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ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRONOMOS | ***NEKAZARITZAKO INGENIARIEN GOI MAILAKO ESKOLA TEKNIKOA***

From ground pollution to soil fertilization:

An environmental assessment of soil amendments derived from organic wastes

Presentado por

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Aurkeztua

Memoria de tesis presentada para optar al grado de Doctor en Agrobiología Ambiental con mención Internacional por la Universidad Pública de Navarra

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NEKAZARITZAKO INGENIARIEN GOI MAILAKO ESKOLA TEKNIKOA
Natura Ingurunearen Zientzien Sailean
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AUTORIZACIÓN DEL DIRECTOR DE TESIS PARA SU PRESENTACIÓN

Dr. Pedro Mª. Aparicio Tejo, Catedrático de Universidad del Área de Fisiología Vegetal del Departamento de Ciencias del Medio Natural de la Universidad Pública de Navarra,
Dr. Ignacio Irigoyen Iriarte, Profesor Contratado Doctor del Área de Producción Vegetal del Departamento de Producción Agraria de la Universidad Pública de Navarra,

INFORMAN:
que el trabajo titulado "**From ground pollution to soil fertilization: An environmental assessment of soil amendments derived from organic wastes**" que presenta la Ing. **Dña. María Eréndira Calleja Cervantes** para optar por el título de Doctor con mención de "Doctor Internacional", ha sido desarrollado bajo nuestra dirección en el laboratorio de Fisiología Vegetal, del Departamento de Ciencias del Medio Natural en la Universidad Pública de Navarra.

Revisado el trabajo, consideran que reúne las condiciones necesarias para su defensa, por lo que,

AUTORIZAN:
La presentación de la citada Tesis Doctoral

En Pamplona, a 15 de abril de 2015

Fdo. Dr. Pedro Mª. Aparicio Tejo Fdo. Dr. Ignacio Irigoyen Iriarte

“Eppur si muove.”

Galileo Galilei

“Scientists often have a naïve faith that if only they could discover enough facts about a problem, these facts would somehow arrange themselves in a compelling and true solution.”

Theodosius Dobzhansky

“No hay nada que enseñe más, que equivocarse.”

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Publicaciones-Publications

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- Calleja-Cervantes Maria E., Irigoyen Ignacio, Gorriz Cristina, Perez-Jaramillo Juan E., Irañeta Jesús, Amorena Alfonso, Aparicio-Tejo Pedro M. , Menéndez Sergio. 2013. Twenty years of continued application of treated sewage sludge: nitrous oxide emissions induced in agricultural soils. *In*: Vallez G., Cambier P., Bacheley H., Cheviron N., Formisano S., Lepeuple AS, Revallier A. and Houot S. (Eds). Recycling of Organic Residues in Agriculture: 15th International Conference. 3rd-5th June 2013. Versailles, France. INRA UMR Environment et Grandes Cultures. ISBN: 978-2-7380-1337-8. Accesible online: https://colloque4.inra.fr/ramiran2013/Post-conference

- Calleja-Cervantes, M.E., Martínez, C., Storino, F., Irigoyen, I., Cibriain, F., Lasa, B., Aparicio-Tejo, P., Menéndez, S. 2014. Metales Pesados en viña tras doce años de aplicación de distintos composts: contenido del suelo, baya y biodisponibilidad para *ray grass*. *In*: Avances en la investigación sobre compost:

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RESUMEN

El crecimiento de la población y sus necesidades de alimentación en las últimas décadas han sido mayoritariamente sustentados por un modelo productivo lineal a escala global basado en consumir recursos y generar residuos. Este modelo en muchas ocasiones ha contribuido de manera crucial al deterioro de agrosistemas y al cambio climático global.

Para mejorar la sostenibilidad de los agrosistemas y mitigar el cambio climático es fundamental potenciar modelos productivos circulares locales basados en aprovechar residuos del entorno como recursos que permitan mejorar la fertilidad y resiliencia de los agrosistemas y desarrollar una agricultura más sostenible.

Es por esto, que esta tesis tiene por objetivo general el estudio del uso continuado de residuos orgánicos en suelos agrícolas como enmiendas. Este trabajo se centra en aspectos clave de la fertilización que son relevantes para el medio ambiente. El objetivo concreto ha sido el de describir el efecto de estas aplicaciones sobre:

a. La fertilidad del suelo y diversas características químicas del suelo
b. El metabolismo de los microbios del suelo, a través del estudio del estado de las enzimas tras la aplicación de los residuos
c. La diversidad bacteriana del suelo
d. Las emisiones de gases de efecto invernadero en cada cultivo y a lo largo de un ciclo productivo

Esta investigación se ha desarrollado en dos ensayos de larga duración ubicados en Navarra (España) donde se realizan distintos manejos de fertilización con residuos orgánicos. El primero de ellos es una viña de DOC Rioja (Bargota) en la que desde 1998 se aplican anualmente 3 compost diferentes. El segundo ensayo es una rotación cerealista

(Arazuri) que desde 1992 recibe anualmente lodos de EDAR a distintas dosis.

Los resultados de estos ensayos muestran un claro efecto beneficioso de la aplicación de distintos residuos orgánicos como fertilizantes en ambos agrosistemas.

En el capítulo 1 y 2 se presentan los resultados de las evaluaciones y análisis hechos en Bargota. El capítulo 3 reporta sobre los posibles efectos en la acumulación de metales pesados tanto en el suelo como en los cultivos de los compost ensayados en Bargota. En el capítulo 4 se presentan los resultados de los análisis hechos en Ararzuri.

En el caso de Bargota se concluye que la mejor enmienda es el estiércol de oveja. En el caso de Arazuri, las dosis ensayadas resultaron excesivas y su efecto en el cultivo estudiado provocaron una disminución de la cosecha de avena, sin embargo, la dosis de 40 toneladas cada tres años, con aporte de fertilizante mineral, beneficia a la fertilidad suelo y contribuye a que la actividad metabólica se incremente sin un aumento significativo de las emisiones a la atmósfera.

Por todo ello se concluye que el aprovechamiento de los residuos orgánicos como enmiendas o fertilizantes en los agrosistemas mediterráneos estudiados presenta importantes beneficios, siempre y cuanto las dosis se ajusten a los cultivos considerados.

Estos estudios a medio plazo de la aplicación de residuos orgánicos como enmiendas son de gran interés a la luz del cambio climático, pues ponen en evidencia que es posible incorporar prácticas para el secuestro de carbono en el suelo, que además fortalecen la fertilidad de suelo, previniendo así el deterioro ambiental y favoreciendo el tránsito de una economía lineal a una economía circular.

LABURPENA

Azken hamarkadetan biztanleriak eta beren janari beharrak hazkunde ikaragarria jasan dute. Hazkunde hori gehienetan ekoizpen eredu global eta lineal batez asetua izan da. Eredu honetan baliabideak kontsumitzen eta hondakinak sortzen dira neurri gabean. Honen ondorioz, askotan nekazaritza sistemen hondamena eta klima-aldaketa globala eragin ditu.

Nekazaritza sistemen iraunkortasuna hobetzeko eta klima-aldaketan eraginak arintzeko ezinbestekoa da tokiz-tokiko ekonomia zirkularrean oinarritutako ekoizpen ereduak indartzea. Hondakin organikoak ongarri bezala erabiltzea aukera ona da, bai nekazaritza sistemen emankortasuna, eta bai bere erresilientzia handitzeko. Baina epe luzera izan ahal dituen zenbait alde negatibo ebaluatu behar dira.

Hori dela eta, tesi honen helburu orokorra lurzoruan epe luzera hondakin organikoen aplikazio jarraiaren zenbait ingurugiro alde aztertzea da. Beraz, inguruarekin erlazionatutako honako alde hauek zehatzago aztertzen dira:

a. Lurzoruaren emankortasuna eta hainbat lurzoru ezaugarri kimiko
b. Lurzoruaren mikrobioen metabolismoa entzimen azterketaren bidez
c. Lurzoruaren bakterio aniztasuna
d. Berotegi-efektuko gasen isurpenak

Ikerketa hau Nafarroan (Espainian) kokatzen diren epe luzerako ongarri organikoen bi entsaioetan burutua izan da. Lehenengoa Bargotako DOC Errioxako mahatsean, zeinetan 1998az geroztik 3 konpost diferente aplikatzen diren. Bigarrena, Arazuriko zereal errotazio batean zeinean 1992az geroztik urtero EDAR-lohi dosi ezberdin aplikatzen diren.

Entsaio hauetan lortutako emaitzek gai organiko hauek epe luzera erabiltzearen abantailak agerian utzi dituzte.

1. eta 2. kapituluetan Bargotan lortutako emaitzak aurkezten dira. 3. Kapituluan Bargotako lurren metal astunen eskuragarritasuna aztertzen da eta azkenik, 4. kapituluan Ararzurin egindako analisien emaitzak aurkeztu dira.

Emaitzen arabera Bargotan konparatu diren ongarrien artean ardi simaur konposta ongarririk onena dela ondorioztatzen da. Bien bitartean, Arazurin probatutako EDAR-lohi dosiak zenbait laboreentzako, oloa kasu, altuegiak direla ikusten da. Halaber, 40 tona hiru urtetan behin aplikatzeak emaitza on-onak lortzen ditu, lurraren emankortasuna eta jarduera biologikoa goratuz eta gaz emisioak nabarmen hazi gabe.

Beraz, ondorioztatzen da hondakin organikoak ongarri bezala erabiltzea Mediterraneo nekazaritza sistematan onuragarria dela dosi egokian aplikatu ezkeroz.

Klima aldaketa aztertzeko epe-ertaineko hondakin organikoen aplikazioaren inguruko azterketa hauek berebiziko garrantzia dute eta haiekin jarraitu beharra dago. Argi eta garbi erakusten dute area mediterraneoetan posiblea dela hondakin organikoekin lurreko C organikoa goratzea, lurraren emankortasuna indartzea eta, era berean ingurugiroari mesede egitea. Hondakin organikoz egindako ongarriak ekonomia zirkular baterantz trantsizioaren abiapuntua dira, behar den bezala erabili ezkeroz.

Abstract

The population growth rate and their diet and the land required to meet this demand has been largely supported by a global model based on a linear economy that consumes resources and generates wastes. This model has often contributed to the deterioration of agricultural systems and global climate change.

The sustainability of agricultural systems can be improved, on one hand, and climate change can be mitigated, on the other, by empowering circular productive models based on local resources and their rational exploitation. If soil fertility is regarded as an important feature of food production, then an important occasion for the contribution of agricultural practices is set to achieve food sustainability.

For this reason, this thesis focuses on studying the continued use of organic wastes in agricultural soils as amendments. This thesis reports on key aspects of fertilization that are relevant to the environment. The specific objective was to describe the effect of this application on:

a. Soil fertility and soil's nutrient characteristics
b. Soil's microbial metabolism, through the study of the enzyme activity in soils after amendment application
c. Soil bacterial diversity
d. Greenhouse gas emission in different crops and during its productive cycle.

This research was conducted in two sites in Navarra (Spain) where long-term trials have been established with different fertilization management and by the use of organic wastes, either composted or treated. The first trial was a vineyard situated in the P.D.O. *La Rioja*, Navarra, in which three different compost have been applied annually since 1992. The second trial is on cereal crops in rotation in Arazuri,

Navarra, that have been receiving Treated Sewage Sludge annually since 1992.

The results of this thesis show a clear beneficial effect after organic wastes have been applied to soil as organic amendments in both agricultural systems.

In chapter 1 and 2 the results of evaluations and analyzes made in Bargota are presented. Chapter 3 reports on the possible effects on the accumulation of heavy metals in the soil and vine tested in Bargota. In chapter 4 results from the analyses made in Ararzuri are presented.

At the Bargota site, the best amendment was sheep manure. In Arazuri, the tested doses were excessive, causing a decline in the harvested oats. However, the dose of 40 tons every three years plus an input of mineral fertilizer benefited soil fertility and increased microbial activity without a significtant increase in GHG emissions.

Therefore, it is concluded that the application of organic waste as amendments in Mediterranean agricultural systems entail important benefits and the mechanisms should be studied in detail.

These medium-to-long-term studies of the application of organic waste, as soil amendments, are of great interest in the light of climate change. This works clearly evidences that it is possible to incorporate practices that sequester carbon in soil, strengthen soil fertility, and prevent environmental degradation, thus favoring the transition from a linear economy to a circular model.

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1. *Current food production challenges.*

Human-driven environmental changes and their ecological impacts have been under scrutiny ever since the causes of climate change were revealed in the 1980s. Due to the urgent need to preserve the Earth's resources and sustain human life, environmental awareness of agricultural activities is rapidly increasing. Broader notions of environmental impacts have been incorporated into our understanding of the chemical, biological and physical implications of food production on Earth cycles.

Agriculture has an important effect on natural nutrient cycles which is proportional to the population growth rate, the population's diet and the land required to meet this demand. Agro-ecosystems are ecosystems that have been modified in their natural functions to produce food and fiber: staples and commodities. In essence, ecological roles are displaced to maximize productivity from input efforts, regardless of whether for profit or for survival.

Since the green revolution, the unpredictable ecological implications of agro-ecosystems have been reduced in a rapid and successful effort to gain maximum control over natural uncertainty. Natural nutrient and water limitations have been restricted where agricultural intensification has been inserted. Genetic, chemical, mechanical and biological resources are constantly developed for the sake of enhancing productivity. Paradoxically, this boost in productivity has neither fully resolved food shortage problems nor

offered a viable action plan for the management of limited resources and the pollution impacts derived from these actions. The food production system based on agricultural intensification is, in many cases, highly destructive as the start of each production process requires ever-increasing levels of resource consumption. Furthermore, the food produced is rapidly transformed into residues that require further investment before being returned to the environment without harmful consequences.

Moreover, considering that agro-ecosystems form part of a greater relationships with the environment (Tilman, 1999), and that the agro-ecosystems themselves constitute yet more complex relationships (Howden *et al*., 2007), then this supposed control over natural uncertainty has encountered important unforeseen problems. At present, disease resistance, genetic homogeneity and animal welfare are just a few of the problems facing today's food production systems (Pretty, 2008). Long-term crop productivity is also threatened by a progressive decline in soil fertility (Gomiero *et al.*, 2011). The problem of limiting nutrients, the associated extraction costs and the subsequent inefficiency in their exploitation are primary focal points on the food sustainability agenda. The enormous displacement of mineral and energy resources across the globe exacerbates the harmful consequences affecting natural ecosystem processes. There is a threat to the natural capacity of these processes to absorb and process the extracted materials, but also to their capacity to supply new raw resources.

Current theories describe the abovementioned situation as a problem inherent to food production in a **linear economy**. Figure 1 shows a schematic representation of the different production phases in a linear economy and the accumulated value corresponding to each stage.

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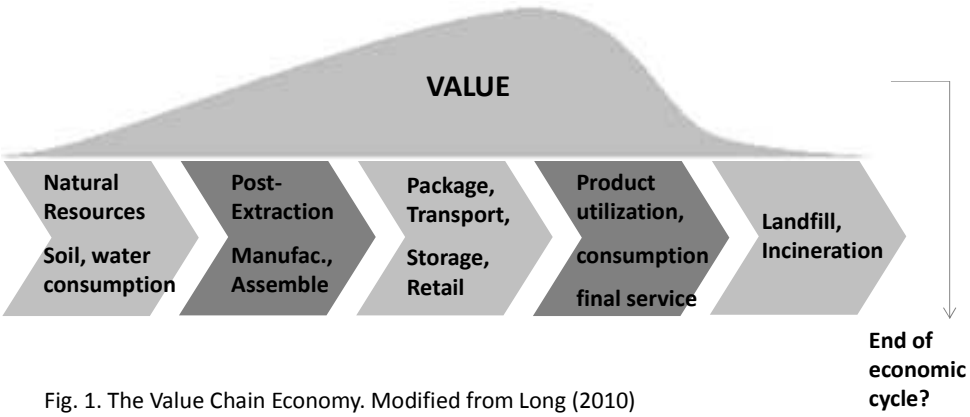


Fig. 1. The Value Chain Economy. Modified from Long (2010)

To understand the influence of this linear scheme in a resource-limited environment it is necessary to describe the natural conditions yielding the resources which are harvested, converted and subsequently disposed of once their value has dramatically depreciated.

2. *A glimpse at nutrient cycling.*

Current knowledge regarding nutrient cycling establishes that naturally mature ecosystems are the result of complex trophic relations which are continuously activated by solar energy. Several "feedback loops" are established in an ideal closed system, for example, there is an increase in microbial activity due to increased levels of organic matter (Xu *et al.*, 2011). Human activity has left significant footprints on these cycles. Before describing these footprints we need to understand what nutrient cycling is (climate change also influences nutrient cycling, but this will be discussed later).

