,3 al 11,9% (FAO, 2019a). Esta mejora del rendimiento productivo ha sido hasta ahora fruto de los desarrollos asociados a la denominada Revolución Verde, la cual introdujo nuevas variedades de cultivos de alto rendimiento así como la instauración del monocultivo, la mecanización progresiva y generalizada, la mejora en los procesos de irrigación y la incorporación del empleo masivo de productos agroquímicos (plaguicidas y fertilizantes minerales o fertilizantes de síntesis química). Este desarrollo trajo consigo la intensificación de las prácticas agrícolas y el modelo de producción agrícola más común de nuestro tiempo, conocido como agricultura convencional o de altos insumos, cuyas consecuencias ambientales han sido obviadas durante muchos años de forma generalizada en virtud del aumento productivo que ofrecen. Sin embargo, el aumento de la productividad agrícola basado en la intensificación acarrea un aumento en la presión del suelo, agravado por el contexto de cambio climático en el que nos encontramos, que se traduce en la degradación a escala global del ecosistema edáfico, limitando su sostenibilidad funcional y, por ende, su capacidad para proporcionar el variado elenco de servicios ecosistémicos de los que depende el bienestar e incluso la supervivencia del ser humano.

Dentro de los citados servicios ecosistémicos se incluyen los servicios de abastecimiento (el suministro de alimento, madera, fibras y otras materias primas, y el sustento físico), regulación (la mitigación de las inundaciones, el filtrado de nutrientes y contaminantes, el almacenamiento de carbono y la regulación de los gases de efecto invernadero, la eliminación de compuestos contaminantes, el reciclaje de residuos, la

regulación de las poblaciones de plagas y enfermedades, etc.) y culturales (la recreación, la estética del paisaje, los valores patrimoniales, la identidad cultural, etc.) (Dominati *et al*., 2014; Haynes-Young y Potchin-Young, 2018). Cuantitativamente, se estima que el valor de los servicios ecosistémicos proporcionados por el suelo es de unos 11,4 billones de dólares al año (McBratney *et al*., 2017). Desgraciadamente, el valor de estos servicios no está reconocido en los mercados económicos y en las políticas de gestión medioambiental, lo que se antoja inexplicable, dado que las necesidades básicas del ser humano como la alimentación y el mantenimiento de la calidad del aire y el agua no son posibles sin la provisión de dichos servicios. De hecho, se estima que el 98.8% de las calorías diarias consumidas por los seres humanos proceden directa o indirectamente del ecosistema edáfico (FAO, 2019a), lo que denota el vínculo intrínseco entre la disponibilidad de alimentos y el suelo. Por ello, la regulación de los servicios del suelo es crucial para la productividad efectiva de los sistemas agrícolas, ya que los criterios y enfoques utilizados para el manejo del suelo van a incurrir inevitablemente en su capacidad para suministrar dichos servicios. En este sentido, la gestión no-sostenible, como la que deriva de la intensificación agrícola convencional, conduce a una devaluación y deterioro de los servicios del ecosistema edáfico, mientras que la gestión sostenible puede mantenerlos o incluso mejorarlos.

Las prácticas agrícolas tradicionales basadas en la intensificación a base de insumos químicos externos inciden directamente en la salud del ecosistema edáfico mediante la disminución de la cantidad de carbono orgánico en el suelo y el incremento simultáneo de procesos tales como la compactación, la erosión, la salinización, la acidificación, las pérdidas de agua, las emisiones de gases de efecto invernadero y la contaminación de compartimentos ambientales, con el consiguiente impacto adverso sobre la biota edáfica (Pereira *et al*., 2018). Debido a los citados efectos, se estima que el 52% de las tierras agrícolas se encuentran moderada o altamente afectadas por la degradación del suelo (ELD, 2015), lo que se traduce en una pérdida de la productividad y un aumento en el precio de la alimentación a escala global. En particular, los fertilizantes minerales (o de síntesis química), utilizados extensivamente bajo el paradigma de la intensificación agrícola, han causado un importante deterioro del medio ambiente y la biodiversidad. Los fertilizantes minerales más demandados son los fertilizantes nitrogenados, cuya producción anual asciende a la friolera de 120 millones de toneladas al año (FAO, 2019a). Sin embargo, la eficiencia de estas bombas químicas de nitrógeno asimilable no llega al 50%, lo que implica que, como mucho, los cultivos

pueden asimilar y convertir en biomasa la mitad del fertilizante aportado. La cantidad restante es por tanto liberada al medio ambiente, donde ejerce efectos perjudiciales sobre los compartimentos ambientales, incluyendo la eutrofización de masas de agua debido a procesos de lixiviación y escorrentía, la emisión de óxido nitroso (un gas de efecto invernadero cuyo potencial calorífico es 298 veces mayor al del anhídrido carbónico), la acidificación del suelo y la pérdida de la biodiversidad. Además, la producción de los fertilizantes nitrogenados depende de un proceso conocido como Haber-Bosch (una de las innovaciones más significativas del siglo XX), cuya abrumadora demanda energética se abastece mediante el consumo de combustibles fósiles.

La externalización de los procesos naturales y su sustitución sistemática por procesos sintéticos reduccionistas conlleva una disrupción de los ciclos biogeoquímicos e incide negativamente en la biota de los ecosistemas y, por analogía, en su salud. Por ello, la incorporación de modelos alternativos y sostenibles, que desliguen la producción agrícola de los modelos de intensificación tradicional y de la degradación generalizada del medio ambiente, es acuciantemente necesaria. Estos modelos han de basarse en un enfoque sistémico en el que los aspectos ambientales, económicos y sociales converjan, junto con la generación de nuevo conocimiento. La búsqueda de modelos de producción capaces de mejorar el rendimiento productivo sin generar presiones sobre el medio ambiente y la salud de los ecosistemas es uno de los grandes desafíos de las próximas décadas.

Bajo las premisas de la integración de la sostenibilidad y los procesos ecológicos junto con la mejora del rendimiento productivo, la intensificación ecológica representa una alternativa al modelo tradicional, orientada a la mejora de las funciones del ecosistema edáfico a fin de promover la provisión de sus servicios ecosistémicos (Bommarco *et al*., 2013). Este paradigma identifica los requisitos básicos de las prácticas agrícolas para mantener la sostenibilidad y la estabilidad del ecosistema edáfico, mediante la combinación efectiva del uso de herramientas avanzadas de mejora y la implementación de procesos de evaluación y perfeccionamiento de las técnicas agrícolas, donde se incluyen, entre otras, el laboreo mínimo, las prácticas de la agricultura orgánica o ecológica, el mantenimiento de la cubierta vegetal y la diversidad de cultivos (Kragt y Robertson, 2014). Dentro de los requisitos identificados se recogen algunos como la promoción de la biodiversidad, el reciclaje de los nutrientes de los residuos agrícolas, la protección contra la erosión del suelo, la conservación y la protección del agua, la

conservación o mejora del carbono orgánico del suelo, la perturbación mínima de la estructura del suelo, la ausencia de productos contaminantes y fertilizantes sintéticos, etc.