Nutrient cycling begins with the decomposition of organic matter. Organic matter is a material produced naturally by living organisms (plants and animals) as well as debris from their decomposition. The process begins when residues and plant and animal remains are deposited in soil involving microbes, minerals and the environmental conditions (Figure 2). The relationships between the quality of the organic matter, the microbes present in the soil, mineral availability and the surrounding environment, determine the rate of decomposition (Brussaard, 1998). In this process resources such as water, energy and carbon dioxide are consumed and released to the atmosphere while synthesizing organic carbon compounds. These compounds are enriched by the incorporation of more dead material and so the initial organic matter evolves into a more complex organic compound called humus (Manlay et al., 2007). The successive production of different

stages of organic matter and its evolution into humus is called humification. Soil properties are affected by the presence of humus: physically (e.g., soil aggregation and aggregate stability are increased); chemically (e.g., nitrogen, phosphorus, potassium and micronutrients are more readily available and better retained by an increase in cation exchange capacity (CEC)); and biologically (microbial action).

Organic matter is not a nutrient in itself, but several organisms feed on it (Saha *et al.*, 2008). Its decomposition releases nutrients in forms that can be absorbed by plants during a process called mineralization. Organic matter then consumes waste generated by microbes, but this material takes longer to decompose than plant and animal debris. The carbon structures within organic matter are disassembled and rebuilt as new ones which store more carbon per unit of biomass. Therefore, soils rely on microbial activity to provide and sustain nutrients for crops and other systems. Organic matter then becomes a succession of different amounts and qualities of organic compounds that have the capacity to sequester carbon from the atmosphere and change soil attributes. As can be implied, losses occur naturally, but their magnitude is limited by the amount of input material and the environment. Nutrients usually flow from one compartment to the other (from soil to atmosphere, to water and back again) through biochemical processes and so the compartment sizes and element enrichment are continuously changing and thus maintaining homeostasis.

It is worth noting the lack of a naturally conditioned soil which produces food and serves as a reference for observing natural nutrient restoration processes in soil. Following some detailed studies where natural conditions were believed to have been preserved, White (1987) proposed an improved understanding of nutrient cycling taking into account some anthropogenic actions. As can be seen in Figure 2, external stimulation is basically provided by the sun and enhanced by human activity. Furthermore, Figure 2 illustrates that the restoration of food production resources (soil, nutrients, water and energy) used to be based on natural feedback loops. The circle was closed following the condition that any material allocated for re-introduction into the cycle must be susceptible to adequate decomposition through environmental and biological actions.

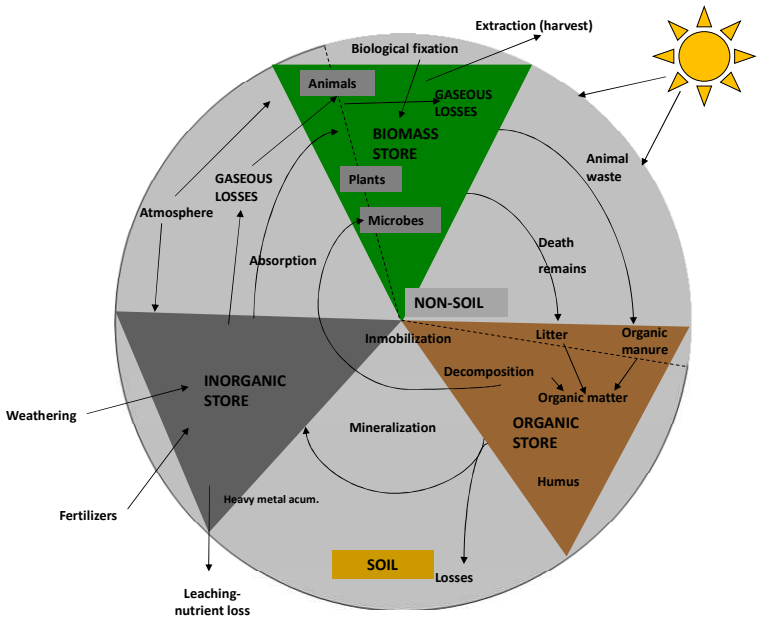


Figure 2. Nutrient cycling components. Source: Modified from White (1987).

The cycle sequence can be altered by two main factors: fertilization and value addition. These two parameters shall be briefly explained in the next section.

3. *How does anthropogenic modification of soil processes affect nutrient cycling?*

In the framework of a linear economy, value addition in food production requires the input of several materials at different stages in a product's economic life-cycle. The most important input is fertilizer, but other materials are continuously introduced and discarded in order to increase product value. For instance, upon harvesting a crop, several materials have already been thrown away, but yet further residues are discarded after crop usage and economic value is lost.

Likewise, technological developments have provided the opportunity to transfer resources from abundant sites to those where there is a natural lack of resources, or the possibility of increasing fertility through agricultural intensification. In essence, human interference in nutrient cycling is designed to increase soil fertility and product value.

In agronomic terms, soil fertility refers to the soil's crop productivity and capacity to improve quality and yields. **Soil fertility** is also used as a general term meaning, "the inherent capacity of a

soil to supply nutrients to plants in adequate amounts and in suitable proportions" (Tomati *et al.*, 1985; Havlicek, 2012). This definition implies that fertility depends on chemical, physical, mechanical and biological factors. Since the green revolution and agricultural intensification, chemical fertility has been based on fertilization inputs. The primary nutrients have all been transformed for application as mineral fertilizers. The process of fixating atmospheric N_2 and phosphorus purification has also caused important deleterious effects in the environment (Galloway *et al*., 1995; Fields, 2004)

Fertility modifying activities cause disruptions in nutrient cycles; in some cases these are beneficial, while in others they exacerbate negative effects. In practice, fertilizing elements (synthetic fertilizers and agrochemicals) applied in amounts that exceed plant requirements will accumulate in the soil or otherwise, will be released to the atmosphere or lost by leaching (Penuelas *et al.*, 2012), as it was indicated in Figure 2.

Eutrophication, leaching and gas emissions are among the undesirable effects intensified by fertilization. Excessively enriching an ecosystem with nutrients, typically nitrogen and phosphorus-containing compounds, is called eutrophication. An excessive accumulation of nutrients in soil increases the probability that they will be dispersed throughout the profile, enriching the subsoil and eventually the groundwater; this process is called leaching. Last but

not least, fertilizer usage is partly responsible for global emissions of the **greenhouse gases N_2O, CH_4 and CO_2** and the atmospheric pollutants NO, NH_3 and SO_3, hence fertilizers play a significant role in global warming, ozone layer destruction and the acidification of soil/water systems. It is important to reaffirm that key ecosystem processes also participate in gas exchange mechanisms (plant photosynthesis and respiration, microbial and animal respiration) and that these have historically contributed to GHG emissions. It is generally accepted that N_2O gas emissions connected to fertilization contribute to 35% of the all N_2O global emission (Isermann, 1994). The relative contribution to greenhouse gas emission in agriculture is about 6% in EUA (Johnson *et al.*, 2007). Notwithstanding the fact that human activities involving the consumption of fossil fuels (including the production of fertilizers and crops) further increase GHG emissions.

Gaseous losses of N can occur as ammonia (NH_3) volatilization or as nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2) emissions. NO is an essential reactant in the formation of acid rain, N_2O contributes to global warming as it is involved in the destruction of the ozone layer, while N_2O is a very important greenhouse gas because it has a mean atmospheric residence time of over 100 years (Dalal *et al.*, 2003). On a global scale, CO_2 is the most important greenhouse gas that contributes to global warming (Paustian *et al.*, 1998). Certainly, as shown in Figure 3, all of these

losses are controlled by microorganisms and affected by both natural and anthropogenic causes.

Thus, ecosystem imbalances are aggravated by the use of fertilizers. Moreover, when land is cultivated continuously, with or without fertilization, soils tend to accumulate an organic matter deficit. This situation worsens if crop remnants are not re-incorporated back into the soil. Machinery also damages soil structure by compacting and eroding it. Ecosystem imbalances are attributable to climate but also to uninterrupted nutrient extraction. Similarly, nutrient extraction is rapidly being limited by resource availability and the energy costs involved in synthesizing nutrients have significant environmental impacts.

As once quoted in *Our Nutrient World* (Sutton *et al.*, 2013), the challenge is to produce more food with less pollution. Several of the problems associated with food production can be tackled if a natural approach is taken which tries to mimic natural conditions. Arguably, current food production is disconnected from the natural rhythm of nutrient cycling. Nutrient cycles are based on long-term processes and so natural approaches can alleviate the problem but will never be as efficient as natural conditions

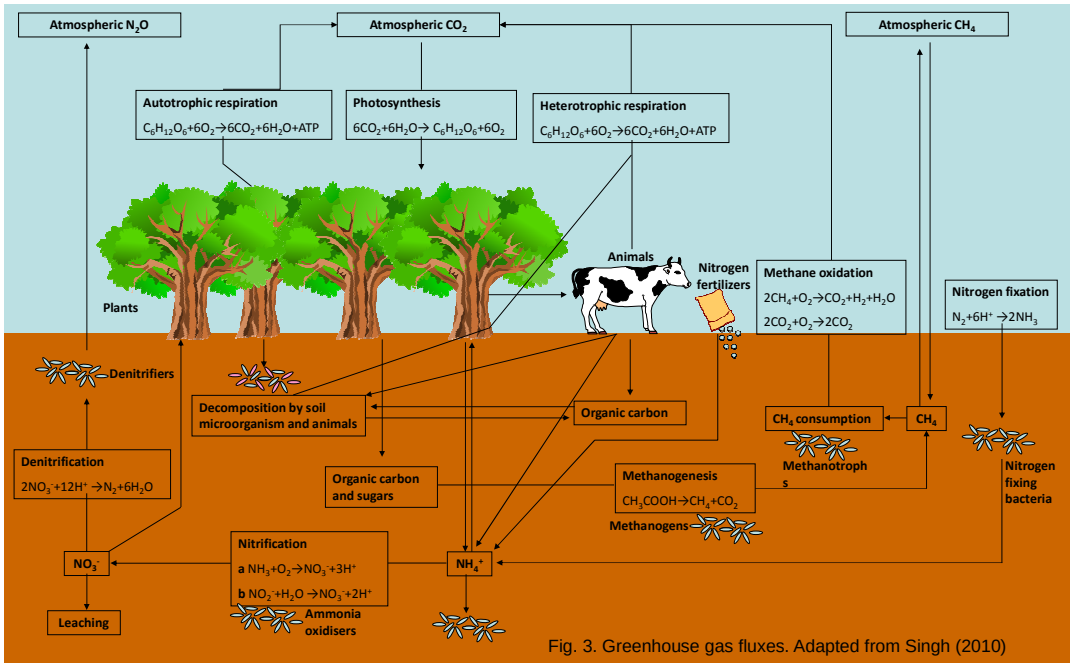


Fig. 3. Greenhouse gas fluxes. Adapted from Singh (2010)

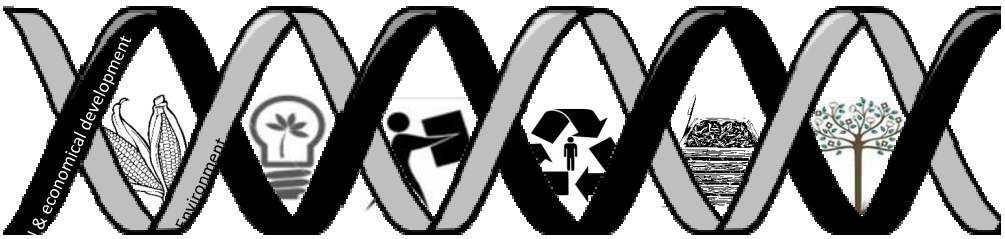
1. *A historic resource for fertilization.*

At present, one approach offering a plan to minimize the agricultural impact of fertilization and value addition in the food-chain production, is the integration of food production activities into a DNA-like structure - the so-called 'Helix of Sustainability'. Figure 4 describes the implications of this development paradigm. In short, food production should be modeled into two strands that act as pillars: economic and social development, and nature conservation. The links between the strands act as principles for product decisions. Each design stage should consider the effects occurring throughout the cycle and especially at the end of the product's useful life. As helices adjust into a spiral, each twist or stage in the food-chain production would offer an opportunity to redesign that particular

stage, so that it reduces the environmental impact of food production accordingly. The approach is different from the linear one, because each stage is tangled together with the preceding and following stages and resources should be limited by the strands to minimize harmful impacts. This approach prioritizes waste reduction, recycling and natural resource protection. Therefore, some of the deleterious impacts mentioned above could potentially be reduced (The Natural Edge Project, 2004).

In this context, the re-utilization of organic waste and residues in agriculture is a historic, common and extended practice in fertilization processes. It has experienced important changes from the practical point of view up to its regulation by government organisms, coupled with scientific advances and knowledge into its impacts.

Specifically, organic waste derived from human settlements, food production and some types of industrial waste have been successfully used as resources with **fertilization value**. However, not all agro-ecosystem surpluses, by-products, residues and wastes can be considered to have such fertilization value. Besides, products from other systems such as those which contain high levels of organic matter, can also, potentially, be integrated into agro-ecosystem cycles.



With sun's energy, plants grow and transform sugars, cellulose, aminoacids, into other complex materials	Food is harvest. Additionally, fuel and other materials can be extracted	Goods are manufactured and its value is based on environmental and social terms	The end-user is benefited as he can repair or reuse, and after total consumption recycling is possible	The product decay begins with its reduction into raw materials through the action of microbes. I.e. compost process	With sun's energy, plants grow and transform sugars, cellulose, aminoacids, into other complex materials

Natural nutrient cycling is mimicked all through, products can be returned to nature by to biological degradation

Fig. 4. The helix of sustainability. Adapted from: (The Natural Edge Project, 2004)

The European Union acknowledges that resource efficiency performance can be improved if a zero waste approach is taken. Zero waste requires a circular economy structure where resources are kept within the economy, thus adding value to the products because their useful life is extended. Waste would then be transformed from a negative factor and now considered as a resource. The European Resource Efficiency Platform considers that the, "Transition to a more circular economy requires changes throughout value chains, from product design to new business and market models, from new ways of turning waste into a resource to new modes of consumer behaviour. This implies full systemic change, and innovation not only in technologies, but also in organisation, society, finance methods and policies"(EREP, 2014). Among the proposed solutions, recycling, reuse and ecological design are preferred over product disposal.

Therefore, it is appropriate to briefly explain the regulatory framework for waste and its current uses.

2. *Waste disposal and its regulation in Europe.*

According to the European Environmental Agency (EEA): "Waste includes all items that people no longer have any use for, which they either intend to get rid of or have already been discarded. Additionally, wastes are such items which people are required to discard, for example, by law, because of their hazardous properties. Many items can be considered as waste e.g., household rubbish, sewage sludge, wastes from manufacturing activities, packaging items, discarded cars, old televisions, garden waste, old paint cans, etc"(EEA, 2013)

The EEA estimates that each European citizen generates around 3.5 ton (including both organic and non-organic material) of waste per year (EEA, 2013). Accordingly, there are different sources ranging from households, commercial activities, industry, agriculture, construction and demolition, mining and quarry and energy generation. The EEA acknowledges that waste management is an environmental and public health issue, because its management poses important risks.

Waste disposal has significant impacts on the environment. Even with current technology, incineration and landfills comprise the

most common pathways for waste and residue disposal. The problems associated with these methods range from air pollution, dangerous ash disposal, landfill availability and extension, to chemical pollution and unwanted leaks into soil and water systems. These problems that translate into environmental costs have created a constantly unsustainable practice for waste management, because of the maintained pollution they create.

Contemporary waste/residue management efforts are based on both local and regional methods. Limitations and practices vary a great deal depending on local restrictions. Even when waste and residue problems are shared among EU countries, the amounts generated and the strategies to reduce them change. There is a wide variety of approaches for managing the different wastes encountered across Europe. Consequently, common European policies have become important channels for mass communication of the effects of residues and wastes, and the benefits gained by reducing these harmful effects and how to reduce them (Fytili and Zabaniotou, 2008).

A series of European Directives have been discussed with the aim of developing an efficient approach to waste reduction. In 1975, EU policy established the principles upon which European environmental protection was based (Fytili and Zabaniotou, 2008).These are: the principle of precaution and prevention, the priority of minimizing pollution at source and the “polluter pays”

principle. Recently, the concept of “greening” has been introduced; a policy directed at encouraging the use of agricultural practices that are known to be environmentally beneficial.

There are currently three major documents addressing the regulation of waste: the revised Waste Framework Directive (WFD) (2008/98/EC), the Thematic Strategy on the Prevention and Recycling of Waste and the 7th Environmental Action Programme (EAP) (EC, 2015). These regulations are based on the **principle of waste prevention** and the **safe reuse** of recycled materials, which in turn is a philosophy founded on the fact that waste reduction at source is the best method of avoiding unwanted residues. But this rationale has been extended and Figure 5 illustrates that the least desirable ending for a product (equally applicable to a surplus, by-product or a residue) is its disposal in a landfill. So, before reaching that stage, prevention, minimization, reuse, recycling and energy recovery are part of the management possibilities available and which should be investigated before discarding a waste material. It is particularly important to protect soil and humans from the spread of pathogens and the persistence of harmful compounds.

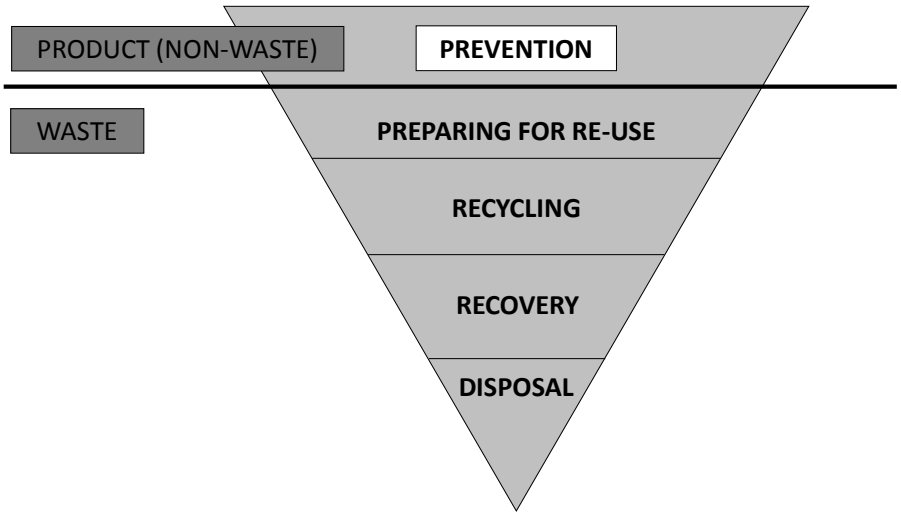


Fig. 5. Europe's approach to waste reduction (EC, 2015)

3. *What types of wastes can be considered fertilizer opportunities?*

Some extra costs are often incurred while attempting to maximize the fertilization value of organic wastes and residues. However, considering the volatile nature of petrol prices, which affects its derivates (fertilizers, fuels, etc.), two main driving forces outweigh such costs: the nutritional value and the pollution impact of alternative management options (e.g., landfill). Furthermore, policy makers, environmental managers and farmers have accepted that such costs provide a method for managing wastes systems: improving the handling techniques, reducing hazardous effects and adding value to food production. Moreover, employing the waste as soil amendment is usually the most economical and natural solution available.

According to the EEA, there are 10 different types of waste, as well as different origins and different sources. In the present case it is only worth mentioning wastes that can be exploited to recover nutrients for food production. These organic wastes are generated within the categories of municipal waste, industrial activities, packaging waste, sewage sludge and agricultural waste, among others (EEA, 2013).

The EEA considers that municipal waste accounts for about 14% of the all waste produced, and 60% of that waste is considered to be “Biodegradable Municipal Waste (BMW)”. Briefly, BMW is, “waste from households and commercial activities that is capable of undergoing biological decomposition” (EEA, 2013).Food waste and garden waste, paper and cardboard are all classified as biodegradable municipal waste.

Additionally, agricultural waste is composed of “organic wastes (mainly, animal excrement in the form of slurries and farmyard manures, spent mushroom compost, soiled water, silage effluent, etc.) and waste such as plastic, scrap machinery, fencing, pesticides, waste oils and veterinary medicines” (EEA, 2013).

Among the disposal options for all types of organic wastes are: i. composting, ii. mechanical-biological pre-treatment, iii. recycling, and iv. incineration (with and without energy recovery), v. muck spreading under strict conditions, vi. anaerobic digestion and

composting. It is worth highlighting the elevated potential fertilization value of these incorporations and waste management methods (EEA, 2013).

While the EU recognizes the opportunity for agricultural use of wastes, the regulation framework has not yet been fully developed, apart for the already described WFD and the other two documents. For instance, compost production, manure handling and animal slurry management are yet to be regulated on a European scale. The regulation of land application of organic wastes and their basic characteristics are strategic in order to avoid over-fertilization or accumulation of harmful substances in arable soils. On the contrary, sewage sludge has been controlled since 1996 by the Sewage Sludge Directive 86/278/EEC (Fytili and Zabaniotou, 2008). This directive was prepared to standardize the agricultural application of sewage sludge in such a way as to, “prevent harmful effects on soil, vegetation, animals and man”.

The Nitrate Directive (1991) is an umbrella regulation and maybe the only one that aims to protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters and by promoting the use of good farming practices (Brouwer and Hellegers, 1996). Although it primarily focuses on mineral fertilization and its effects in water resources, key practices, such as manure application management, are currently affected by this directive.

In contrast to lack of policies that regulate agricultural use of organic wastes across all EU member states, Spain has a unique legislation on soil fertilizers that incorporates the regulation of organic wastes and their further use in agriculture. Two different Spanish laws (RD 506/2013 on organic fertilizers and RD 865/2010 concerning substrates) (Ministerio de la Presidencia, 2010; Ministerio de la Presidencia, 2013) classify, typify and regulate composted waste use in agriculture. The effort to provide a regulatory framework for agricultural use of organic wastes is recognized within Europe. Although, there is still a need for studies that cover the behavior of soil composts that have already been regulated, registered in the Spanish fertilization registry, commercialized and applied to soil. The knowledge on the impacts and effect of using compost in soil would serve so recommendations can be made to improve these strategic resources.

4. *Which quality parameters should be considered? Which quality parameters should be considered?*

The opportunity to consider soil fertility affords an important occasion for the contribution of fertilizers attained from organic waste to achieve food sustainability. Wastes can be transformed into materials rich in organic matter, but the safe use of these materials has to be guaranteed and they must have a standardized quality to be considered fertilizers. Nutrient contents, hazardous ingredients, hygiene standards, physical composition, maturity and

plant growth performance are among the most common parameters subjected to continuous evaluation (Matteson and Sullivan, 2006).

Most EU countries have different ideas about the parameters which should be included, but it is commonly accepted that nutrient content should be declared. Also, the contents of undesirable and hazardous ingredients (including PCBs, PAHs, dioxins and heavy metals), where appropriate, must be within the limits of the corresponding legislation. As compost is a material rich in organic matter, pathogens and toxic elements tend to proliferate. Sanitization procedures, therefore, have to be considered to reduce associated risks for workers and during application to the soil. An ideal physical composition should avoid the inclusion of non-biodegradable material, for example, glass, plastic, metals; as well as other undesirable matter such as weed seeds, high salt concentrations, and so some limits have to be established. The completeness of the composting process is characterized by the compost's maturity (Wichuk and McCartney, 2010). This later affects the evolution of the compost once it has been applied to the soil and subsequently plant growth performance.

Several studies have focused on plant performance and the hazardous ingredients content of transformed organic wastes. But, given the current framework and scientific developments, there are still some gaps in our knowledge about the agricultural application of different composted organic wastes.

In the midst of ongoing climate change and awareness that the process is anthropogenic, especially in terms of transforming nutrient cycles, as described above, then evaluation of the warming potential of these applications also becomes relevant. Moreover, it is widely accepted that microorganisms play a key part in determining the atmospheric concentrations of greenhouse gases (Singh et al., 2010).The mechanisms of microbial communities that regulate gas exchange after the application of organic wastes could also shed some light on the diversity and possible functions that drive nutrient cycling in soil. In summary, microbial life not only mineralizes organic matter but is also responsible for the fate of nutrients.

In the past, these enhanced parameters have been evaluated separately and by employing different methods; the following section presents a brief description of some technologies developed relatively recently.

5. *Emerging tools for environmental assessment of fertilization practices that involve waste recycling*

As described in previous sections, agriculture has a significant impact on the environment, and this impact has been studied extensively. Nevertheless, very little is known about the interactions between long-term fertilization practices and the soil's microbial community, which has a key role in nutrient cycling (Morales et al.,

2010). A thorough evaluation of the soil's environmental condition should also include soil enzyme measurements since they are good indicators of soil disturbances. Enzymes also have an essential part to play in nutrient cycling processes and are frequently regulated as a response to exogenous soil conditions (Naseby and Lynch, 2002). Moreover, bearing in mind that agriculture contributes significantly to atmospheric GHG emissions, the fullest possible evaluation of gas emissions is relevant for all types of greenhouse gases together, since it is no trivial matter to estimate budgets from field-fluxes. Some other parameters can be introduced into these studies, namely $\delta^{15}N$ and soil fertility evaluations in terms of chemical characterization.

A brief explanation of some of the methods used in this thesis is presented below.

The microbial community in a soil can be directly evaluated through the use of genomic tools. Developments in genomic sciences have provided an opportunity to begin to construct our knowledge of soil microbiology, which has long been an enigma to researchers. Soil biodiversity can be surveyed using metabarcoding. “Metabarcoding is a molecular approach based on the assumption that each Operational Taxonomic Unit (OTUs) can be unequivocally identified through a specific sequence of DNA (barcode)”(Orgiazzi *et al.*, 2015). This approach, simplified in Figure 6 involves: (a) the direct isolation of DNA from a soil sample; (b) the amplification of a

specific DNA sequence, e.g., 16s gene plus its V5 and V6 regions which are taxonomically valuable; (c) sequencing the corresponding DNA; (d) analysis of the obtained sequences through a pipeline; and (e) evaluation of the taxonomic diversity. (Orgiazzi *et al.*, 2015). Covering all genomes has logistical and cost constraints; metagenomics is useful if the aim of a study is to learn about the composition and functional potential of a [particular] microbial community (Singh et al., 2010).

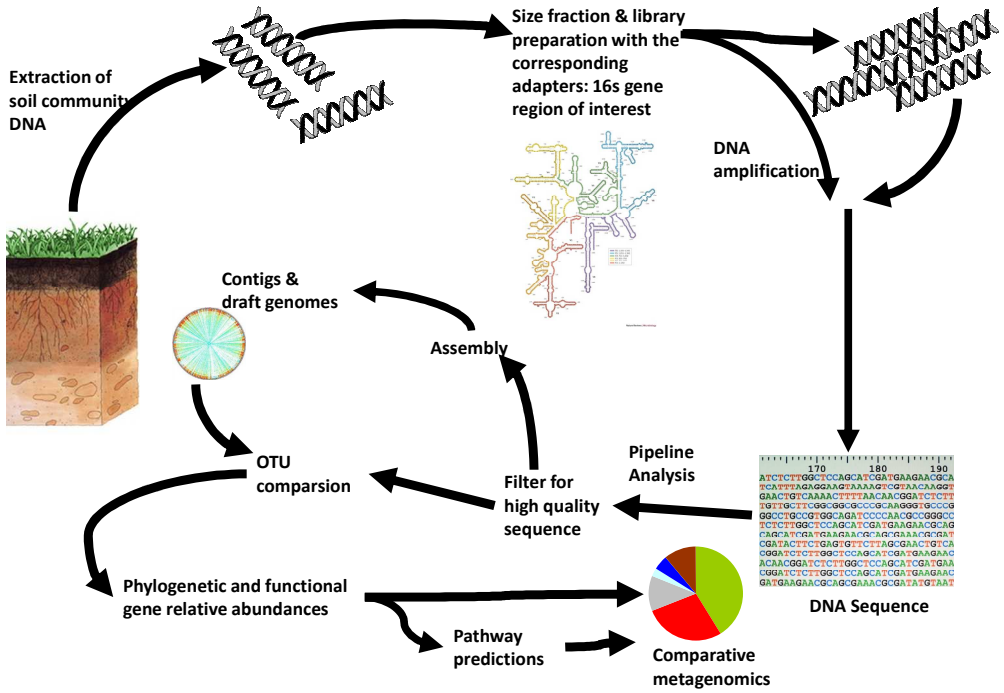


Fig 6. Soil metagenomics. Adapted from: Microbe (Jansson, 2011)

Additionally, this technology establishes an unprecedented opportunity to derive a deep understanding of the structure of soil biodiversity (Orgiazzi *et al.*, 2015). Metagenomics also provides a

chance to observe species diversity and variations within species, thus it becomes possible to perform an untargeted screening of functional genes ([Anonymous], 2009).

Soil enzyme activity has been regarded as a soil quality indicator in terms of the soil's metabolic activity (Burns *et al.*, 2013). Soil enzyme activities help to understand microbial function in soil and the environmental significance. Enzyme activities reflect the functions of bacterial communities, depending on metabolic requirements and on nutrient availability (Caldwell, 2005). Even a small shift in the activity of enzymes can significantly alter decomposition rates or organic matter (Chen *et al.*, 2014). It can be considered a good indicator of management impacts in soil.

The natural abundance of N isotopes in higher plants can be used to compare and relate plant physiology and environmental effects of composted organic wastes. The natural variation in stable N isotopes has been used as a powerful tool in several studies investigating plant and ecosystem N dynamics (Handley and Raven, 1992). Some studies assume that the $\delta^{15}N$ of leaf tissue reflects that of the source in the soil (Denton *et al.*, 2001). This assumption implies that the isotope ratio of the N source is preserved during N absorption, assimilation and translocation. However, it is clear that physiological processes and biological mechanisms, such as N-uptake, assimilation, internal N recycling in the plant and gaseous N exchange can discriminate against ^{15}N (Werner and Schmidt, 2002).

To summarize, human activity has a profound impact on nutrient cycling. Agriculture, in particular, is responsible for several disruptions. An effective strategy to reduce environmental effects is the transformation of organic wastes into compost or other materials that can be applied to soil as fertilizer alternatives. These applications still require long-term evaluation. Since microorganisms mediate the processes that affect the environment, it makes sense to assess microbial soil communities, and the contribution of these fertilization practices to greenhouse gas emissions. Further fertility characteristics can also be evaluated in order to complete the description of the possible effects that these applications may have on soils.

Objectives

Considering the arguments expressed in the introduction, regarding organic waste application to agricultural soils, the present work proposes the following objectives:

- To evaluate the effect of the long-term application of composted organic wastes on soil fertility of different agricultural soils under Mediterranean conditions and using local materials,

- To study the microbial community structure and microbial activity through soil enzyme activities after a continued application of organic wastes, and

- To determine levels of GHG emissions (N_2O, CO_2 and CH_4) from different crops under Mediterranean conditions, as well as the influence of different fertilization strategies.

To achieve these objectives, the following research has been structured around two experiments which are presented in four independent chapters. The project includes soils from two different agricultural sites, a vineyard (Bargota, Navarra, Spain) and cereal crops in rotation (Arazuri, Navarra, Spain).

In chapter 1 and 2, soil status after a 12/13-year period of continued addition of different composted materials (organic amendments) was evaluated

In chapter 3, a brief exploration into the heavy metal contents studied in chapter 1 and 2 is presented.

In chapter 4, soil status after 20 years of treatment application of treated sewage sludge application to an arable soil was compared to an unfertilized control and a mineral fertilized soil.

CHAPTER 1

Changes in soil nutrient content and bacterial community after 12 years of organic amendment application to a vineyard

1

Changes in soil nutrient content and bacterial community after 12 years of organic amendment application to a vineyard

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1.1 ABSTRACT

An interesting alternative to landfills for disposing of organic residues is their addition to soil as composted organic residues. There is little information available about the long-term benefits following prolonged periods of application. After 12 years of annual incorporation of organic amendments to the soil of a vineyard, three soil characteristics were analysed: mineral content, bacterial community and soil GHG gas emissions. The organic amendments were (i) a pelletized organic compost (PEL) made from plant, animal and sewage sludge residues, (ii) a compost made from the organic fraction of municipal solid waste (OF-MSW) and (iii) a stabilized sheep manure compost (SMC). Mineral fertilizer (NPK) and an unaltered control treatments were also included. Our results showed that long-term application of treated residues as compost changed soil nutrient content, bacterial community and gas emission rates. For instance, SMC increased nutrients and soil organic matter (OM) throughout the experiment. There was a change bacterial community structure, with an increase in the phylum Proteobacteria was observed for all four treated soils and in Bacteroidetes for PEL, OF-MSW and SMC treatments. Among the organically-amended soils, the amount of *Adhaeribacter* increased by a factor of 2.5 times more than the control, which had an increase of 2.0% of the total community compared with 5.6% for PEL, 5.2% for specializes in the degradation of residues in the different composts. The SMC treatment had the largest Chao1 estimator and was the most biodiverse of all treatments. These changes in bacterial community

structure did not correlate with the observed GHG gas emission rates. The application of amendments did not affect N_2O fluxes. However, the application of treatments reduced slightly the capacity of CH_4 sequestration by soil with respect to the untreated soils. Compost is an effective method to increase soil fertility. Soil GHG emissions should be further evaluated.