Por otra parte, la agricultura de conservación y la agricultura orgánica son algunos de los modelos incluidos dentro del paradigma de la intensificación ecológica. La primera se rige según tres principios fundamentales: laboreo mínimo, cubierta vegetal continua y rotación de cultivos (Palm *et al*., 2014). Este modelo promueve la preservación de la estructura del suelo con el objetivo de mejorar sus propiedades físicas como la capacidad de retención hídrica y de nutrientes, así como la mínima alteración de las comunidades microbianas. Sin embargo, no establece limitaciones en el uso de agroquímicos. Por su parte, la agricultura orgánica se basa en los procesos ecológicos y en la preservación de la integridad del ecosistema edáfico y su biodiversidad, al objeto de maximizar el suministro de servicios ecosistémicos (Reganold *et al*., 2016). Al contrario que la agricultura de conservación, este modelo sí excluye la utilización de fertilizantes sintéticos, sustituyéndolos por enmiendas orgánicas y contribuyendo a la reutilización y revalorización de los residuos agrícolas (Misselbrook *et al*., 2012). Se trata de un modelo que combina tradición, innovación y ciencia en beneficio del medio ambiente cuyo requerimiento energético es sustancialmente menor al del modelo productivo convencional imperante (Clark y Tilman, 2017).

En lo referente a las enmiendas orgánicas, hemos de volver al inicio de este Capítulo, en referencia al crecimiento demográfico e industrial, para entender que dicho crecimiento también repercute en la generación masiva e incesante de residuos, incluyendo residuos urbanos, agrícolas, industriales, etc. La necesaria transición hacia sistemas de producción más sostenibles exige cambiar el modelo que actualmente prevalece de producción lineal (recurso – producto – residuo) por un modelo circular en el que los residuos y subproductos se integren de manera efectiva en la cadena de valor, de forma que se reduzcan conjuntamente la explotación intensiva de los recursos naturales y la generación de nuevos residuos (Murray *et al*., 2015). Se trata de una transición lógica que las instituciones políticas europeas han tratado de impulsar mediante varios documentos y marcos legislativos, como el Plan de Acción de la UE para la Economía Circular (European Commision, 2015), o la Directiva (UE) 2018/851 sobre residuos, que establece objetivos ambiciosos en materia del reciclaje de residuos tales como la necesidad de que los Estados Miembros reutilicen y reciclen más de un 55% de los residuos municipales generados para el año 2025, y un 65% para 2035 (European Parliament and European Council, 2018). En este sentido, el aprovechamiento y

valorización de los residuos orgánicos mediante su utilización como enmiendas de suelo tiene un gran potencial, ya que plantea una alternativa rentable y ambientalmente racional frente al depósito y almacenamiento de los residuos en vertedero (el último recurso en el principio jerárquico de la gestión de residuos) (European Parliament and European Council, 2018). El amplio rango de residuos orgánicos utilizados como materias primas incluyendo, entre otros, los restos vegetales de los cultivos, las deyecciones ganaderas y los lodos de las estaciones de tratamiento de aguas residuales, se traduce en una amplia gama de enmiendas orgánicas de suelo con distintas propiedades y potencial agronómico. Dada su naturaleza orgánica, todas ellas aportan en mayor o menor medida materia orgánica al ecosistema edáfico, lo cual es fundamental para el mantenimiento de la fertilidad química, física y biológica del suelo (Hijbeek *et al*., 2017). Además, este aporte de materia orgánica contribuye al secuestro de carbono y a la iniciativa internacional "4 por 1000: Los Suelos para la Seguridad Alimentaria y el Clima" adoptada en la COP21 en el año 2015, cuyo objetivo es aumentar las reservas mundiales de carbono orgánico en el suelo un 0,4% anualmente, en aras de compensar las emisiones globales de gases de efecto invernadero y mitigar el cambio climático.

No obstante, al igual que ocurre con los productos agroquímicos, la utilización excesiva de estas enmiendas puede ejercer efectos perjudiciales sobre la salud del ecosistema edáfico y el medio ambiente, tales como emisiones de gases de efecto invernadero, contaminación de masas de agua por exceso de nutrientes, y procesos de acidificación o salinización del suelo (Alvarenga *et al*., 2015). Por otra parte, algunas enmiendas orgánicas pueden albergar ciertos riesgos intrínsecos tales como la presencia de contaminantes tradicionales y/o emergentes (Mohapatra *et al*., 2016). En este sentido, la presencia de antibióticos, bacterias resistentes a antibióticos, genes de resistencia a antibióticos y elementos genéticos móviles implicados en la transferencia horizontal de genes puede promover la resistencia a antibióticos en el medio ambiente, lo que actualmente supone una de las mayores amenazas para la salud mundial (Comisión Europea, 2017). La baja tasa de metabolización de los compuestos antibióticos por parte de los seres humanos y animales de granja convierte residuos orgánicos con alto potencial agronómico (*e.g.*, las deyecciones ganaderas y los lodos procedentes de las plantas de tratamientos de aguas residuales) en reservorios de bacterias y genes resistentes a los antibióticos (Rizzo *et al*., 2013; Zhu *et al*., 2013) cuya aplicación al suelo agrícola supone una vía de exposición y diseminación de dicha resistencia al medio ambiente y, en concreto, a los cultivos de los que posteriormente se alimentará al ganado o al ser humano.

Además, la presencia de metales pesados en las enmiendas orgánicas puede promover la resistencia simultánea a metales y antibióticos, debido al mecanismo evolutivo de co-selección a través de los procesos de co-resistencia (distintos genes que codifican para resistencia a metales y antibióticos se encuentran en el mismo elemento genético) o resistencia cruzada (un mismo gen confiere resistencia a metales y antibióticos) (Baker-Austin *et al*., 2006), lo que puede aumentar la proliferación de resistencias a antibióticos incluso sin la presencia de dichos contaminantes emergentes.

Por tanto, el tratamiento efectivo de los residuos orgánicos es crucial e indispensable a fin de garantizar la sostenibilidad y seguridad a largo plazo de la aplicación de enmiendas orgánicas en la producción de alimentos. A este respecto, el compostaje y la digestión anaerobia son los métodos más comunes de tratamiento de los residuos orgánicos, combinando aspectos de bioseguridad con aspectos ambientales y económicos. El compostaje hace referencia al proceso de degradación biológica de la materia orgánica en condiciones aerobias que da lugar a un producto estable conocido como compost (St. Martin y Brathwaite, 2012). Durante dicho proceso, las fracciones más lábiles de los compuestos orgánicos son metabolizadas y dan paso a moléculas orgánicas más recalcitrantes, lo que dota a la enmienda de una estabilidad que permite que su mineralización en el suelo sea más lenta, manteniendo la fertilidad biológica del suelo a largo plazo (Diacono y Montemurro, 2010). Además, las altas temperaturas alcanzadas durante el compostaje, debido al incremento de la actividad metabólica al principio del proceso, permiten la reducción de la carga de organismos potencialmente patógenos, así como la disminución de los determinantes de resistencia a antibióticos y otros contaminantes orgánicos (Qian *et al*., 2018). Por ello, el compost constituye la enmienda orgánica más utilizada dentro de los sistemas de producción agrícola sostenibles (Scotti *et al*., 2015). Por su parte, la digestión anaerobia es otro proceso de descomposición biológica de la materia orgánica que, de forma contraria al proceso de compostaje, se realiza en condiciones de ausencia de oxígeno, dando lugar a la formación de biogás (principalmente, metano y dióxido de carbono) y a un subproducto orgánico conocido como digestato (Tambone *et al*., 2009). El biogás producido puede aprovecharse como fuente de energía, mientras que el digestato, rico en macro- y micronutrientes y en compuestos orgánicos parcialmente degradados, tiene potencial como enmienda orgánica. Debido a la generación de energía, en los últimos años las plantas de biogás se han convertido en una alternativa atractiva para la gestión sostenible de los residuos en Europa, donde actualmente existen algo más de 18.000 plantas en

funcionamiento (EBA, 2019). El proceso de digestión puede realizarse en condiciones mesófilas o termófilas. Ambos procesos resultan en la higienización efectiva de la enmienda, si bien es verdad que en cuanto a la eliminación de los determinantes de resistencia a antibióticos la digestión termófila es más efectiva (Miller *et al*., 2016). Al igual que en el proceso de compostaje, la estabilización de la materia orgánica puede influir en los procesos de biodisponibilidad de los compuestos orgánicos, incluidos contaminantes, por adsorción (Zheng *et al*., 2019).

Por otro lado, además de los residuos orgánicos, cabe destacar la posibilidad de utilizar otro tipo de agentes promotores del crecimiento vegetal dentro de las prácticas de producción agrícola sostenibles, leáse, los inóculos microbianos o bioestimulantes (tal y como vienen recogidos en el Reglamento UE 1009/2019 sobre fertilizantes). En este sentido, el aislamiento, la caracterización y posterior inoculación de microorganismos con rasgos fenotípicos interesantes desde el punto de vista de la competitividad ecológica y/o la producción agrícola tiene un potencial muy valioso en la agricultura moderna, dada la relevancia de la microbiota edáfica en el mantenimiento de la funcionalidad y la fertilidad del suelo.

Independientemente de la técnica de fertilización utilizada, la promoción de un cambio en el modelo de producción agrícola convencional debe basarse en una mejora efectiva de la salud del ecosistema edáfico, a fin de garantizar la sostenibilidad de un nuevo paradigma basado en la intensificación ecológica. En consecuencia, es indispensable disponer de un conjunto de indicadores fiables y relevantes que nos permitan evaluar la salud del ecosistema edáfico. En este sentido, el papel determinante de la microbiota edáfica en el funcionamiento del suelo convierte a las propiedades microbianas del suelo en los indicadores más sensibles y de mayor relevancia ecológica para la evaluación y monitorización de la salud del ecosistema edáfico.

En este contexto, el presente trabajo se ha centrado en la investigación de los efectos que algunas prácticas propias del paradigma de la intensificación ecológica ejercen sobre el ecosistema edáfico, a través del estudio de parámetros microbianos que reflejan la biomasa, actividad y diversidad de las comunidades microbianas edáficas, así como del potencial agronómico de dichas prácticas, con el objetivo de disminuir o sustituir los insumos procedentes de la síntesis química. Para ello, en el Capítulo 4, estudiamos el efecto de la incorporación al suelo agrícola del rastrojo de maíz como alternativa a su retirada, una práctica habitual dentro de la agricultura de conservación, en un ensayo de 6 años. En comparación con la retirada del rastrojo, la incorporación

mejoró el rendimiento productivo del cultivo de maíz de forma significativa (33,8 Mg ha^{-1} frente a 28,4 Mg ha^{-1} en las parcelas donde el rastrojo fue retirado). Además, la incorporación del rastrojo mantuvo la cantidad de carbono orgánico del suelo en valores constantes desde el comienzo del estudio, mientras que la retirada del rastrojo supuso una reducción significativa en dichos valores, desde el comienzo del estudio (20,1 g kg^{-1}) hasta el tiempo final (14,7 g kg^{-1}). La cantidad de carbono orgánico se correlacionó positivamente con los parámetros de actividad y biomasa microbianas, resultando en un incremento del Índice de la Calidad del Suelo (el cual integra el valor de una variedad de indicadores microbianos; Mijangos *et al*., 2010) del 27% en las parcelas con incorporación frente a las parcelas donde el rastrojo había sido retirado. En cuanto a la diversidad estructural de las comunidades microbianas edáficas, el tipo de manejo del rastrojo no resultó en diferencias significativas, aunque sí se observaron cambios en la composición de taxones procariotas y eucariotas no dominantes. Por otro lado, el tipo de manejo resultó en una diferenciación del perfil fisiológico-metabólico de las comunidades heterótrofas cultivables del suelo estimado con placas Biolog EcoPlates™, por el cual la incorporación del rastrojo aumentó la tasa de utilización de xilosa y manitol.

Sin embargo, como se ha comentado previamente, la agricultura de conservación permite la utilización de fertilizantes de síntesis química. De hecho, en el estudio para la evaluación de los efectos de la gestión de los residuos vegetales sobre el suelo agrícola del Capítulo 4, se utilizó fertilizante mineral a la misma dosis. Por ello, de cara a limitar y/o sustituir la utilización de fertilizantes minerales, en el Capítulo 5 realizamos varios ensayos utilizando una enmienda orgánica obtenida a partir de la fermentación de residuos agrícolas, y evaluando sus efectos sobre la salud del suelo agrícola en comparación con un fertilizante de síntesis química, igualando la cantidad de N aportado por la enmienda orgánica y el fertilizante mineral. Dichos ensayos se realizaron en cámara de crecimiento controlado y en campo, utilizando dos cultivos distintos: la lechuga y el maíz. Además, para testar la reproducibilidad de las enmiendas orgánicas fermentadas, se aplicaron dos enmiendas distintas: una comercial y otra fabricada en una explotación agrícola siguiendo las directrices de la empresa de la que se adquirió la enmienda comercial. Las enmiendas se utilizaron en dos dosis de aporte: igualando la cantidad de nitrógeno a los requerimientos nutricionales demandados por el cultivo; y a una dosis menor, recomendada por la empresa que comercializa la enmienda. Los resultados difirieron según la escala del ensayo y el cultivo utilizado. Así, en los ensayos en campo, la utilización de enmiendas líquidas fermentadas a la cantidad de nitrógeno demandada