1.2 INTRODUCTION

In the current environment of urban development and the concomitant agricultural intensification, large quantities of wastes and organic residues are generated annually. An alternative to the traditional disposal methods used for these residues is to compost them for re-use as soil amendments, as the resulting material is rich in organic matter (OM) (Santos *et al.*, 2010). There is a broad consensus that the addition of OM enhances the desirable agricultural properties of soil. This s mainly related to the fact that the benefits of OM addition are in improving the soil's nutrients and physical and biological functions (Evanylo *et al.*, 2008). Composted residues are not merely fertilizers, but they also provide important advantages to the ecosystem while maintaining crop yields.

The addition of large quantities of OM promotes an active soil ecosystem, which may decrease nutrient input requirements, depending on the extraction rate and need of each crop (Mäder *et al.*, 2002). This is mainly a result of an enhanced microbial biomass that promotes an increase in microbial activity, and in the best of the cases, a greater rate of nitrogen mineralization (Paul, 2007; Birkhofer *et al.*, 2008). Further studies into the potential amount of carbon that can be stored, after the continued application of amendments, in a given crop system are still needed to enhance our understanding of the key processes that drive carbon sequestration in soil. This knowledge will help to define new ways to mitigate the effects of global warming in crop production.

The compost process is based mainly on a bio-oxidative transformation of heterogeneous solid OM into humified material (Zmora-Nahum *et al.*, 2007) or more stable carbon (C) compounds (Paul, 2007). Nitrogen (N) in organic amendments is mainly present in organic forms, so it has to be mineralized before becoming plant-available. Therefore, if the compost process is not completed before being applied to the soil, problems from nitrate leaching or gaseous losses can occur. The latter occurs during OM decomposition as nitrous oxide (N_2O), carbon dioxide (CO_2) or methane (CH_4) emissions. These gasses are involved in global warming, so their examination after long-term organic amendment addition is relevant for a balanced picture of nutrient cycling after compost application.

The process of nutrient release through decomposition and exchange reactions mediated by microbes is a direct result of the addition of inputs (Harris *et al.*, 2011). The rate of nutrient release is determined by the diversity of microorganisms, which in turn depend on OM for energy and nutrients (White, 2006). Furthermore, organically managed soils may have more stable systems and have improved environmental properties compared to soils with mineral fertilization (van Diepeningen *et al.*, 2006). Compost application to soil prompts direct reactions from its biota: studies on compost addition should include an examination of its effects on microbial diversity and other indicators of soil fertility. It is worth remembering that soil biota is one of nature's most complex ecosystems and that scientists have struggled to understand, describe and catalogue it. A community sequencing analysis to

assess the bacterial changes that occur after compost application to soil may shed light on the interaction between biological diversity and soil fertility (Mocali and Benedetti, 2010).

Finally, knowledge of the crop's influence on the soil also needs to be improved. In the present study, we studied permanent crop (the grape vine) on their effects on the soil. Steenwerth and Belina (2008a) argue that viticulture soil management has a unique influence on the soil's physical properties and microbial communities, because vineyard soils are usually fertilized and tilled less than other crops of equally intensive farming. Morlat and Chaussoud (2008) claim that vineyards have a relatively poor frequency of nutrient replacement leading to small contents of OM. This difference is crucial when soil processes are studied. Research in vineyards must apply a long-term view in order to demonstrate different soil transformations.

The main objective of this study was to observe soil status (impacts and effects in its nutrient status after a 12-year period of continued addition of different composted materials (organic amendments). This was achieved by evaluating the influence of the different amendments on the soil bacterial community when compared with a normally managed vineyard. We compared these effects with the simultaneous measurement of soil's greenhouse gas emissions after long-term application of different amendments.

1.3 MATERIALS AND METHODS

1.3.1 Site description

A long-term field experiment was conducted in Bargota, Navarra, Spain within the wine-producing area of La Rioja Protected Designation of Origin (P.D.O.). The site is located on a 5% slope in the catchment basin of the River Ebro. The location has a Mediterranean climate with a mean annual rainfall of 442 mm and mean temperature of 13.8º C. According to Soil Taxonomy System (Soil Survey Staff, 2010) the soil is classified as Inceptisol (Typical Calcixerept) with a silt loam texture. Basic soil characteristics were measured prior to conducting the experiment and the results are reported as Control 1998 (Table 1).

The experiment began in 1998, two years after the grapevines (*Vitis vinifera* L. c.v. Tempranillo.) grown on Richter 110 rootstock were planted at a density of 0.3 stocks m^{-2}. The grapevines were trained in a midway bilateral cordon system (*Cordon de Royat*) to a height of 0.8 m. Plants were pruned every year in January. All residues were removed from the ground around and then land was ploughed to a depth of 0–30 cm. Depending on the weather conditions, a sprinkler irrigation system was used when necessary throughout the year to supplement rainwater. Chemical weed control was carried out in February and the soil was left exposed and without vegetation.

Table 1. Physical and chemical properties of the soil[a] at the Bargota site (1998 and 2010) (n = 3).

	Control 1998	Control 2010	PEL 2010	OF-MSW 2010	SMC 2010
pH	7.6±0.03	7.6±0.24	7.5±0.12	7.6±0.10	7.6±0.14
EC / dS m^{-1}	0.3±0.01	0.3±0.04	0.5±0.19	0.4±0.02	0.4±0.09
O.M. / %	1±0.15	1.2±0.07	1.7±0.34	1.6±0.50	1.9±0.14
N Kjendahl / %	0.08±0.01	0.08±0.00	0.11±0.01	0.11±0.03	0.11±0.08
Olsen P / mg kg^{-1}	26.5±2.80	20.0±2.70	50.4±12.94	57.2±3.01	52.3±10.43
K / mg kg^{-1}	169.2±11.39	204.7±10.20	253.9±11.56	287.0±30.97	359.8±34.42
C:N	7.6±0.43	8.5±0.25	8.6±0.78	7.9±0.94	9.8±0.28
$CaCO_3$ / % DW	37.4±1.14	36.9±1.92	35.6±3.44	37.2±2.61	37.6±2.68

[a]Pelletized organic compost (PEL), organic fraction of municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK), soil without fertilization treatment (Control). DW = dry weight.

1.3.2 Treatments and experimental design

We used three composted organic residues and a synthetic fertilizer. The amendments (Table 2) were applied two years after planting the vines. The following amendments were used: a pelletized organic compost (PEL) made from plant, animal and sewage sludge residues; a compost made from the organic fraction of municipal solid waste (OF-MSW); a compost from sheep manure (SMC); a NPK 5–10–15 mineral fertilizer (nitrogen, phosphorous and potassium); and finally a control soil without addition was included. These amendments and represent three residues commonly generated in Spain (industrial, urban and livestock) and used in this region. The mean annual rates applied from 1998 to 2010 were 3700 kg ha^{-1} in fresh weight (FW) of PEL, 4075 kg ha^{-1} FW of OF-MSW, 4630 kg ha^{-1} FW of SMC and 340 kg ha^{-1} of NPK treatment. These amounts represent the amount of bulk product applied: the specific amounts of the individual macro-nutrients are shown in Table 3. These additions represent a basal dressing application of a common NPK fertilizer for a vineyard in this region. A plot of 108 m^2 with 15 vines per row was selected for each treatment. There were three replicates of each treatment in a random experimental design with three blocks. Organic amendments were applied to the soil surface between the rows every February and tilled into the soil to a depth of 0–30 cm with a chisel plough.

Table 2. Physical and chemical properties of fertilisers applied at the Bargota site (2010).

	PEL	OF-MSW	SMC	NPK
pH	8.8	8.3	9.0	N.A.
EC / dS m^{-1}	8.3	8.1	6.6	31.6
Dry matter / % w	86.2	69.8	59.1	98.1
Organic matter / %DW	31.8	56.9	29.1	N.A.
TOC % C / DW	14.9	32.3	14.3	N.A.
Total humic extracts	10.4	18.8	9.5	N.A.
Fulvic acids	2.8	6.5	1.7	N.A.
Humic acids	6.1	12.7	7.1	N.A.
Nitrogen Kjeldahl / %	2.8	2.5	1.4	5.0
Organic N / % DW	2.5	2.1	1.3	N.A.
C:N	7.0	13.0	12.0	N.A.
P / % DW	3.3	2.4	0.6	4.3
K / % DW	2.5	2.1	3.5	12.4
Ca / % DW	13.4	12.9	18.2	5.7
Mg / % DW	1.1	0.9	1.4	N.A.
Na / % DW	1.3	0.6	0.8	N.A.
N-NH_4 / % DW	0.3	0.3	0.0	N.A.
N-NO_3 / % DW	0.0	0.1	0.1	N.A.
δN^{15}	11.2	10.5	18.8	0.1
δC^{13}	-24.4	-25.7	-27.2	N.A.

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral (NPK) fertilizer. N.A. (Not applicable). DW = dry weight.

1.3.3 Soil sampling and analysis

Four individual soil samples were collected from different places within each replicated plot, in October after harvesting the grapes, at a depth of 0–20 cm. These samples were combined for to provide a single sample for each replicate. Afterwards, the samples were air-dried at room temperature and then stored at 4° C. Physical

and chemical properties of the soil were determined after screening the samples through a 2 mm sieve. Soil pH was measured after suspending in deionized water at a wt./wt. ratio of 1:2.5. Soil OM was determined by colorimetry after using the method described by Walkley and Black (1934). Total nitrogen was measured using the Kjendahl method and phosphorus according to the Olsen method. Available K content was determined by colorimetry after extraction with a 0.1 M ammonium acetate. Electrical conductivity was measured by suspending a portion of soil in water in a 1:5 soil:solution ratio with an EC-meter (model CDM2010, Radiometer Analytica, Lyon, Francel).

In addition to these measurements, in the year 2010, on a specific sampling day in early summer before the soil became too dry (June 2010), gaseous emissions were measured and individual soil samples were collected for DNA extraction. The date was chosen because the amendments were applied in March and the rainfall season had already finished, giving the bacterial communities enough time to stabilize and adapt to steady conditions and without being limited by water.

1.3.4 Gaseous emission sampling

Emissions of N_2O, CO_2 and CH_4 were measured just before collecting soil for DNA analysis at the same sampling locations as for the DNA, with the closed chamber technique (Chadwick *et al*., 2000). In each plot, two PVC cylinders (12 cm diameter and 15 cm height)

were placed as replicates and inserted approximately 2 cm into the soil. All cylinders were located randomly and were open to the atmosphere. To determine gas fluxes the cylinders were covered and hermetically closed with a PVC lid. Soil gas exchange rates were calculated from gas concentration changes during 45 min of closing. Headspace samples of 20 ml were taken during this period and stored in evacuated 12 ml glass vials. During the following two days the samples were analysed by gas chromatography (GC) (Agilent, 7890A, Santa Clara, CA, USA) (Menéndez *et al.*, 2008) with an electron capture detector (ECD) for N_2O detection and a flame ionization detector (FID) for CH_4. For the determination of CO_2, the GC was equipped with a methaniser to reduce the CO_2 to CH_4. A capillary column (IA KRCIAES 6017; Ingeniería Analítica S.L., Sant Cugat del Vallès, Cataluña, Spain; 240 ºC, 30 m x 320 mm) was used to separate each gas from the samples. The column's temperature was increased from 40º C to 80º C and the ECD temperature was 350º C. A mixture of Ar with 5% CH_4 was used as the carrier gas with N_2 as the remainder (15 ml $minute^{-1}$). A headspace auto-sampler (Teledyne Tekmar HT3, Mason, Ohio, USA) was connected to the gas chromatograph to automate the process. Standards were stored under the same conditions and analysed at the same time as the field-collected samples.

1.3.5 DNA extraction

Soil samples for DNA extraction were collected from a 0–20 cm deep soil layer at a distance of 50 cm from the corresponding

vine row. Two individual soil samples were collected from random points in each replicate and were pooled to obtain one representative sample for each replicate treatment. Samples were stored for less than 24 hour at 4° C and sieved through a 2 mm screen immediately before DNA extraction.

Soil DNA was extracted from each individual soil sample with the PowerSoil™ DNA Isolation kit (MoBio, Laboratories Inc. Carlsbad CA, USA) according to the manufacturer's instructions. Briefly, the method involved chemical lysis of microbial cells with gentle beating by beads: the released DNA was bound to a silica spin filter which was subsequently washed, and the DNA was recovered in an elution buffer solution. The DNA yields and quality were checked under a UV light after gel electrophoresis in 0.8% (w/v) agarose stained with ethidium bromide (Sambrook *et al.*, 1989). Measurements DNA concentration in the obtained samples were made with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

1.3.6 DNA amplification and pyrosequencing

Partial prokaryotic 16S rRNA gene sequences were obtained from the analysis of each individual sample with the coded-primer approach to multiplex pyrosequencing (Binladen *et al.*, 2007). The PCR amplification of the hypervariable regions of the 16S rRNA gene was performed on each individual soil DNA extraction with universal primers U519F and U926R (Baker *et al.*, 2003). The PCR was performed with an 8 bp bar-coded sequence joined to the specific

primers (Parameswaran *et al.*, 2007). PCR mixtures (25 µL) contained 25 pmol of each primer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 1 X of the corresponding Taq buffer, 1 U of Taq Master (5 Prime, Gaithersburg, MD, USA) and 10 ng of the DNA template. The PCR programme consisted of an initial denaturation step at 94º C for four minutes, 25 cycles of denaturation at 94º C for 15 s, primer annealing at 55º C for 45 s and extension at 72º C for one minute, followed by a final stage of heating at 72º C for ten minutes. For each sample, amplicons were generated in several replicate PCRs. Amplicons of the same treatment were pooled to reduce per-PCR variability and purified with Ultracentrifugal Filter Units with Ultracel-100 K membranes (Amicon, Cork, Ireland) according to the manufacturer's instructions. After quantification by agarose gel electrophoresis and with a Nanodrop 1000 spectrophotometer, the samples were combined in equimolar amounts and subjected to pyrosequencing with the Genome Sequencer Titanium GS-FLX system (454 Life Sciences, Branford, CT, USA) at LifeSequencing S.L. (Valencia, Spain). The sequence file was submitted to the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) and are available with the accession number PRJNA269011.

1.3.7 Taxonomic assignment of sequence reads and diversity indices

Raw sequences were processed through the Ribosomal Database Project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu) release 10 (Cole *et al.*, 2009). Sequences

were excluded from the analysis according to four criteria: if the length read was less than 150 bp; if the forward primer sequences contained more than two errors; if the sequences had one or more Ns; and if the quality index, according to the .qual file generated during the pyrosequencing process, was less than 20.

Qualified sequences were clustered into operational taxonomic units (OTUs); classified on the basis of a distance of 3%, by complete linkage clustering. Then, they were assigned to phyla by the RDP-II classifier, using an 80% confidence threshold (Wang *et al.*, 2007). Sequences that could not be classified to a phylum at this level of confidence were excluded from subsequent analyses.

Each unique sequence was further aligned with the RDP pyrosequencing function Aligner to generate phylogenetically ordered rRNA sequences. Aligned data sets were clustered with the default parameters for the RDP Clustering function. The resulting clusters were used to calculate the Shannon-Weaver index, Chao 1 estimator and rarefaction curves using the pyrosequencing analysis tools from RDP at the level of 3% species-level dissimilarities.

1.3.8 Statistical analysis

The data are the mean values of, at least, three independent replicates per treatment (for each sample, four sub-samples were taken and pooled). Standard errors are presented to determine the signification of difference between treatments. ANOVA was used to

compare differences between treatment (P <0.05). Correlations between variables were analysed, where appropriate, and the Pearson value was also calculated.

Table 3. Results of anova testing the effects of the addition of different fertilisers at the Bargota site (2010).