por la planta resultó en un rendimiento productivo comparable al obtenido con la fertilización mineral para los cultivos de lechuga y maíz. Dicha aplicación mejoró los parámetros de actividad y biomasa microbianos en comparación al fertilizante mineral, pero solamente en el cultivo de maíz. A este respecto, es importante mencionar que el ensayo en campo con maíz se realizó durante dos campañas y el aporte de enmienda resultó en un aumento de la cantidad de materia orgánica frente al tratamiento mineral, mientras que el ensayo en campo con lechuga se prolongó durante un solo ciclo de cultivo y no mostró dicho aumento en la cantidad de materia orgánica del suelo. Por otro lado, en el ensayo en la cámara de crecimiento controlado con el cultivo de lechuga, la aplicación de enmiendas líquidas fermentadas resultó en rendimientos productivos inferiores a los obtenidos con el fertilizante mineral (incluso igualando la dosis de nitrógeno aportada), pero indujo una mejora en los parámetros de actividad y biomasa microbianas con respecto al tratamiento mineral. Estas diferencias con respecto al cultivo de lechuga entre el ensayo en la cámara de crecimiento y el campo pueden ser debidas a los distintos suelos utilizados en uno y otro ensayo (un suelo arenoso con una cantidad de materia orgánica menor al 1% en el ensayo de campo, frente a un suelo franco-limoso con una cantidad de materia orgánica de entre el 1 y el 2%). En cuanto a la diversidad estructural de las comunidades procariotas edáficas, el aporte de enmienda orgánica fermentada únicamente mejoró los parámetros de alfa-diversidad en el ensayo en campo con lechuga. Por el contrario, en lo que respecta a la composición de la comunidad microbiana, el único ensayo donde el tipo de fertilización supuso una alteración significativa de la comunidad fue el ensayo en cámara de crecimiento. Estos resultados denotan ciertas mejoras en cuanto a la salud del ecosistema edáfico derivadas de la utilización de enmienda con respecto a la fertilización mineral; sin embargo, los resultados varían enormemente según la escala del ensayo, el tipo de suelo utilizado y la dosis de aporte de enmienda.

En el Capítulo 6 evaluamos los efectos de la utilización de otro tipo de residuo orgánico ampliamente utilizado en las prácticas de agricultura orgánica, el estiércol. Sin embargo, debido a los problemas derivados de su utilización en fresco en la emergencia y diseminación de resistencias a antibióticos, sometimos dos estiércoles de distinto origen (estiércol equino y gallinaza) a distintos grados de maduración (fresco, compost y bokashi) y los aplicamos a un suelo agrícola para la evaluación de la salud del suelo, el estudio del rendimiento productivo de las distintas enmiendas, y la abundancia de varios genes de resistencia a antibióticos y elementos genéticos móviles. En este sentido, las

enmiendas frescas ofrecieron un mayor rendimiento productivo, así como una mayor actividad y biomasa microbianas. Como se ha comentado anteriormente, la maduración de los residuos orgánicos conlleva la descomposición de los compuestos más lábiles, incorporando al suelo una enmienda más recalcitrante que se mineraliza de forma mucho más lenta, contribuyendo así a la fertilidad del suelo a largo plazo. A este respecto, un cultivo de ciclo corto como la lechuga necesita una mineralización más rápida de los sustratos orgánicos aportados por la enmienda. Además, una fuente de carbono más lábil puede suponer el crecimiento masivo de algunos microorganismos saprófitos o incluso el enriquecimiento selectivo, lo que puede ser la razón del aumento en la actividad y biomasa microbianas. Este hecho también puede ser el causante de la menor diversidad estructural observada en las enmiendas frescas. En lo que a contaminación potencial se refiere, ninguna de las enmiendas implicó una concentración de metales pesados superior a los valores VIE-B de la Ley 4/2015 de contaminación del suelo del País Vasco. No obstante, todas las enmiendas orgánicas condujeron al aumento de la abundancia relativa de, al menos, un gen de resistencia a antibióticos y un elemento genético móvil, en comparación con el suelo sin enmendar. En cualquier caso, el grado de maduración del estiércol se tradujo en una reducción muy significativa en la abundancia de genes de resistencia a los antibióticos, en comparación con el estiércol fresco, sobre todo con respecto a la gallinaza fresca que mostró un aumento significativo en la abundancia de todos los genes estudiados frente al suelo sin enmendar. En este sentido, la aplicación de una enmienda más estable, como el compost o el bokashi, puede suponer una pérdida en cuanto al rendimiento productivo, pero minimiza el riesgo para la salud humana y del medio ambiente que supone la utilización del estiércol fresco como fuente de fertilización agrícola.

De la misma forma que el estiércol, los lodos provenientes del tratamiento de aguas residuales también han sido descritos como reservorios de resistencias a los antibióticos (Pruden *et al*., 2013), además de contener cantidades de otros contaminantes como, por ejemplo, metales pesados (McBride *et al*., 1997). Sin embargo, debido a su riqueza en nutrientes asimilables por las plantas y materia orgánica, su valorización como enmienda orgánica es una práctica agrícola bastante común. De hecho, según los datos del Registro Nacional de Lodos (2012), aproximadamente el 80% de los lodos de depuradora tiene como destino final su aplicación en suelo agrícola. Dado este uso extendido y el potencial riesgo de diseminación de resistencias a los antibióticos que de él se deriva, en el Capítulo 7 realizamos un estudio de evaluación de la salud del suelo

agrícola, así como de la abundancia y riesgo de diseminación de genes de resistencia a antibióticos, tras la aplicación prolongada (24 años) a suelo agrícola de distintas cantidades y frecuencia de aplicación de lodos de depuradora urbana digeridos anaeróbicamente y deshidratados, frente a un testigo sin enmendar. En este sentido, la aplicación a largo plazo de los lodos resultó en el aumento de la concentración total de zinc y cobre en el suelo agrícola, el cual sin embargo no se tradujo en un aumento de la fracción biodisponible. De hecho, la medida de la fracción biodisponible, mediante el empleo de distintos extractantes, se mantuvo en todos los casos y para todos los metales y metaloides estudiados por debajo del límite de cuantificación de la técnica. La aplicación reiterada de lodos mostró un aumento en la cantidad de materia orgánica total del suelo agrícola y en el fósforo disponible, pero no incrementó la cantidad de nitrógeno mineral ni de potasio disponible en comparación con el suelo sin enmendar. Además, la aplicación de lodos resultó en una mejora de los parámetros de actividad y biomasa microbiana, pero no en una mejora de la diversidad estructural microbiana ni en una alteración significativa de la composición de las comunidades microbianas. En lo que respecta a los genes de resistencia a los antibióticos, la aplicación periódica de lodos de depuradora digeridos anaeróbicamente y deshidratados, incluso a la menor dosis de aporte (40 t ha^{-1} cada 4 años), resultó en el aumento de varios genes de resistencia a antibióticos y elementos genéticos móviles, en comparación al suelo sin enmendar, lo que implica un riesgo potencial para la salud humana y del medio ambiente. El hecho de que las comunidades procariotas no se vieran significativamente alteradas por el aporte reiterado de lodos sugiere que la transferencia horizontal de genes es la principal responsable del aumento en la abundancia de estos genes. Además, las concentraciones de cobre y zinc se correlacionaron positivamente con la abundancia de genes de resistencia, lo que sugiere la existencia de mecanismos de co-selección para ambos tipos de contaminantes. Los lodos utilizados en este capítulo sufrieron un tratamiento de digestión anaerobia mesófila antes de ser deshidratados y aplicados, si bien se ha demostrado que la carga de resistencias se reduce de forma más efectiva tras la digestión termófila (Miller *et al*., 2016) o el compostaje (Masse *et al*., 2014). Queda patente la necesidad de implementar tratamientos efectivos para la reducción de determinantes de resistencia a los antibióticos, de cara a garantizar una valorización correcta y segura de los lodos de depuradora como enmiendas orgánicas para suelo agrícola.

Por último, dada la relevancia de los microorganismos para el correcto funcionamiento del ecosistema edáfico, en el Capítulo 8 se estudiaron los efectos de la

agricultura orgánica, frente a suelo natural, sobre las poblaciones de micorrizas, así como los efectos agronómicos derivados de la utilización de inóculos micorrícicos, en un ensayo de biofertilización de dos ciclos de crecimiento de lechuga en cámara de crecimiento controlado. A este respecto, las prácticas de la agricultura orgánica, léase, el arado mínimo y la aplicación de enmiendas compostadas, ejercieron un efecto negativo en la abundancia y diversidad de organismos micorrícicos. Por su parte, el aislamiento e inoculación de micorrizas arbusculares resultó en una mejora del rendimiento productivo de la lechuga, tras el segundo ciclo de crecimiento del cultivo, en comparación al suelo sin inocular. Sin embargo, la inoculación de micorrizas no resultó en la alteración de la composición o la diversidad de las comunidades micorrícicas en los suelos inoculados frente al suelo sin inocular. La utilización de este tipo de organismos como bioestimulantes necesita de investigación básica para conocer las condiciones y los mecanismos bajo los cuales la inoculación estos organismos puede contribuir a mejorar el rendimiento de los cultivos y su calidad nutricional.

Como se ha mencionado anteriormente, la presión a la que estamos sometiendo actualmente al ecosistema edáfico no tiene precedentes. El argumento de la necesidad de alimentar a una población humana en constante crecimiento no debe justificar prácticas agrícolas que se han demostrado insostenibles desde el punto de vista medioambiental y que además pueden causar efectos perjudiciales sobre la salud humana. Los límites productivos nunca deben superar los límites ecológicos, ya que la degradación a gran escala del ecosistema edáfico pone en jaque su capacidad para proveer a la población humana un conjunto de servicios ecosistémicos cruciales para su bienestar y desarrollo. Por otra parte, la seguridad alimentaria a escala global no se encuentra relacionada, hoy por hoy, con una insuficiencia en el volumen de producción agrícola mundial, sino que, tristemente, depende de la distribución del alimento producido (la FAO nos recuerda habitualmente que, mientras cada año mueren millones de personas por desnutrición, se produce en el planeta más comida de la necesaria para alimentar a todos sus habitantes (FAO, 2019b) y que, de hecho, la cantidad de alimento desperdiciado anualmente supone 4 veces la cantidad necesaria para alimentar a los millones de personas que padecen desnutrición a escala global (BCFN, 2012)). A este respecto, cada vez son más los agricultores que, por un motivo de concienciación o por ser conscientes de la pérdida de la fertilidad edáfica originada por prácticas agrícolas intensivas, se esfuerzan por preservar la salud de sus suelos optando por prácticas más respetuosas con la

funcionalidad del ecosistema edáfico y que permitan mantener la provisión de sus servicios ecosistémicos.

Dichos servicios aportados por el ecosistema edáfico están intrínsecamente unidos a la sostenibilidad, y el perjuicio o mejora en los mismos, derivados de las actividades antrópicas, se ven reflejados en la sociedad y en la economía. Sin embargo, el valor de estos servicios no está reconocido adecuadamente en los mercados económicos y en las políticas de gestión ambiental. De hecho, el ecosistema edáfico fue ignorado por la Comisión Europea en la clasificación de los tipos de ecosistemas para su evaluación económica en la iniciativa TEEB (La Economía de los Ecosistemas y la Biodiversidad, 2008). Por ello, es necesario destinar mayores esfuerzos a la creación de marcos legislativos capaces de analizar los servicios ecosistémicos del suelo.

Mientras tanto, la búsqueda de prácticas agrícolas sostenibles que eludan la simplificación del ecosistema edáfico mediante la externalización de los procesos naturales por insumos químicos, y que, por el contrario, se basen en procesos ecológicos como los ciclos biogeoquímicos o las dinámicas de las comunidades edáficas, al tiempo que generen buenos rendimientos productivos de cultivos sanos y de buena calidad, sean resilientes y contribuyan al desarrollo sostenible de la sociedad, representa uno de los grandes retos de las próximas décadas. En este sentido, la diversidad de los sistemas agrícolas representa un valor añadido a la hora de combinar la eficiencia productiva y la sostenibilidad agrícola. La unión entre conocimiento y práctica se ha traducido en varios principios rectores de la intensificación ecológica que convergen en torno a las prácticas agrícolas sostenibles, incluyendo: (i) la rotación de cultivos; (ii) la diversidad de cultivos para mejorar la estabilidad del ecosistema; (iii) el mantenimiento de la estructura del suelo mediante la reducción del trabajo mecánico de la superficie cultivable; (iv) la mejora y el mantenimiento de la cubierta vegetal, mediante cultivos o residuos orgánicos, para la protección de la superficie del ecosistema, el mantenimiento de la materia orgánica, la retención de agua y nutrientes, la promoción de la actividad biológica, y la contribución a la gestión integrada de plagas y malas hierbas; y (v) la utilización optimizada de enmiendas orgánicas como forma de devolución de compuestos orgánicos al ecosistema edáfico y como fuente de energía y alimento para la microbiota edáfica y las comunidades vegetales.

Unido a este último punto, la revalorización de la excesiva carga de residuos que generamos a nivel de sociedad mediante su utilización como enmiendas agronómicas tiene un potencial enorme en el marco de la economía circular, la minimización de

residuos, el ahorro energético, y la sostenibilidad de la agricultura. Sin embargo, dentro de las prácticas agrícolas sostenibles, es de importancia sustancial evitar aquellas que conlleven un riesgo de contaminación del ecosistema edáfico. Por ello, la intrusión en el sistema de cualquier contaminante u organismo potencialmente dañino para el medio ambiente y la salud humana ha de ser evitado mediante tratamientos efectivos de los residuos orgánicos. A este respecto, a pesar del enorme avance de las últimas décadas en el marco legislativo y en las tecnologías orientadas al tratamiento, reciclaje y reutilización de los residuos, los contaminantes emergentes apenas se mencionan en los marcos legislativos actuales en materia de residuos (Directiva (UE) 2018/851), subproductos ganaderos (Reglamento (CE) Nº 1069/2009), lodos (Directiva 86/278/CEE), fertilizantes (Reglamento (UE) 2019/1009), etc. Actualmente, la resistencia a los antibióticos pone en jaque la medicina moderna. El desarrollo de tecnologías para la minimización de la emergencia y diseminación de este tipo de determinantes de resistencia a los antibióticos es de máxima urgencia en la actualidad. En este sentido, el compostaje y la digestión anaerobia termófila han demostrado ser técnicas útiles para la minimización del riesgo de resistencia a los antibióticos, pero a su vez se han mostrado insuficientes para la eliminación completa de los genes de resistencia. Se han propuesto tratamientos adicionales previos tales como alcalinización de los residuos, o tratamientos de radiación por microondas, ultravioleta o gamma, que puedan incidir en la degradación de los microorganismos y sus componentes genéticos. El estudio de la inhibición de la conjugación bacteriana, el mecanismo más común de transferencia horizontal de genes, o la eliminación selectiva de determinados genes y elementos genéticos móviles mediante la técnica CRISPR/Cas, son alternativas de gran interés para minimizar la diseminación de resistencias a los antibióticos. No obstante, es necesario primero dedicar esfuerzos a la investigación básica para evaluar con el requerido rigor científico las ventajas y posibles limitaciones de estas alternativas. En concreto, la tecnología CRISPR actualmente ha de superar obstáculos tanto éticos como legislativos antes de considerarse una alternativa potencial.