	Source	Degrees of freedom	Sum of Squares	Mean Square	*F* ratio	*P*
pH	Treatment	4	.027	.007	.269	.891
	Residuals	10	.251	.025		
EC / dS m^{-1}	Treatment	4	.068	.017	1.781	.209
	Residuals	10	.095	.010		
O.M. / %	Treatment	4	1.116	.279	3.029	.071
	Residuals	10	.921	.092		
N Kjendahl / %	Treatment	4	.003	.001	3.182	.063
	Residuals	10	.002	.000		
Olsen P / mg kg^{-1}	Treatment	4	4535.4	1133.8	6.084	.010
	Residuals	10	1863.7	186.3		
K / mg kg^{-1}	Treatment	4	57463.4	14365.8	9.960	.002
	Residuals	10	14423.6	1442.3		
C:N	Treatment	4	7.347	1.837	3.321	0.05
	Residuals	10	5.531	.553		
$CaCO_3$ / % DW	Treatment	4	7.814	1.953	.313	.863
	Residuals	10	62.31	6.231		
g N_2O-N ha^{-1} day^{-1}	Treatment	4	.377	.094	.931	.476
	Residuals	13	1.315	.101		
kg CO_2-C ha^{-1} day^{-1}	Treatment	4	21.593	5.398	4.157	.024
	Residuals	13	15.582	1.299		
g CH_4-C ha^{-1} day^{-1}	Treatment	4	5.333	1.333	4.164	.022
	Residuals	13	4.162	.320		

Comparison of bacterial communities was performed with the RDP sample abundance statistics tool to calculate Jaccard's index and compare the five treatments (Chao *et al.*, 2005). Aligned data sets from each treatment were merged into a single cluster file, which was used to construct a distance matrix at 3% dissimilarity. With this information a tree-diagram was produced using the unweighted pair group method with arithmetic mean (UPGMA). The unweighted Unifrac algorithm (Lozupone and Knight, 2005) was applied to explore differences in phylogenetic diversity among samples (10 000 iterations). Principal coordinates analysis (PCoA) was performed in accordance with the default parameters in Fast Unifrac Server (http://unifrac.colorado.edu/). Unifrac PCoA calculates the distance matrix for each pair of samples using the UniFrac metrics based on phylogenetic lineages they contain. The 3D plot was visualized with the KING applet contained in the same server.

The analysis of molecular variance (AMOVA, mothur, v. 1.21.0, Schloss *et al.*, 2009) was also applied to obtain significant differences between the five bacterial communities studied. The default option of 1000 iterations in the mothur program was used. This method is widely used in population genetics to test the hypothesis that genetic diversity within two populations is not significantly different from that which would result from pooling the two populations (Excoffier *et al.*, 1992).

The five different libraries generated from each soil treatment were subjected to pair-wise comparison with the Lib-compare tool (Cole *et al.*, 2009) of the RDP, which is a comparison made by estimating the likelihood that the frequency of membership of a given taxon (phylum, class, order) is the same for the different libraries.

1.4 RESULTS

In order to compare all of the treatments at the end of the experiment, results are shown for fertilizers and soil for the twelfth year only.

1.4.1 Effect on soil nutrient status

At the beginning of the experiment and throughout the 12-year study, clay, sand, silt, $CaCO_3$ contents and pH did not vary between each successive application, nor between the different types of treatment. The variation between 1998 and 2010 for characteristics such as pH, $CaCO_3$ and OM are shown in Table 1 and Table 5. The OM content was significantly greater by 2010 in the soils that received SMC (1.9%) and PEL (1.7%) than in NPK (1.1%) and control (1.2%) treatments. The SMC treatment received the least amount of OM (797 kg ha^{-1} $year^{-1}$) among the three composted materials (Tables 2 and 4). The OF-MSW treatment increased the soil's OM content by 1.6% over the 12-year period, which was not significantly different to the other compost treatments.

There were no significant differences in total N contents (0.11%) observed between those treatments with organic amendments despite the different amounts of organic N provided by each treatment, the annual mean for different treatments ranging from 38 kg ha^{-1} $year^{-1}$ to 90 kg ha^{-1} $year^{-1}$ (Table 4). As expected, organic amendments resulted in a significant increase in total soil

nitrogen (26%) when compared with the control (0.08%) and NPK (0.08%) treatments.

Table 4. Annual doses and total amounts of fertilizers applied over a 12-year period (1998– 2010).

	PEL		OF-MSW		SMC		NPK	
	Annual mean	**12-year total**	Annual mean	**12-year total**	Annual mean	**12-year total**	Annual mean	**12-year total**
	kg ha^{-1} year^{-1}	**kg ha^{-1}**	kg ha^{-1} year^{-1}	**kg ha^{-1}**	kg ha^{-1} year^{-1}	**kg ha^{-1}**	kg ha^{-1} year^{-1}	**kg ha^{-1}**
Dose	3,700	**44,400**	4,080	**48,960**	4,630	**55,560**	340	**4 080**
OM	1,015	**12,176**	1,623	**19,472**	797	**9,563**	N.A.	**N.A.**
N total	90	**1,077**	72	**859**	38	**456**	16	**200**
Org N	79	**950**	61	**729**	34	**414**	N.A.	**N.A.**
P	107	**1,280**	67	**807**	17	**204**	14	**175**
K	79	**950**	60	**715**	97	**1,163**	41	**499**
Ca	429	**5,145**	367	**4,409**	500	**5,997**	19	**229**
Mg	38	**456**	27	**322**	38	**460**	N.A.	**N.A.**

Pelletized organic compost (PEL), organic fraction of municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK). N.A. (Not applicable)

Phosphorus (P) content, initially 26.5 mg kg^{-1}, doubled with organic amendments in all cases (Table 1 and Table 3). The largest mean content at the end of the 12-year period was in the OF-MSW treatment with 57.2 mg kg^{-1}, followed by SMC and PEL with 52.3 mg kg^{-1} and 50.4 mg kg^{-1}, respectively. The differences for the control and NPK treatments were insignificant, the contents being 20 mg kg^{-1} and 16.4 mg kg$^{-1,}$ respectively, in 2010.

The effect on potassium (K) content in soil varied between the treatments (Table 1 and Table 3). Each of the organic amendments increased K content from the initial value of 169.2 mg kg^{-1}.The SMC treatment contained 359.8 mg kg^{-1} of K after the 12-year period, and in the OF-MSW replicate the content was 287.0 mg kg^{-1} and PEL 253.9 mg kg^{-1}. The smallest value was observed for the NPK treatment with 186.7 mg/kg, followed by the control with 204.7 mg kg^{-1}.

The application of SMC again produced the largest effect on the C: N ratio, with a final value of 9.8, but it was not significantly different from that for PEL (8.6) and the control (8.5). Significantly smaller values were observed for the OF-MSW and NPK treatments, with a C: N ratio of 7.9. The application of treatments significantly increased the conductivity of the sol.

1.4.2 Effect on gaseous emissions

Nitrous oxide fluxes ranged from 2.1 g N_2O-N ha^{-1} day^{-1} to 3.2 g N_2O-N ha^{-1} day^{-1} (Table 3 and Table 5). Fluxes of carbon dioxide varied from 2.5 kg CO_2-C ha^{-1} day^{-1} up to 5.6 kg CO_2-C ha^{-1} day^{-1} (Table 4 and Table 5). The control and OF-MSW treatments had larger emission rates than all other treatments. The methane fluxes were negative showing that the soil acted as a sink (Table 4 and Table 5): the rates varied from -1.6 g CH_4-C ha^{-1} day^{-1} to -0.03 g CH_4-C ha^{-1} day^{-1}. The application of treatments reduced the amount of CH_4 oxidized by the soil, as treated soils had smaller negative fluxes (less CH_4 was

sequestered). CO_2 and CH_4 fluxes correlated negatively (r^2 = 0.538).

Table 5. Nitrous oxide, carbon dioxide and methane emissions from the fertilised soils at the Bargota site (2010) (n = 4).

	Control	PEL	OF-MSW	SMC	NPK
g N_2O-N ha^{-1} day^{-1}	2.9±0.69	3.0±0.59	3.2±0.51	2.1±0.16	2.3±0.14
kg CO_2-C ha^{-1} day^{-1}	4.3±0.12	2.9±0.32	5.6±1.04	2.9±0.27	2.5±0.48
g CH_4-C ha^{-1} day^{-1}	-1.6±0.29	-0.7±0.03	-1.2±0.22	-0.03±0.26	-0.50±0.31
kg CO_2eq ha^{-1}	17.2±0.12	11.9±0.91	22.1+1.99	11.7+1.73	10.2±1.73

Pelletized organic compost (PEL), organic fraction of municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK), soil without fertilizer treatment (Control).

1.4.3 Diversity and taxonomic composition of bacterial communities

Pyrosequencing-based analysis and subsequent statistical inference provided up to 20,214 prokaryotic sequences in the soil from the NPK treatment, which after the trimmed process resulted in 15,023 useful sequences. These were clustered in operational taxonomic units (OTUs), on the basis of a distance of 3%, obtaining values of up to 1,920 (Table 6). Chao's index had a richness of up to 2,955 different OTUs for the SMC treatment; while the Shannon-Weaver index (H') had diversity values of over 6.5 with the trimmed results obtained for each treatment (Table 6).

Table 6. Trimmed and normalized (at the lowest number of sequences of OF-MSW) values of different soil bacterial diversity estimators under different fertilizer treatments.

	Control		PEL		OF-MSW		SMC		NPK	
	Trimmed (T)	Normalized (N)	T	N	T	N	T	N	T	N
Distance	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
No. seq	9,393	9,138	10,254	9,138	9,138	9,138	10,782	9,138	15,023	9,138
OTUs	1,616	1,596	1,698	1,607	1,685	1,685	1,822	1,675	1,920	1,522
Chao1	2,725	2,676	2,628	2,514	2,617	2,617	2,955	2,783	2,865	2,436
LCI95	2,523	2,479	2,458	2,345	2,449	2,449	2,759	2,585	2,699	2,263
UCI95	2,972	2,918	2,837	2,722	2,821	2,821	3,192	3,023	3,067	2,650
H'	6.385	6.382	6.532	6.505	6.533	6.533	6.504	6.468	6.498	6.401
Var H	0.00023	0.00024	0.00019	0.00021	0.00022	0.00022	0.0002	0.00023	0.00014	0.00021
E	0.86434	0.86528	0.87827	0.88118	0.87926	0.87926	0.86637	0.87132	0.85955	0.87358
Coverage	92.26%	92.13%	93.25%	92.67%	92.18%	92.18%	92.58%	91.74%	95.03%	92.90%

Pelletized organic compost (PEL), organic fraction of municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK), soil without fertilizer treatment (Control).
No. seq = number of sequences; OTUs = total number of detected OTUs at a distance of 3% (0.03); Chao1 = Chao1 richness index; LCI95 and UCI95: Lower and upper 95% confidence intervals of the Chao1 index; H' = Shannon-Weaver diversity index; Var H= variance of H'; E = Evenness considering H'; Coverage is according to the Good's index.

The number of sequences for all the treatments were normalized against the smallest number observed that of OF-MSW amended soil with 9,138 sequences. In every case, for treatments NPK and the control, the coverage of the prokaryotic diversity was greater than 91% of the total, for trimmed and normalized data. Distribution of more than 93% of the sequences between different taxa is represented in Figure 1 (and Supplementary Material: resource 1). Figure 1 shows that the most represented phylum was the Bacteroidetes (on average 25% of the total bacterial population) in all samples except for the NPK treatment where Proteobacteria was the predominant phylum (25.2%). Other represented phyla were Acidobacteria, Actinobacteria, Gemmatimonadetes and Verrucomicrobia. These six phyla comprised more than 83% of the total bacterial population (Figure 1 and Table 7). Other phyla representing about 1% of the total included Firmicutes, Chloroflexi and Planctomycetes, while phyla such as Nitrospira or Cyanobacteria were around 0.5% or less. The unclassified bacteria represented between 8.4% and 10.4% of the total bacterial population.

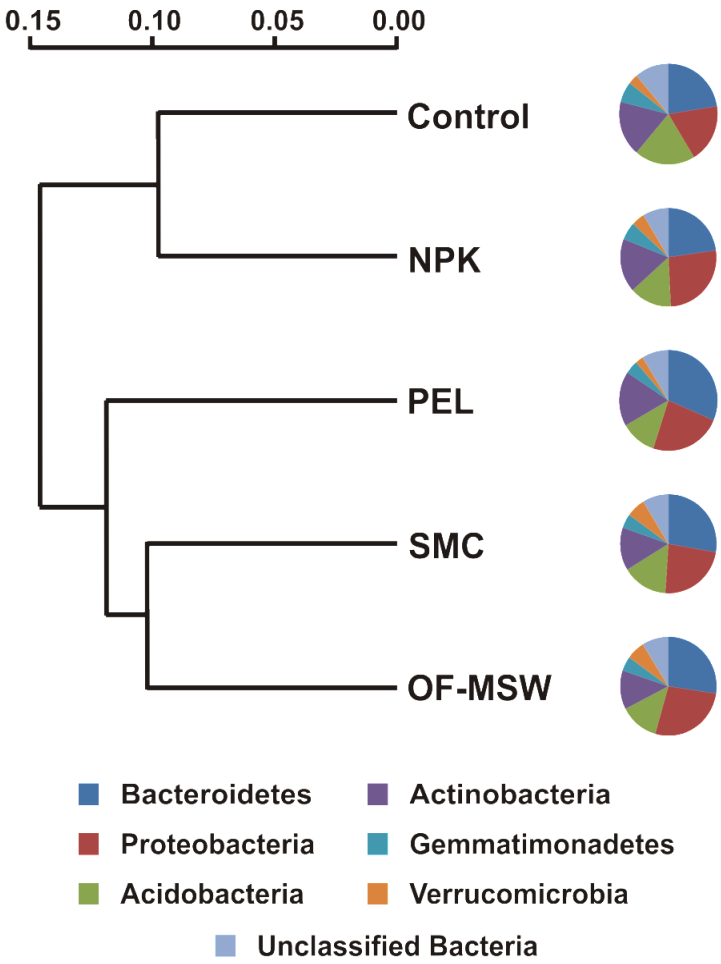

Figure 1 Cladogram of the five different soil bacterial communities based on Jaccard distance (3% dissimilarity). All bacterial communities were different among each other according to the analyses of molecular variance (AMOVA) and unweigthed Unifrac algorithm Pie charts below each branch represent the relative abundance of the different phyla. Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), and sheep manure compost (SMC) mineral fertilizer (Mineral), soil without fertilization treatment (Control).

Table 7. Percentage distribution of the detected phyla between the different soil treatments.

Phylum	Control	PEL	OF-MSW	SMC	NPK
Bacteroidetes	21.2%	29.7%	25.8%	26.4%	21.7%
Proteobacteria	17.8%	22.2%	25.5%	22.1%	25.2%
Acidobacteria	18.8%	11.1%	12.3%	14.5%	13.5%
Actinobacteria	16.7%	16.6%	12.0%	13.4%	16.8%
Gemmatimonadetes	6.3%	4.0%	4.5%	4.4%	5.6%
Verrucomicrobia	3.0%	2.5%	5.8%	6.0%	4.2%
Firmicutes	1.1%	1.8%	1.7%	1.1%	0.8%
Chloroflexi	2.0%	1.7%	1.5%	1.3%	1.8%
Planctomycetes	1.3%	1.3%	1.4%	1.3%	1.0%
Nitrospira	0.4%	0.4%	0.6%	0.4%	0.5%
TM7	0.1%	0.1%	0.3%	0.3%	0.4%
Cyanobacteria	0.4%	0.2%	0.1%	0.2%	0.3%
Deinococcus-Thermus	0.0%	0.0%	0.0%	0.0%	0.0%
OP10	0.1%	0.0%	0.0%	0.1%	0.0%
Tenericutes	0.0%	0.0%	0.0%	0.0%	0.0%
BRC1	0.0%	0.0%	0.1%	0.0%	0.0%
Chlamydiae	0.0%	0.0%	0.0%	0.0%	0.0%
Spirochaetes	0.0%	0.0%	0.0%	0.0%	0.0%
OP11	0.0%	0.0%	0.0%	0.0%	0.0%
OD1	0.0%	0.0%	0.0%	0.0%	0.0%
WS3	0.0%	0.0%	0.0%	0.0%	0.0%
Unclassified Bacteria	10.6%	8.3%	8.4%	8.3%	8.3%

Pelletised organic compost (PEL), organic fraction of municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK), soil without fertilization treatment (Control).

1.4.4 Effects of different treatments on bacterial communities

The AMOVA analysis demonstrated that the bacterial communities were significantly different between all treatments ($P < 0.001$), even though the alpha diversity indexes (Chao1 as richness index and Shannon-Weaver as diversity index) for the five treatments were not significantly different when comparing the normalized data (Table 6). The normalized NPK treatment had the smallest number of OTUs (1,522) and the smallestt Chao1 estimator (2,436) representing a decrease with respect to the control treatment. However, the richness index was also smaller for the PEL treatment than in the control. In contrast, OF-MSW the treatment produced an increase in the number of the detected (1685) and SMC-increased the Chao1 richness estimator (2783), compared with the control treatment. However, the Shannon-Weaver diversity (H') and equity (E) indices for the different treatments were always larger than the corresponding values for the control treatment, with the largest diversity observed for the OF-MSW treatment and the largest equity for the PEL treatment (Table 6).