De nuevo, es necesario enfatizar que el suelo es un recurso no renovable a escala humana del cual obtenemos infinidad de beneficios y cuya integridad es esencial para el mantenimiento de los ecosistemas terrestres y el desarrollo humano. Por ello, las prácticas agrícolas sostenibles han de demostrar su capacidad para garantizar el correcto funcionamiento del suelo. La monitorización de la salud del ecosistema edáfico mediante el empleo de indicadores fiables es crucial. En particular, las propiedades microbianas del

suelo tienen un gran potencial como indicadores de su salud debido a la enorme relevancia de las comunidades microbianas para el correcto funcionamiento del ecosistema edáfico, así como por su sensibilidad y rapidez de respuesta. Sin embargo, la interpretación de los datos biológicos resulta, en ocasiones, extremadamente complicada debido a la enorme complejidad, a nivel funcional, estructural y de respuesta, del ecosistema edáfico. Y es que, hoy por hoy, existe todavía un gran desconocimiento acerca de las complejas relaciones ecológicas entre los microorganismos, las plantas y el suelo. En este sentido, el desarrollo de las denominadas técnicas "ómicas" (representadas en este trabajo por el análisis de librerías de genes estructurales mediante de DNA metabarcoding, pero que abarcan un espectro mucho mayor: metagenómica, metatranscriptómica, metaproteómica y metabolómica) ha evolucionado a un ritmo asombroso en los últimos años y revolucionado el campo de la ecología microbiana edáfica. Estas nuevas técnicas permiten profundizar en las consecuencias funcionales que conllevan los cambios en la composición y diversidad microbiana que se derivan de los distintos tipos de manejo del suelo agrícola. Sin embargo, todavía hoy este tipo de técnicas muestran limitaciones debido, entre otros factores, a la dificultad de la interpretación de la abrumadora carga de datos que generan. Además, dependen fuertemente del desarrollo de tratamientos bioinformáticos, precisan de muestras biológicas de alta calidad, requieren la existencia y disponibilidad de bases de datos, etc. Por otro lado, en el caso particular de la matriz edáfica, la extracción del material genético se realiza habitualmente partiendo de una muestra de 250 mg de suelo que previamente ha sido extraído, procesado, desestructurado, etc., por lo que no podemos pretender que el análisis genético de las comunidades microbianas de esa muestra sea representativo de la realidad edáfica. No obstante, el valor y potencial de estas herramientas en el campo de la ecología microbiana es enorme y tremendamente valioso. Ciertamente, una parte importante de los esfuerzos dedicados a la investigación en el campo de la funcionalidad del suelo ha de orientarse a elucidar los roles funcionales y la complejidad de las comunidades biológicas que lo componen. Una interpretación mecanística de esta información podrá contribuir enormemente a la evaluación y monitorización adecuada de la salud del suelo.

Una vez que seamos capaces de entender mejor los mecanismos responsables de la sostenibilidad funcional del ecosistema edáfico, podremos transferir con mayor rigor recomendaciones y pautas a los agricultores y responsables de las políticas de gestión agrarias, de cara a la implementación de prácticas agrarias sostenibles y saludables. Asimismo, es indispensable educar a la sociedad sobre el enorme valor del suelo y, en

particular, sobre la relevancia de los microorganismos en los procesos que soportan la funcionalidad de dicho recurso.

Finalmente, las tecnologías venideras en materia de alimentación y agricultura pueden suponer grandes cambios a escala global. Así, el informe "Rethinking Food and Agriculture 2020-2030" realizado por RethinkX, un grupo de expertos independiente que analiza y pronostica la velocidad del cambio promovido por la tecnología, sugiere que la generación de biomasa microbiana mediante fermentación de precisión permitirá la producción de proteína a gran escala y de forma económica, lo que supondrá, según el informe, el declive del sistema agroganadero que hoy en día prevalece. Este proceso de fermentación "reemplazará un sistema ineficiente que requiere enormes cantidades de insumos y produce enormes cantidades de residuos por uno que será preciso, específico, eficiente y manejable" (RethinkX, 2019). Además, el informe asegura que para 2035 cerca del 60% de la tierra que actualmente se utiliza para pastos y la producción de materias primas para los piensos que nutren a nuestra ganadería se liberará para otros usos, lo que representa una oportunidad de restauración ambiental a gran escala. En este sentido, siendo conocedores de los desafíos de la alimentación mundial y de la degradación del medio ambiente, lograr una alimentación que no dependa del suelo se presenta como una oportunidad novedosa y atractiva para lograr algunos de los objetivos de la seguridad alimentaria a nivel global. Por otro lado, la tecnificación y digitalización de la agricultura también se ha presentado como una solución potencial al deterioro a escala global del ecosistema edáfico. En particular, la difusión de tecnologías como la agricultura de precisión, la robótica y la inteligencia artificial, junto con la mejora de la conectividad en el ámbito agroalimentario, se han propuesto a fin de aumentar los rendimientos productivos y reducir los residuos, incrementando así la eficiencia de los procesos agrícolas. No obstante, el problema de las promesas de la tecnología venidera es que pueden tentarnos a posponer las actuaciones y decisiones necesarias en el momento actual. Podemos y debemos tomar mejores decisiones de inmediato, integrando las prácticas agrícolas sostenibles como principal modelo de la producción de alimentos, al tiempo que se promueven políticas que valoren el ecosistema edáfico y los servicios que proporciona, y se siga investigando en técnicas de tratamiento de residuos orgánicos, para su utilización segura como enmiendas agronómico, así como en la mejor comprensión de los roles y relaciones entre los microorganismos edáficos, las plantas y el suelo.

10| CONCLUSIONES Y TESIS

10.1. CONCLUSIONES

1-. La incorporación del rastrojo de maíz al suelo es una práctica beneficiosa para el ecosistema edáfico que incrementa su fertilidad y, en concreto, la actividad y biomasa de las comunidades microbianas del suelo. Asimismo, dicha incorporación modifica la capacidad metabólico-funcional de las comunidades bacterianas heterótrofas cultivables.

2-. Los beneficios potenciales sobre la salud del suelo derivados de la aplicación de enmiendas líquidas obtenidas a partir de la fermentación de residuos orgánicos dependen, entre otros factores, del tipo de suelo y la dosis de aporte de enmienda. Desde el punto de vista de la producción agrícola, dicha dosis debe ajustarse a los requerimientos de nitrógeno del cultivo.

3-. La caracterización físico-química y biológica en profundidad de las enmiendas orgánicas es indispensable para su correcta selección y posterior utilización, al objeto de optimizar su adecuación para cada contexto y casuística (léase, tipo de suelo, factores climatológicos, cultivo, etc.).

4-. Las enmiendas orgánicas suministran energía y carbono a la microbiota edáfica heterótrofa, así como macro- y micronutrientes esenciales para el crecimiento idóneo de los cultivos. La sostenibilidad medioambiental de esta práctica agrícola (*i.e.*, aplicación de enmiendas orgánicas a los suelos) se encuentra supeditada a la eliminación o, en su defecto, minimización de los potenciales riesgos asociados a su utilización, tales como el riesgo de diseminación de resistencias a antibióticos, la incorporación de contaminantes, la presencia de potenciales patógenos humanos, etc.

5-. La aplicación de estiércol fresco al suelo agrícola aporta energía y carbono lábil para las comunidades microbianas edáficas, con el consiguiente aumento de su biomasa y actividad metabólica, e incrementa el rendimiento productivo del cultivo de lechuga. No obstante, esta aplicación conlleva un aumento en la abundancia de genes de resistencia a antibióticos y elementos genéticos móviles, con el consiguiente riesgo potencial para la salud humana y el medio ambiente.

6-. Tanto el compostaje como la fermentación anaerobia (bokashi) del estiércol conllevan una reducción notable en la carga de determinantes de resistencia a antibióticos.

7-. La aplicación a largo plazo de lodos de depuradora urbana digeridos anaeróbicamente y deshidratados conlleva una mejora en las propiedades físico-químicas y biológicas de los suelos agrícolas, la cual no se traduce en una modificación significativa de la composición de las comunidades microbianas edáficas.

8-. La aplicación reiterada de lodos de depuradora urbana digeridos anaeróbicamente y deshidratados aumenta la concentración total de cobre y zinc en el suelo. No obstante, este incremento no conlleva un aumento en la fracción biodisponible de dichos elementos traza. Por otra parte, dicha aplicación aumenta la abundancia de genes de resistencia a antibióticos y elementos genéticos móviles de forma correlacionada con la concentración total de los citados elementos traza, lo que sugiere la existencia de mecanismos de co-evolución.

9-. Algunas prácticas agrícolas propias de la agricultura orgánica (*i.e.*, laboreo mínimo y empleo de enmiendas orgánicas) ejercen un efecto negativo sobre la abundancia y diversidad de hongos micorrícicos arbusculares. Por otra parte, la inoculación de hongos micorrícicos arbusculares mejora el rendimiento productivo del cultivo de lechuga sin ejercer una alteración notoria en las comunidades edáficas de dichos hongos.

10-. Debido a su sensibilidad, rapidez de respuesta, carácter integrador y relevancia ecológica, las propiedades microbianas edáficas son indicadores biológicos de gran valor para la evaluación y monitorización del impacto de prácticas agrícolas sobre la salud del suelo.

10.2. TESIS

La aplicación de enmiendas orgánicas al suelo es una práctica agrícola enmarcada dentro de los paradigmas actuales de minimización de residuos y economía circular promovidos por las instituciones europeas que, frente a la utilización tradicional de insumos de síntesis química, aporta beneficios contrastados al ecosistema edáfico, incrementando la biomasa, actividad y diversidad funcional de las comunidades microbianas, las cuales son responsables, en gran parte, del funcionamiento y la fertilidad del suelo. No obstante, es indispensable diseñar e implantar medidas de eliminación o, en su defecto, minimización del riesgo potencial de diseminación de contaminantes asociado al uso de las citadas enmiendas, de cara a garantizar su sostenibilidad medioambiental. A este respecto, la utilización de propiedades microbianas con potencial bioindicador de la salud del suelo representa una opción idónea para la evaluación y monitorización del impacto potencial de la aplicación de enmiendas orgánicas a los suelos agrícolas.

10.1. ONDORIOAK

1-. Arto-uztondoa lurzoruan gehitzea jarduera onuragarria da ekosistema edafikoarentzat, lurzoruko komunitate mikrobianoen aktibitatea eta hazkuntza sustatzen baitu, haren emankortasuna handituz. Era berean, jarduera horrek bakterio heterotrofo kultibagarrien gaitasun metaboliko-funtzionalaren aldaketa dakar.

2-. Hondakin organikoen hartziduraren bidez lortutako medeapen likidoak aplikatzeak lurzoruaren osasunean dakartzan onura potentzialak, besteak beste, lurzoru-motaren eta aplikazio dosiaren araberakoak dira. Nekazaritza-ekoizpen ikuspegitik dosi hori laborearen nitrogeno-eskakizunetara egokitu behar delarik.

3-. Medeapen organikoen karakterizazio fisiko-kimiko eta biologiko sakona ezinbestekoa da, testuinguru espezifiko bakoitzerako (hau da, lurzoru mota, faktore klimatologikoak, laborantza, etab.) hobekien egokitzen dena hautatu eta erabiltzeko.

4-. Medeapen organikoak energia eta karbono iturri dira lurzoruko mikroorganismo heterotrofoentzako eta, aldi berean, makro- eta mikronutriente esentzialez hornitzen dituzte laboreak. Nekazaritza-jarduera honen (hau da, medeapen organikoen erabilpena) jasangarritasuna aplikazioari lotutako arrisku potentzialak, hala nola, antibiotikoekiko erresistentziak barreiatzeko arriskua, kutsatzaileen sarrera, giza patogeno potentzialak egotea, etab. ezabatzearen edo minimizatzearen menpe dago.

5-. Simaur freskoa energia eta karbono iturri labila da lurzoruko komunitate mikrobianoentzat, hortaz, nekazal lurzoruan aplikatzen denean komunitate mikrobianoen hazkuntza eta aktibitate metabolikoa sustatzen du eta, aldi berean, letxuga laborearen ekoizpena handitu egiten du; hala ere, aplikazio horrek antibiotikoekiko erresistentzia-geneak nekazal lurzoruetan eta ingurumenean zabaltzeko arriskua dakar, giza osasunerako eta ingurumen osorako arrisku potentziala suposatuz.

6-. Simaurraren konpostatzeak edo hartzidura anaerobioak (bokashi) nabarmenki murrizten du antibiotikoekiko erresistentzia-determinatzaileen karga.

7-. Anaerobikoki digeritutako eta deshidratatutako hiri-araztegiko lohiak epe luzean aplikatzeak nekazal lurzoruen propietate fisiko-kimikoak eta biologikoak hobetzen ditu, lurzoruko komunitate mikrobianoen konposizioan aldaketa nabarmenik eragin gabe.