The Jaccard distance matrix analyses of the similarity of communities at the OTU level indicated that bacterial communities from the control and NPK treatments occupied a node that was distinguishable from that formed by communities taken from OF-MSW, SMC and PEL treatments (Figure 1). This suggests that the OTU composition of the bacterial communities being treated with organic substances were more similar amongst themselves and

different from the control and NPK treatments. The bacterial communities from SMC and OF-MSW soils were most similar to each other with the PEL community becoming similar later. Surprisingly the NPK treatment contained a bacterial community very close to that of the control, despite the observed increase in the H' and E indices coupled with the decreases in the number of OTUs and the Chao estimator with respect to the control. These results were also observed in the PCoA representation obtained after a UniFrac analysis (Figure 2), confirming a close relationship of the bacterial communities from SMC, OF-MSW and PEL soils in the first dimension, but a clear separation of the PEL community in the second dimension. Generally, applying a compost treatment to the soil resulted in an increase of the copiotrophic bacteria, which in PEL, SMC and OF-MSW resulted from an increase in Bacteroidetes and Proteobacteria phyla, while in the NPK treatment, it was only from increase of Proteobacteria.

At a lower taxonomic level, differences in community composition were observed between the treatments. The bacterial community of vineyard soils treated with compost (SMC, OF-MSW and PEL) had a significant increase ($P < 0.01$) of the order Sphingobacteriales (phylum Bacteroidetes). In the five treatments the more abundant genera of this order were *Adhaeribacter, Pedobacter, Flavisolibacter* and *Ferruginibacter*. There was no difference between treatments and the control in the proportions of the last three genera, but *Adhaeribacter* was increased by more than

2.5 times with respect to the control. This represents 1.96% of the total community in the control compared with 5.60% in the PEL bacterial community, 5.16% in OF-MSW and 5.03% in SMC. Another important genus of the phylum Bacteroidetes was *Ohtaekwangia*, which represented around 3.5% in each of the five bacterial communities, with no significant differences ($P < 0.01$) between treatments.

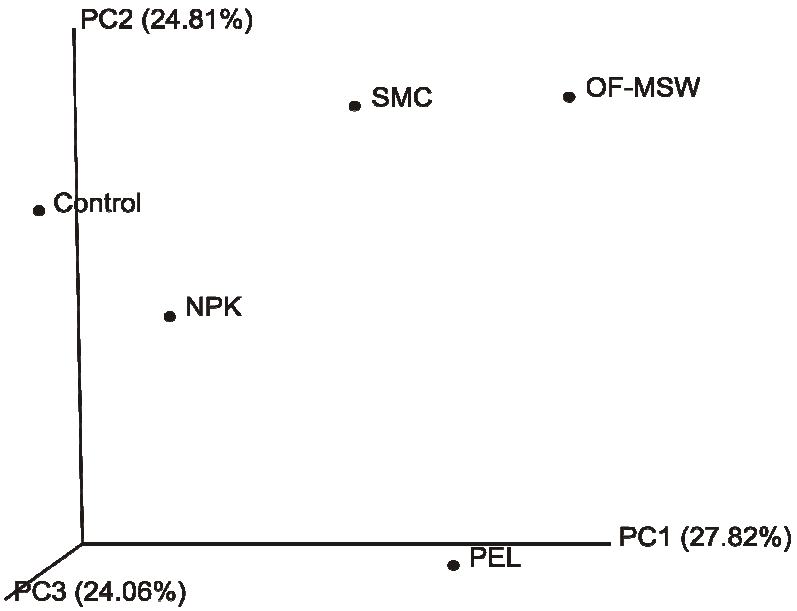

Figure 2. Plot representing the eigenvectors for the principal coordinates analysis (PCoA) of the five microbial communities. Pelletised organic compost (PEL), municipal solid waste compost (OF-MSW), and sheep manure compost (SMC) mineral fertilizer (Mineral), soil without fertilizer (Control).

The order Actinomycetales (phylum Actinobacteria) increased in the bacterial communities of PEL and NPK treatments, and decreased in the soil treated with SMC and OF-MSW. The main genera of this order were *Arthrobacter, Nocardioides, Blastococcus* and *Streptomyces*, but there was no significant difference ($P < 0.01$) between the five treatments (Table 8). Other genera which did have a significant difference were the cellulolytic bacteria *Cellvibrio* (order Pseudomonadales, class γ-Proteobacteria) representing 1.69% of the OF-MSW community and *Nitrosospira* (order Nitrosomonadales, class β-Proteobacteria) which represented 2.24% of the community from the NPK treatment and 0.48% of the PEL vineyard treatment (data not shown). This latter genus plays an important role in the oxidation of ammonia.

Table 8. Percentage distribution of the detected genera between the different soil treatments.

Genera	Control	PEL	OF-MSW	SMC	NPK
Adhaeribacter	1.959%	5.598%	5.165%	5.027%	1.531%
Gp3	0.916%	0.507%	0.547%	0.640%	0.466%
Gp4	9.741%	4.067%	5.603%	6.251%	7.176%
Gp6	7.016%	5.588%	5.297%	6.659%	5.199%
Gp7	-	-	-	-	0.433%
Gemmatimonas	6.026%	3.774%	4.344%	4.331%	5.665%
Ohtaekwangia	3.598%	3.657%	3.349%	3.255%	3.668%
Arthrobacter	2.193%	1.697%	1.215%	1.577%	1.784%
Opitutus	2.161%	1.531%	2.725%	2.347%	1.864%
Nocardioides	1.235%	2.731%	1.346%	1.391%	2.303%
Flavisolibacter	1.203%	1.590%	1.062%	1.725%	1.172%
Solirubrobacter	1.160%	0.868%	0.777%	0.974%	1.078%
Flavobacterium	1.160%	2.467%	2.200%	2.235%	1.551%
Ferruginibacter	1.097%	0.878%	1.269%	0.992%	1.138%
Streptomyces	0.926%	0.995%	0.646%	-	0.872%
Blastococcus	0.852%	-	-	0.631%	0.865%
Flavitalea	0.724%	0.692%	0.569%	0.621%	0.652%
Massilia	0.703%	0.936%	0.766%	0.742%	1.551%
Skermanella	0.703%	0.527%	-	0.584%	0.606%
Steroidobacter	0.639%	0.624%	0.788%	0.816%	0.885%
Saccharothrix	0.617%	-	-	-	-
Subdivision3_genera_incertae_sedis	0.607%	0.653%	2.583%	3.153%	1.571%
Armatimonas/Armatimonadetes_gp1	0.596%	-	-	-	0.513%
Microvirga	0.564%	0.722%	-	0.714%	-
Nitrosospira	-	0.478%	-	-	2.243%
Nitrospira	-	-	0.547%	-	0.539%
Cellvibrio	-	1.685%	-	-	0.473%
Sphingomonas	-	-	1.105%	1.345%	1.618%
Pedobacter	-	2.253%	1.948%	0.927%	1.338%
Hymenobacter	-	-	-	0.482%	0.779%
Rhizobium	-	-	-	-	0.479%
Pseudomonas	-	-	0.668%	-	0.473%
Lysobacter	-	1.404%	-	-	0.439%
Devosia	-	-	-	-	0.413%
Dyadobacter	-	-	-	-	0.373%
Rubrobacter	-	-	-	0.705%	0.353%
Spartobacteria_genera_incertae_sedis	-	-	-	-	0.346%
Pontibacter	-	1.043%	-	-	-
Brevundimonas	-	0.644%	-	-	-
Blastococcus	-	0.614%	-	-	-
Rhizobacter	-	0.556%	0.668%	-	-
Rhodococcus	-	0.507%	-	-	-
Terrimonas	-	0.488%	-	-	-
Arenimonas	-	-	0.547%	-	-
Terrimonas	-	-	-	0.566%	-

Pelletised organic compost (PEL), organic fraction of municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK), soil without fertilization treatment (Control).

1.5 DISCUSSION

Each treatment had a different effect with respect to increases in fertility, expressed as the quantity of nutrients (N, P and K) available to plants. For example, in the case of phosphorous, to induce a 1 μg g^{-1} increase in P in the soil (Olsen), 6 kg ha^{-1} $year^{-1}$ is required over 12 years of continued application of the SMC treatment. To achieve the same gain with PEL or OF-MSW, 21 or 42 kg ha^{-1} $year^{-1}$, respectively, would have to be applied. This difference is probably related to the differences in the original material in the soil as well as the composting process (Gattinger *et al.*, 2012).

The evaluation of OM content and how it changes in soil reflects the soil's C sequestering potential and so, its capabilities to mitigate or reduce global warming. Under the vineyard's management practices described, for instance, we can deduce the quantities of compost required to increase the soil OM content by 0.1% (at a depth of 0–20 cm) over a 12-year period. The amount that needs to be applied depends on the type of compost. From our data, an increase of 0.1% can be achieved if 1,517 kg, 2,590 kg or 5,727 kg of OM from SMC, PEL and OF-MSW, respectively, are added. This is indicative of the different maturity states of the OM in each amendment and suggests that SMC has gone through a longer composting process and that its OM is composed of more complex and recalcitrant molecules (mature and stable) (Kubat and Lipavsky, 2006; von Lützow, M. *et al,.* 2008). In contrast, the OM in PEL is presumably labile and is more easily degradable. Thus, nutrients in

PEL tend to be lost easily. The capacity of any compost to yield net gains in C stocks is linked to the nature, different characteristics and quality of the composting process (Senesi, 1989). Gattinger *et al.* (2012) recently studied the ability of organic amendments to enhance C stocks in soils. Their thorough review suggested that carbon sequestration is more likely to occur if mixed fertilizer techniques are used. However, in our study SMC addition alone, regardless of the crop management, also increased OM stocks. The difference in maturity states is further indicated by the differences in the C: N ratio. Carbon sequestration capacity is optimal when C: N ratios range from 10–14, and anything above or below this threshold reduce sequestration capacity (Raviv, 2005): the SMC material falls into this category. If improved carbon sequestration-oriented management is put in place further detrimental effects of GHG emissions may be attenuated (Lal *et al.*, 2007) and ideally, it would achieve a reduction in the energy required to produce grapes. However, this assumption has to be further evaluated by a specific study on the complete cycling of nutrients.

It has been widely suggested that the application of organic fertilisers increases N_2O emissions (Snyder *et al.*, 2009). However, in our case increases did not appear to be related to fertilizer application. This was probably because of the long period between application of the fertilizer and the measurement of N_2O emissions (three months), in addition to the small N application rate (Table 4). The nitrogen was mainly applied in organic forms and so the

availability of mineral nitrogen for nitrification and/or denitrification would be very small, even in the fertilizer treatments. Under our conditions, this would explain the lack of differences between treatments with regard to N_2O emissions. Although to confirm this measurements for a longer period of time should be carried out. The addition of labile C with the different amendments may have also reduced N_2O emissions (Sanchez-Martin *et al.*, 2008), by promoting denitrification to N_2. Pearson correlations between N_2O fluxes and the main bacterial genera were evaluated but no significant correlations were observed.

Carbon dioxide and methane emissions were negatively correlated. In addition, neither CO_2 nor CH_4 showed any correlation with the bacterial communities. It is well known that the main factor which controls soil respiration (Linn and Doran, 1984) and CH_4 oxidation by methanotrophs (Dutaur and Verchot, 2007) is O_2 availability. Therefore the differences in CO_2 emission and CH_4 consumption should be a consequence of variation in O_2 availability between the different treatments. Because all soils were under the same rainfall/irrigation conditions, it is reasonable to assume that the variation in O_2 availability resulted from the different effects of each treatment on the physical properties of the soil.

With the bacterial community structure with diversity coverage greater than 91%, the different treatments had a minor effect on the total number of OTUs observed: this was less than

3,000 in accordance with other studies on agricultural soils (Acosta-Martínez *et al.*, 2008). In relation to the normalized data, the Pielou's equity and the Shannon's diversity increased slightly with the treatments, but only the SMC treatment had a clear increase in the Chao1 richness estimator. The β diversity showed minor differences between treatments, with amendment application providing a slight improvement in the ecological indices.

The phyla composition reveals a clear effect of treatment; an increase in copiotrophic bacteria of the phyla Proteobacteria and Bacteroidetes was observed, and a decrease of the more oligotrophic phyla Actinobacteria and Acidobacteria. Clustering with the Jaccard distance matrix and PCoA analysis showed that the bacterial communities from the NPK and control treatments were very similar, while those of the organic amendments were grouped together in a second cluster.

The increase in OM, N and P contents, when PEL, SMC and OF-MSW were applied, were correlated with the increase of the phylum Bacteroidetes. These organisms are chemoorganotrophic and have been described as efficient in the degradation of a great variety of organic compounds (Kirchman, 2002). *Adhaeribacter* showed the greatest increase in this phylum. This genus has been isolated from water, air, forest soils and soils of the Antarctica. It has been described as a generator of large amounts of extracellular fibrillar material, a concrete corrosion agent that is able to degrade

polymers (Zhang *et al.*, 2009; Li *et al.*, 2012; Aislabie *et al.*, 2013). Thus, *Adhaeribacter* could be a specialised genus in the degradation of complex products derived from different composts. In the case of mineral NPK fertilizer addition, the Bacteroidetes phylum was present in the same proportion as in the control treatment.

Also in NPK fertilisation, Proteobacteria increased and the genus *Nitrosospira* constituted 2.24% of the total sequences analysed for this treatment. This genus plays an important role in the oxidation of ammonia, which was present in the NPK fertilizer. A second treatment which showed a similar result was the PEL amendment, in which *Nitrosospira* constituted 0.48% of the total Proteobacteria. This suggests that ammonia is probably present in the PEL because the proportion is larger than in the other treatments where the proportion was never larger than 0.08%. The differences observed at a bacterial community level reflect the state of the soil at the time of sampling and it does not necessarily reflect accurately how these communities interact to yield different changes. However, this information is useful as a record of the steady-state of the soil and further analysis should be carried out to understand seasonal changes or changes closely related to fertilizer practices.

1.6 CONCLUSIONS

In this region, the available organic amendments vary significantly in their composition. Their uniqueness in material sources and composting process is underscored by this study. The expression of differences after twelve years fills in the knowledge gaps on the biological implications of organic matter behaviour in soil.

Likewise, twelve years of differentiated fertilization are sufficient to yield different outcomes. Under this farming system, an important increase in soil OM was found. This has particular relevance since Mediterranean conditions tend to be vulnerable to other climate change impacts and OM increases soil's resilience.

Given the studied management, SMC is the most promising organic amendment; it has shown to be the most effective under this agro ecosystem. Its application substantially improves nutrient availability in soil and enhances soil O.M actively. It supports a rich biodiversity and increases soil biota. Copiotrophic bacterial community is favoured over oligotrophic community. Being Bacteriodetes the dominant phylum, in opposition to Proteobacteria that dominated NPK community. Furthermore, SMC and OF-MSW shown similar bacteria communities compared to PEL most probably as an indication of industrial products within PEL. *Adhaeribacter* could be a genus that is specialized in the degradation of complex products derived from the compost process since its abundance

increase more than 2.5 times in SMC, OF-MSW and PEL. Moreover the genus Nitrosospira, involved in ammonia oxidation, only increased in the mineral treatment.

More importantly, the use of SMC is a very effective form to integrate animal manure into vineyard systems, with a proven long-term capture of organic C in the form of OM. The sequestration rate has been shown to be active and it is suggested to monitor if a saturation point has been reached. Its usage in similar agroecosystems has to be studied to verify these results. The study highlights the need to integrate different economic sectors in the region, so that the growing needs for alternative sources of fertilization is coupled together with the disposal needs of residues that have been adequately composted.

A great potential for agricultural mitigation of climate change, in a vineyard system, is indicated by the results. The captured O.M highlights a key process for climate change mitigation. The results of this study lead to the development of further studies to understand the active contributions of fertilization practices to climate change alleviation. Furthermore, these conclusions are of general interest since Mediterranean climates are widely cultivated and their sustainability could be benefited from similar practices.

CHAPTER 2

Thirteen years of continued application of composted organic residues in a vineyard modify soil quality characteristics.

2

Thirteen years of continued application of composted organic wastes in a vineyard modify soil quality characteristics

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2.1 ABSTRACT

A solution for environmentally wiser agriculture is the use of composted organic wastes as soil amendments. Just as this alleviates the problem of recycling organic residues, it provides necessary nutrient input for food production. The objective of this work was to study the effect that 13 years of applying three different composted organic wastes or organic amendments have had on soil quality, GHG emissions and the dynamics of its microbial communities 15 days after the annual application. For this purpose, in 1996 a field trial was set up in a Tempranillo vineyard. Since 1998, the applied organic amendments have been as follows: 1. a pelletized organic compost (PEL) made from plant, animal and sewage sludge residues; 2. a compost made from the organic fraction of municipal solid waste (OF-MSW); 3. a compost made of stabilized sheep manure (SMC); 4. a mineral fertilizer (NPK); and 5. an unaltered control. The mean annual doses applied since 1998 have been 3700 kg ha^{-1} fresh weight (FW) of PEL, 4075 kg ha^{-1} FW of OF-MSW, 4630 kg ha^{-1} FW of SMC, and 340 kg ha^{-1} of NPK treatment. Soil quality was consistently enhanced by amendment application over the 13 years. Total nitrogen was significantly increased in PEL (0.1%), OF-MSW (0.09%) and SMC (0.1%) compared to control (0.06%). Nutrient content was also improved in a similar way, e.g. the most significant increase in P Olsen (80.7 mg kg^{-1}) and K_2O (473.8 mg kg^{-1}) was found on SMC. The overall enzyme activity was also increased 15 days after the annual application and OF-MSW had the highest rate (95.9) compared to control (51.3). This increase in metabolic activity was also recorded

in GHG emissions. CO_2 equivalents per hectare were 1,745 for OF-MSW and the only significant difference found. PEL with 1,598 and SMC with 1,591 were not different from the Control (1,104). Even though GHG emissions in the soil increased because of the application, soil organic matter content increased significantly (at least 35% more in all organic treatments compared to control) and this rise in organic matter was consistent over the years. According to the results, 85% of the sequences corresponded to 5 main phyla: Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria and Gemmatimonadetes, with unclassified material making up for 10.9% (average) of the sequences. Bacterial diversity by Shannon and Chao1 indices was not affected 15 days after the application. However, slight changes in the bacterial community were recorded 15 days after application only in OF-MSW treatment. Assessing soil quality using these three factors allows the relevant agronomical capabilities of the soil to be integrated with the potential effect of this practise on global warming.

2.2 INTRODUCTION

Climate change poses an important challenge for food production. Agricultural systems must meet the goal of ensuring productivity by effectively adapting to changing climatic conditions and enabling mitigation practices, in other words, agriculture has to be more resilient (Steenwerth *et al.*, 2014).

To achieve the goal of a wiser agriculture, the quality of the soil that is being used must be preserved or enhanced through fertilization. Soil quality has been described as the degree of fitness of a soil for a specific purpose (Gregorich *et al.*, 1994). Also, soil quality is understood as a series of ecological functions, such as nutrient cycling, energy and water storage, and biological transformations (Doran and Zeiss, 2000; Havlicek, 2012). Moreover, the ability of soil to function as a viable component in food production, according to the requirements of each crop and its surrounding environment, has to be the central focus for evaluating soil quality.

The assessment of soil quality should consider the purpose of each particular soil. In this case, as previously stated by Aranda *et al.* (2011) soil management practices in traditional Spanish viticulture neglect soil quality and lead to its deterioration. One reason is the frequent ploughing to keep the land bare for the majority of the time. This practice is aimed at reducing competition with weeds and supressing pathogens (Francia Martínez *et al.*, 2006). Additionally,

large quantities of phyto-sanitary products are applied to the vines, which seriously erode and degrade soil (Milgroom *et al.*, 2007). These practices deplete the organic matter content, noticeably affecting firstly soil quality and eventually the quality of the grapes, as well as the sustainability of the vineyard (Morlat and Symoneaux, 2008).

An interesting alternative to traditional mineral fertilization, which improves soil quality in vineyards, is the application of composted organic wastes. Residues and surplus materials from different stages of food production, such as organic waste and manure, provide agricultural systems with nutrient resources that would otherwise be discarded (Westerman and Bicudo, 2005). These residues already cause an important management problem as disposal options are limited and not necessarily environmentally-friendly (i.e., landfills and incineration) (Kumar *et al.*, 2012). Moreover, after composting, these materials are rich in nutrients, and contain an important quantity of organic matter. Compost performance in soil is closely linked to the composting process (Ceustermans *et al.*, 2010). A mature compost increases the possibility of preventing or reducing the greenhouse gas emission potential of waste, and a beneficial feedback loop is generated as resources are used more efficiently (with less losses). More importantly, composted organic wastes prevent the deterioration of soil functions. Previous studies on the effect of composted organic-residues in vineyard soils have demonstrated that significant

changes are observed from the 6th year of trial onwards (Morlat and Chaussod, 2008). Also, previous research into the physical properties of soil revealed that the addition of compost, from the organic fraction of municipal waste, improved the organic matter content without posing a risk of heavy metal phyto-toxicity (Pinamonti, 1998).

In the case of long-term application of compost from organic wastes, soil quality should be studied in addition to other parameters. A good starting point is the traditional soil quality measurement that includes indicators such as enzyme activity, nitrogen mineralization, nitrification rates, total nitrification and denitrification. All these processes depend greatly on microorganisms and their community structure. Also, as incorporating composted organic-residues into soil fosters intense short-term microbial activity (Zaccardelli *et al.*, 2013), an examination of the soil's microbial structure is also an appropriate addition to soil assessments. This latter can be analysed through the use of novel techniques based on the abundance of the 16s gene through the extraction of microbial DNA (Robe *et al.*, 2003). The advantage of this technique is that a high proportion of the community can be recovered (Vega-Avila *et al.*, 2015).

Lastly, greenhouse gas emissions (CO_2, CH_4, and N_2O) from soils are important, as, for example, fertilization is the origin of 35% of global N_2O emissions (Isermann, 1994). Gaseous emissions are

usually researched and used as the sole indicator of how a fertilization practice influences climate change. The rationale behind climate change mitigation is bound to the idea of reduction (or even removal) of greenhouse gas emissions (Shcherbak *et al.*, 2014) within a context of mineral and short-term fertilization. Where composted organic residues are applied this objective is not fulfilled because the practice itself is an emissions trigger. Conversely, over some years, long-term carbon sequestration is achieved by maintaining and even increasing C stocks in soils (Gattinger *et al.*, 2012) as a consequence of applying a fully mature compost (Johnson *et al.*, 2007). For this reason, the application of composted organic residues offers a new mitigation opportunity and this indicator should also be analysed.

North-western Spain has a long tradition of viticulture and wineries. From ancient times, these industries have been considered one of the most important economic activities in the Navarra region (Unwin, 1996). The extensive vineyards in the area cover approximately 27,447 ha, and more importantly, wine making represents around 8.8% of the total agro-related income (INE, 2009). The economic importance of wine production is much more far reaching than just the area covered by vineyards: the Protected Designation of Origin (P.D.O.) *D.O.C. La Rioja* in Navarra, establishes a quota per hectare of produced grape. However, the focus of this study is not efficiency in terms of yield, but rather a comparison of the effects long term application of various fertilization practices

have on soil quality.

In this context, the aim of this work is to characterize the quality of the soil after 13 years of continued treatment using organic amendments. In addition, we have analysed the effect of this application in the microbial activity and the GHG emissions. We were particularly interested in observing the composition of bacterial communities 15 days after amendment's last application. Additionally, this study sought to understand if these fertilization practices serve as sources or sink for carbon.

2.3 MATERIALS AND METHODS

2.3.1 Site description

In accordance with the aforementioned objectives, this research was carried out at a long-term experimental site established in 1996 in Bargota, which is situated within the P.D.O. *La Rioja,* in Navarra, Spain. The area is located on a 5% slope in the catchment area of the Ebro River. The climate, according to the classification system of Papadakis (1961), is Mediterranean, with hot summers and annual rainfalls of between 450 mm and 490 mm; the mean annual temperature is 13.8°C. Figure 1 shows the precipitation levels and air temperatures recorded during the assayed period. Soil texture in this area is loamy-clay and it is classified as a typical Calcixerept. The characteristics of the control soil for the year 2011 are found in Table 1.

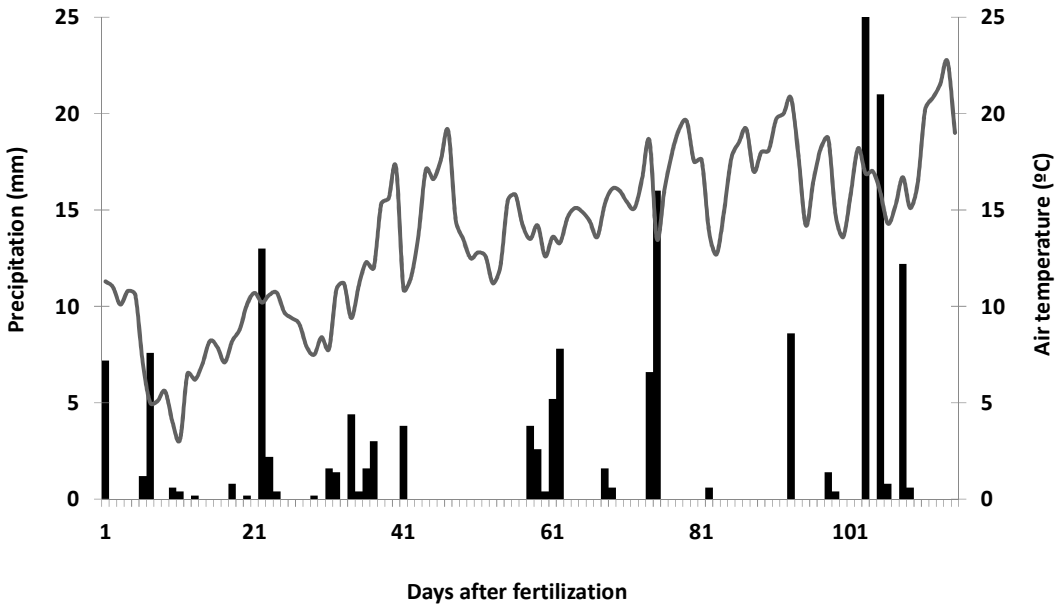

Figure 1. Precipitation and Air temperature during the experiment at the Bargota site (2011).

Table 1. Physical and chemical properties of the soil at the Bargota site (2011)

	pH	EC (dS m^{-1})	O.M. (%)	N Kjendahl (%)	P Olsen (mg kg^{-1})	K_2O (mg kg^{-1})	C/N	$CaCO_3$ (%)
Control 2011	7.36	0.37	1.19	0.06	29.5	231.7	11.07	38.1
	n.s.	b	b	b	b	b	a	n.s.
PEL	7.37	0.89	1.65	0.1	68.6	429.3	8.97	36.2
	n.s.	a	ab	a	a	ab	b	n.s.
OF-MSW	7.39	0.57	1.6	0.09	64.7	302.5	9.89	36.4
	n.s.	ab	ab	ab	a	ab	bc	n.s.
SMC	7.41	0.48	1.79	0.1	80.7	473.8	10.5	36.0
	n.s.	b	a	a	a	a	ab	n.s.
NPK	7.34	0.49	1.12	0.06	28.5	252.8	9.88	37.5
	n.s.	b	b	b	b	b	bc	n.s.

Different letters within a column indicate Duncan test results between treatments ($P<0.1$; n=3). Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK).

In the year 1996, grapevines (*Vitis vinifera* L c.v. Tempranillo) were planted over a Richter 110 rootstock at a density of 0.3 stocks m^{-2} (3 x 1.15 m). The vines were trained using a midway bilateral cordon system (*Cordon de Royat*) to a height of 0.8 m. Each year, in January, the vines were pruned and two buds per spur were left. The pruning remains were removed and the land was plowed to a depth of 0-30 cm. Chemical weed control was implemented each February. During the vineyard cycle, the soil was left exposed with no vegetation. When necessary, and depending on the weather conditions, a sprinkler irrigation system provided supplementary water. The agricultural management did not differ between the various treatments.

2.3.2 Treatments and experimental design

This study involved the application of three different

composted organic residues, or 'organic amendments', along with a mineral fertilizer and an unfertilized control. The annual application of the treatments began in 1998 (Table 2). The amendments were applied to the soil surface and incorporated into the soil to a depth of 0-30cm each February, using a chisel plough. The fertilizers were: PEL, a pelletized organic compost made from plant, animal and sewage sludge residues; OF-MSW, a compost made from the organic fraction of municipal solid waste (separate collection); SMC, a compost made of sheep manure weathered for at least 1 year; and the mineral fertilizer NPK 5-10-15 (NPK). The mean annual doses applied from 1998 onwards were 3700 kg ha^{-1} FW of PEL, 4075 kg ha^{-1} FW of OF-MSW, 4630 kg ha^{-1} FW of SMC and 340 kg ha^{-1} of NPK. The specific doses of the relevant macronutrients are shown in supplementary Table 1. For each treatment, an experimental plot of 15 vines (108 m^2) was designated. The experiment followed a randomized complete block design with three blocks.

Table 2. Physical and chemical properties of fertilizers applied at the Bargota site (2011)

	PEL	OF-MSW	SMC	NPK
pH	8.8	8.3	9.0	N.A.
E.C. (dS m^{-1})	8.3	8.1	6.6	31.6
Dry matter (% p/p)	86.2	69.8	59.1	98.1
Organic matter (%sss)	31.7	56.9	29.1	N.A.
TOC (% C DW^{-1})	14.9	32.2	14.2	N.A.
Nitrogen Kjeldahl (% N DW^{-1})	2.81	2.51	1.39	5
Organic N(% DW^{-1})	2.48	2.13	1.26	N.A.
C/N	7	13	12	N.A.
P_2O (% P DW^{-1})	3.34	2.36	0.62	4.3
K_2O (% K DW^{-1})	2.48	2.09	3.54	12.4
CaO (% Ca DW^{-1})	13.4	12.9	18.2	5.7
MgO (% Mg DW^{-1})	1.1	0.9	1.4	N.A.
Na (% Na DW^{-1})	1.3	0.6	0.8	N.A.
$N\text{-}NH_4$(% P DW^{-1})	0.30	0.28	0.04	N.A.
$N\text{-}NO_3$(% P DW^{-1})	0.04	0.10	0.10	N.A.

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral (NPK). N.A. (Not applicable). D.W. (Dry weight)

2.3.3 Soil sampling for general soil analysis

At the end of 2011, four soil samples were collected from four randomly chosen spots within each experimental plot, from the 0-30 cm layer. The four soil samples were bulked for each experimental plot. The samples were then air-dried at room temperature and stored at 4°C. The physical and chemical properties of the soil were determined after the samples had been sieved through a 2 mm mesh. Soil pH was measured in suspension with a deionized water (w/w) ratio of 1:2.5. Soil organic matter was determined using the Walkley–Black organic C method. Total

nitrogen was measured with the Kjeldahl digestion-distillation-titration method, and phosphorus via the Olsen method. Available K content was determined after extraction with a 0.1 M ammonium acetate solution. Electrical conductivity (EC) was determined with an EC-meter from a water suspension of soil using a 1:5 soil/solution ratio (EC1:5 water extract). Grape yield values are not shown because P.O.D. *La Rioja* establishes a quota per hectare of produced grape, in this case 7 tons. No further yield comparisons are made as the evaluated treatments met that demand and any excess is removed from the vines early in the season.

2.3.4 Soil quality: fertility and enzyme activity measurements

Soil samples were taken from all plots to a depth of 30 cm and analysed for ammonium and nitrate content on days 1, 3, 7, 10, 15, 21, 42, 74, 115, after the fertilizer was applied to the soil. Fresh 100 g samples were extracted with 200 mL KCl (2M). The extracts were filtered and stored at -20ºC until they were analysed. The method used was the Gries-Illosvay colorimetric method modified by Barnes and Folkard (1951), by means of a Bran & Luebbe II AutoAnalyzer. Amino acids were analysed using the fluorometric method of Jones *et al.* (2002).

In order to evaluate enzyme activities in soil, 15 days after fertilization soil samples were obtained from four random spots on each plot. Samples were bulked and sieved through a 2 mm mesh and stored at 4º C to await enzyme activity assays. Protease, urease,

phosphomonoesterase, and β-glucosidase, as well as the hydrolysis of fluorescein diacetate, were assayed.