8-. Anaerobikoki digeritutako eta deshidratatutako hiri-araztegiko lohien aplikazio konstanteak areagotu egiten du nekazal lurzoruetako kobre eta zink metal astunen kontzentrazio totala. Hala ere, hazkuntza horrek ez dakar metal astun horien frakzio bioerabilgarriaren areagotzea. Bestalde, lohien aplikazioak nekazal lurzoruen antibiotikoekiko erresistentzia-geneen eta elementu genetiko mugikorren ugaritasuna handitzen du, aipatutako metal astunen kontzentrazio totalarekin korrelazioan dagoena, koeboluzio mekanismoen presentzia iradokiz.

9-. Nekazaritza organikoko berezko jarduera batzuek (hala nola, gutxieneko laborantza eta medeapen organikoen erabilpena) eragin negatiboa dute onddo mikorriziko arbuskuskularren aniztasun eta ugaritasunean. Bestalde, onddo mikorriziko arbuskularren inokulazioak letxuga laborearen ekoizpena hobetzen du, lurzoruko onddo horien komunitatetan aldaketa nabarmenik eragin gabe.

10-. Beraien sentsibilitate, azkar erantzuteko ahalmen, izaera bateratzaile eta garrantzi ekologikoa dela eta, lurzoruko propietate mikrobiologikoak balio handiko bioadierazleak dira medeapen organikoen aplikazioak lurzoruan izan dezakeen eragina ebaluatzeko eta monitorizatzeko.

10.2. TESIA

Medeapen organikoen aplikazioa Europako erakundeek sustatutako hondakinen minimizazioaren eta ekonomia zirkularraren egungo paradigmen barruan dagoen nekazaritza-jarduera bat da, zeinak, sintesi kimikoko ongarrien erabilpenean oinarrituta dagoen nekazaritza tradizionalaren aurrean, onura egiaztatuak ekartzen dizkio ekosistema edafikoari, lurzoruko komunitate mikrobianoen biomasa, aktibitate eta dibertsitate funtzionala sustatuz, hein handi batean, lurzoruaren funtzionamenduaren eta emankortasunaren erantzule direnak. Hala ere, medeapen organikoen erabilerari lotutako kutsatzaileak ingurumenean barreiatzeko arrisku potentzialak ezabatzeko edo minimizatzeko neurri konkretuak diseinatu eta ezartzea ezinbestekoa suertatzen da, nekazaritza-jarduera honen jasangarritasuna bermatze aldera. Zentzu horretan, lurzoruko osasunaren bioadierazle izan daitezkeen propietate mikrobiologikoen erabilpenak, medeapen organikoen aplikazioak izan dezakeen eragina ebaluatzeko eta monitorizatzeko aukera egokia suposatzen du.

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PUBLICACIONES GENERADAS

Yo, Julen Urra Ibañez de Sendadiano, declaro que:

Esta tesis doctoral ha generado cinco artículos ya publicados en revistas indexadas JCR así como un artículo aceptado con correcciones y uno más que actualmente se encuentra bajo revisión:

1. Urra J, Alkorta I, Garbisu C, 2019. Potential benefits and risks for soil health derived from the use of organic amendments in agriculture. *Agronomy*, 9, 542 (ver Capítulo 1).
2. Urra J, Mijangos I, Lanzén A, Lloveras J, Garbisu C, 2018. Effects of corn stover management on soil quality. *European Journal of Soil Biology*, 88, 57-64 (ver Capítulo 4).
3. Urra J, Alkorta I, Mijangos I, Garbisu C, 2020. Commercial and farm fermented liquid organic amendments to improve soil quality and lettuce yield. *Journal of Environmental Management*, 264, 110422 (ver Capítulo 5.1).
4. Urra J, Mijangos I, Epelde L, Alkorta I, Garbisu C. Impact of the application of commercial and farm-made fermented liquid organic amendments on corn yield and soil quality. *Applied Soil Ecology, accepted with corrections* (ver Capítulo 5.2).
5. Urra J, Alkorta I, Lanzén A, Mijangos I, Garbisu C, 2019. The application of fresh and composted horse and chicken manure affects soil quality, microbial composition and antibiotic resistance. *Applied Soil Ecology*, 135, 73-84 (ver Capítulo 6).
6. Urra J, Alkorta I, Mijangos I, Epelde L, Garbisu C, 2019. Application of sewage sludge to agricultural soil increases the abundance of antibiotic resistance genes without altering the composition of prokaryotic communities. *Science of the Total Environment*, 647, 1410-1420 (ver Capítulo 7).
7. Epelde L, Urra J, Anza M, Gamboa J, Garbisu C. Inoculation of arbuscular mycorrizhal fungi increases lettuce yield without altering natural soil communities. *Archives of Agronomy and Soil Science, under revision* (ver Capítulo 8).

Asimismo, el presente trabajo ha generado un artículo en una revista no indexada por JCR pero incluida dentro Scopus y de SJR:

1. Urra J, Alkorta I, Mijangos I, Garbisu C, 2018. Data on links between structural and functional prokaryotic diversity in long-term sewage sludge amended soil. *Data in Brief*, 20, 1787-1796 (ver Capítulo 7.7).

Además, de este presente trabajo también son fruto las siguientes comunicaciones a congresos:

1. Epelde L., Urra J, Anza M, Lanzén, Garbisu C. Characterization and inoculation of arbuscular mycorrhiza of different origins in organic orchard soil. 1st Meeting of the Iberian Ecological Society - XIV AEET Meeting - Ecology: an integrative science in the Anthropocene. Barcelona, 2019.
2. Urra J, Martín I, Epelde L, Lanzén A, Blanco F, Mijangos I, Garbisu C. Long-term influence of sewage sludge on the presence and abundance of mobile genetic elements and antibiotic resistance genes in soil. 17th International RAMIRAN Conference: Sustainable utilisation of manures and residue resources in agriculture. Wexford (Irlanda), 2017.
3. Urra J, Mijangos I, Epelde L, Lanzén A, Blanco F, Garbisu C. Effect of liquid organic amendments on soil microbial communities. FEMS, 7th Congress of European Microbiologists. Valencia, 2017.
4. Urra J, Blanco F, Lanzén A, Lloveras J, Mijangos I, Epelde L, Garbisu, C. Long-term effects of the incorporation of corn stover on the health of agricultural soil. CONSOWA, 1st World Conference on Soil and Water Conservation Under Global Change. Lleida, 2017.
5. Urra J, Lanzén A, Martín I, Blanco F, Anza M, Epelde L, Mijangos I, Garbisu C. Influence of manure amendments on the presence and abundance of antibiotic resistance genes. BAGECO 14, Bacterial Genetics and Ecology. Aberdeen (Escocia), 2017.
6. Urra J, Lanzén A, Epelde L, Mijangos I, Blanco F, Garbisu C. Evaluation of organic fertilization using "bioles" on soil health and microbial community structure. ISME, International Symposium on Microbial Ecology. Montreal (Canada), 2016.
7. Urra J, Garbisu C, Becerril JM, Artetxe U, Anza M, Mijangos I. Organic amendment effects on lettuce production and rhizosphere soil. Rhizosphere 4, Streching the Interface of Life. Maastricht (Holanda), 2015.