Protease activity was assayed by determining the tyrosine released after a 1-hour incubation of 0.4 g of soil with 1 ml of 200 mM THAM buffer (pH 8.0) and 1 ml of 2% Na-caseinate at 50ºC for 2 hours. The remaining substrate was precipitated with 0.92 M trichloroacetic acid and measured colorimetrically using Folin–Ciocalteu reagent at 700 nm. Urease activity was measured using 1 g of soil with 1.75 ml of 100 mM Borate buffer (pH 10.0) and 0.25 ml of 820 mM urea solution at 37ºC for 1 hour. Excess urea was extracted with KCl solution and estimated colorimetrically at 670nm. Phosphomonoesterase (alkaline phosphatase) activity was assayed using 1 g of soil (wet equivalent), 1.6 ml of 20 mM modified universal buffer (pH 11), and 0.25 ml of 50 mM p-nitrophenyl phosphate. After incubation the enzyme reaction was stopped and centrifuged. After centrifuging, the absorbance was measured in the supernatant at 410 nm. β-glucosidase activity was determined using p-nitrophenyl-b-D-glucopyranoside (PNG, 0.05 M) as the substrate. The assay is based on the release and detection of p-nitrophenol (PNP) according to Tabatabai (1982). The amount of PNP was determined using a spectrophotometer at 410 nm. The hydrolysis of fluorescein diacetate [3',6'-diacetylfluorescein (FDA)] was determined using 2 g of soil with 50 ml of 60 mM sodium phosphate buffer at pH 7.6, and 0.50 ml of 4.9 mM FDA lipase substrate solution were added. After mixing, the samples were incubated, the

reaction was stopped and the samples were filtered. The absorbance was measured on a spectrophotometer at a wavelength of 490 nm.

The geometric mean of the values of all enzyme activities (Overall Enzyme Activity, OEA) was calculated according to the formula [1]:

$$[1]\ OEA = \sqrt[5]{\beta glucosidase \times protease \times alkaline\ phosphatase \times urease \times FDA}$$

This indicator has been applied as an overall indicator of soil quality (García-Ruiz *et al.*, 2008). The relative increase (%) in the activity of each enzyme was calculated with respect to that of the control treatment and represented by means of a web chart. This method is an advantageous way to clearly distinguish different soil enzyme-fingerprints.

2.3.5 Soil GHG emissions

On days 1, 3, 7, 10, 15, 21, 42, 74, 115 after applying the treatment (over a period of 115 days) N_2O, CO_2 and CH_4 were measured. Gaseous emissions were determined using the closed chamber technique (Chadwick *et al.*, 2014) and with a methodology in accordance with Global Research Alliance guidelines (de Kleine and Harvey, 2012). Ten ambient air samples (20ml) were collected in pre-evacuated vials of 12 mL prior to closing the chambers for 45 min. After this time a 20 mL sample of gas was then extracted from

the chamber using a syringe and stored in pre-evacuated 20 mL glass vial so that it was under pressure. Emission rates and cumulative emissions were calculated, taking into account the concentration increase with time (Menéndez *et al.*, 2008). The samples were analysed using gas chromatography (GC) (Agilent, 7890A) with an electron capture detector (ECD) for N_2O detection and a flame ionization detector (FID) for CH_4. For determining CO_2, the GC was equipped with a methanizer to reduce CO_2 to CH_4. A capillary column (IA KRCIAES 6017: 240ºC, 30 m x 320 mm) was used. The column temperature ramped from 40ºC to 80ºC and the temperature of the ECD was 350ºC; a 5% mixture of Ar with CH_4 was used as the carrier with N_2 as make up (15 mL min^{-1}). A headspace autosampler (Teledyne Tekmar HT3) was connected to the gas chromatograph. Standards were stored and analysed at the same time as field samples. Previous to the start of the experiment, several proofs were made in order to check that fluxes were linear for 45 min. The cumulative gas production during the experiment was estimated by averaging the fluxes of two successive determinations, multiplying that average flux by the length of the period between the measurements, and adding that amount to the previous cumulative total.

N_2O production was determined 15 days after fertilization had taken place (Estavillo *et al.*, 2000). Three soil cores per experimental plot (2.5 cm diameter×30 cm depth) were incubated in a tightly closed one litre glass bottle. The bottles were then

incubated at the ambient temperature of the soil, in a hole dug in the ground adjacent to the experimental plots. Samples from the air headspace were taken at the beginning of the incubation and again after 24 hours, and analyzed using gas chromatography, checking that the accumulation of N_2O was linear during this time.

Fifteen days after fertilization, the potential nitrification of the soil was measured following Norton and Stark (2011). Briefly, 15 g of soil was incubated at 24 ºC for 24 h in a solution of 0.2 *M* KH_2PO_4, 0.2 *M* K_2HPO_4 and 0.05 *M* NH_4SO_4. The solution was sampled up to eight times during the incubation period. The samples were subsequently analyzed for NO_3^- and the rate of NO_3^- production was calculated by linear regression of the solution concentration over time.

Denitrification potential was determined as denitrifying enzyme activity (Phase 1), as described by Smith and Tiedje (1979), following the method described by Tiedje *et al.* (1989). 25 g of fresh soil and 25 mL of a solution containing glucose 1 *M*, KNO_3 1 mM and 1 gL^{-1} chloramphenicol were put into 125 mL flasks. The flasks were sealed with a rubber stopper and repeatedly flushed with N_2 for 10 min in order to create anaerobic conditions. The flasks were separated into two groups (with or without further addition of 5% acetylene) in order to be able to determine denitrification potential to N_2O or N_2O+N_2 by difference (Estavillo *et al.*, 2002). The flasks were incubated in an orbital shaker at 20º C and 1 mL of the

headspace air was sampled for N_2O determination at 1 and 3 hours incubation time, with the increase in N_2O concentration over this time being linear. N_2O was analyzed using gas chromatography.

2.3.6 Soil bacterial community

In order to discover the status of the microbial communities, soil samples for DNA extraction were collected 15 days after fertilization, from the 5-30 cm layer at four random spots in each experimental plot. Samples were bulked stored at 4°C and sieved through a 2 mm mesh, before DNA extraction within 24 h from sampling. Soil DNA was extracted from each individual soil sample using the PowerSoil™ DNA Isolation kit (MoBio, Laboratories Inc., CA) as recommended by the manufacturer. For this technique DNA extraction begins with the chemical lysis of microbial cells with gentle bead-beating, released DNA is bound to a silica spin filter which is subsequently washed, and DNA recovered in elution buffer solution. The DNA yields and quality were checked after electrophoresis in 0.8% (w/v) agarose gel stained with ethidium bromide under UV light (Sambrook *et al.*, 1989) and measured with a Nanodrop 1000 Spectrophotometer (Thermo Scientific).

Partial prokaryotic 16S rRNA gene sequences were obtained from the analysis of each individual sample using the coded-primer approach to multiplex pyrosequencing (Binladen *et al.*, 2007). PCR amplification of the hypervariable V4-V5 regions of the 16S rRNA gene was performed for each individual soil DNA extraction using

universal primers with an 8 bp bar-coded sequence (Curiel Yuste *et al.*, 2012). The PCR mixtures (25 µl) contained 25 pmol of each primer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 1 X the corresponding Taq buffer, 1 U of Taq Master (5 Prime, USA) and 10 ng of the DNA template. The PCR program involved an initial denaturation step at 94ºC for 4 min, 25 cycles of denaturation at 94ºC for 15 sec, primer annealing at 55ºC for 45 sec and extension at 72ºC for 1 min, followed by a final step of heating at 72ºC for 10 min. For each sample, amplicons were generated in several replicate PCRs. Amplicons from the same treatment were pooled to reduce per-PCR variability and purified using Ultracentrifugal Filters with Ultracel-100 K membranes from Amicon (Cork, Ireland) according to the manufacturer's instructions. After quantification by agarose gel and Nanodrop 1000, the samples were combined in equimolar amounts and subjected to pyrosequencing with the Genome Sequencer Titanium GS-FLX system (454 Life Sciences, Branford, CT, USA) at LifeSequencing S.L. (Valencia, Spain). Sequence files were submitted to the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) and are available with accession number PRJNA269011.

For the taxonomic assignment of sequence reads, the raw sequences were processed through the Ribosomal Database Project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu) release 10 (Cole *et al.*, 2009). Those sequences that met any of the following four criteria were left out of the analysis: 1. If the length read was less than 150 bp; 2. If the forward primer sequences contained more

than two errors; 3. If the sequences had 1 or more Ns; and, 4. If the quality index, according to the .qual file generated during the pyrosequencing process, was less than 20. Eligible sequences were clustered into Operational Taxonomic Units (OTUs), based on a distance of 3%, by complete linkage clustering. The phyla were assigned by the RDP-II classifier, using an 80 % confidence threshold (Wang *et al.*, 2007). Sequences that could not be classified to a phylum at this level of confidence were excluded from subsequent phylum composition analyses. Each unique sequence was further aligned using the aligner function of the RDP, to generate phylogenetically ordered rRNA sequences. Aligned data sets were clustered using the default parameters for the RDP clustering function. The resulting clusters were utilized to calculate the Shannon index, Chao 1 estimator, rarefaction curves, and Good's coverage index using the pyrosequencing analysis tools from RDP at the level of 3% dissimilarity, this being approximate to species level.

2.3.7 Statistical analysis

The data presented in this work are the mean values of, at least, three independent samples per treatment. In the case of the statistical analyses of organic amendments, one-way ANOVA tests were used. To compare differences between treatments, Duncan tests were performed with a significance level of $P > 0.05$ using the SPSS software, version 21.0.0.0 (IBM Corp., Released 2012). Correlations were analysed among variables where appropriate, and the Pearson value between them was also calculated. In the case of

CO_2 emissions a general linear model was used to determine the influence of WFPS and temperature.

Comparison of bacterial communities was performed with the RDP tool complete-linkage clustering to obtain the OTUs at 3% dissimilarity. Using the cluster file format conversion the corresponding matrix was obtained in table form. The distance matrix was completed with the Bray-Curtis index in the Ginkgo software package (De Cáceres *et al.*, 2007). This software was used to produce an illustration of the clustering using the unweighted pair group method with arithmetic mean (UPGMA). Finally, a hierarchical clustering was obtained that took into account the abundance of each OTU. Moreover, the five different libraries generated from the individual soil treatments were pair-wise compared with the STAMP software (Parks and Beiko, 2010) using the parameters recommended by the developers (the statistic test was the two-sided type Fisher's exact test, with a confidence interval of 95% evaluated using the differential Newcombe-Wilson proportions and the FDR multiple test correction of Storey).

2.4 RESULTS

To compare the effect on soil quality of 13 years of treatment application, the result data presented for fertilizers and soil corresponds to the 13th year of fertilization.

2.4.1 Effects on soil quality

Some of the organic amendments improved soil characteristics (Table 1). No significant changes were recorded in pH among treatments; the values ranged from 7.34 (NPK) to 7.41 (SMC). PEL increased electrical conductivity, with a recorded value of 0.89 dS m^{-1}. This is a significant gain compared to 0.49 dS m^{-1} with NPK treatment and 0.37 dS m^{-1} in the control. Neither OF-MSW nor SMC significantly increased EC values. SMC application raised organic matter content in soil by 1.79%, about 40% more than the control (1.19 %) and NPK (1.12 %). The increase due to PEL and OF-MSW, about 30%, was not significant. Organic amendment application resulted in higher levels of N, P and K in the soil. The main changes in N were found for PEL (0.1 %) and SMC (0.1 %), compared to a 0.06% content for the control and NPK treatments. SMC yielded the most important increase in P (Olsen), with 273% more (80.7 mg kg^{-1}) than the control. At the same time, PEL (68.6 mg kg^{-1}) and OF-MSW (64.7 mg kg^{-1}) treatments doubled the amount of P compared to the control (29.5 mg kg^{-1}) and NPK (28.5 mg kg^{-1}). K content increased significantly in soil treated with SMC (473.8 mg kg^{-1}). Although PEL (429.3 mg kg^{-1}) and OF-MSW (302.5 mg kg^{-1}) did increase K content, the levels were not significantly different from

those for the control (231.7 mg kg^{-1}). The C/N ratio of the control treatment showed the highest value with 11.07, followed by SMC (10.5), OF-MSW (9.89) and NPK (9.88).

Given that N was applied at different rates to soil (Table 3), the total N content (Kg-N ha^{-1}) in soil and the percentage of different forms of N in soil, such as NH_4^+, NO_3^- and total AA differed between treatments (Fig 2).

Table 3. Annual doses and total amounts of fertilizers applied over a 13-year period (1998-2011)

			DOSES	O.M	N total	N org	P_2O	K_2O	CaO	MgO
PEL	**Annual mean**	kg ha^{-1} yr^{-1}	3700	1015	90	79	107	79	429	38
	Total (13-year)	**kg ha^{-1}**	**48100**	**13190**	**1029**	**1166**	**1386**	**1029**	**5574**	**494**
OF-MSW	**Annual mean**	kg ha^{-1} yr^{-1}	4080	1623	72	61	67	60	367	27
	Total (13-year)	**kg ha^{-1}**	**53040**	**21095**	**789**	**930**	**874**	**774**	**4776**	**348**
SMC	**Annual mean**	kg ha^{-1} yr^{-1}	4630	797	38	34	17	97	500	38
	Total (13-year)	**kg ha^{-1}**	**60190**	**10360**	**448**	**495**	**221**	**1259**	**6497**	**498**
NPK	**Annual mean**	kg ha^{-1} yr^{-1}	340	N.A.	16	N.A.	14	41	19	N.A.
	Total (13-year)	**kg ha^{-1}**	**4420**	**N.A.**	**217**	**N.A.**	**189**	**540**	**248**	**N.A.**

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral (NPK). N.A. (Not applicable). D.W. (Dry weight)

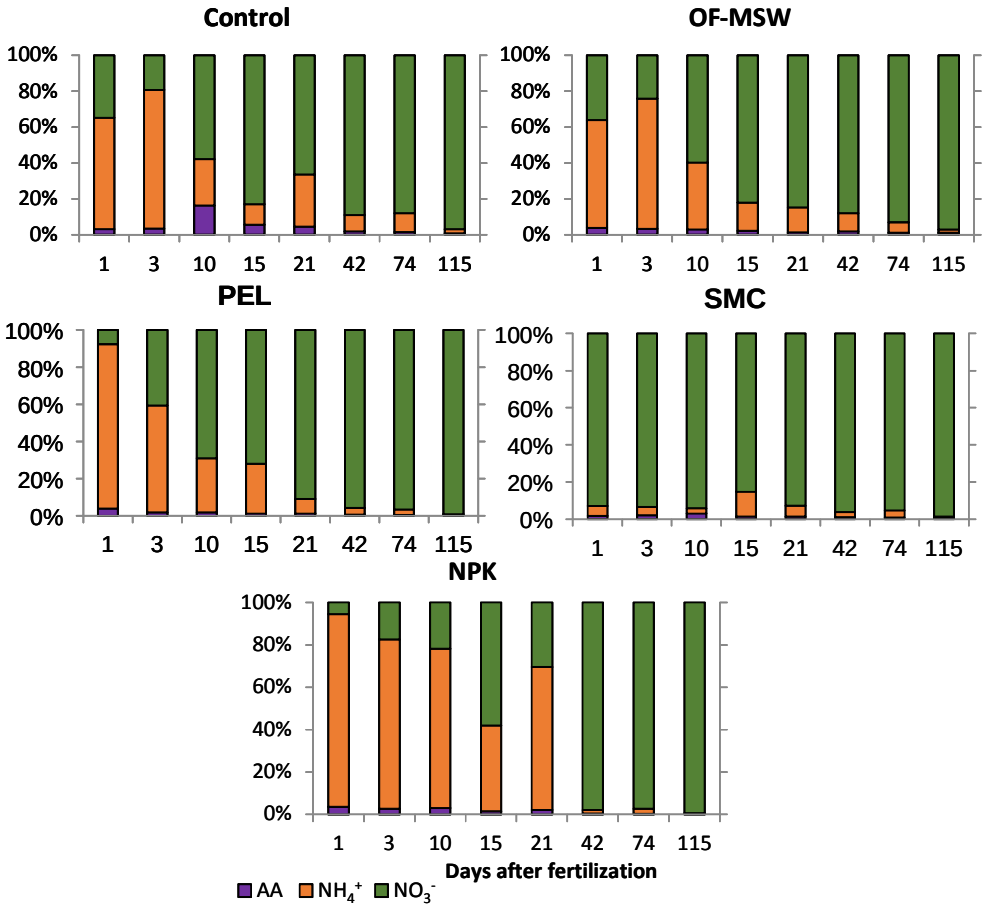

Figure 2. Different N forms (Aminoacid-N%, NH_4^+-N% and NO_3^--N% per kg of soil on each sampling day) in soil during the sampling period Feb-July at the Bargota site (2011). Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), and sheep manure compost (SMC) mineral fertilizer (NPK).

The total N content in soil (Fig 3) for the control treatment was found to be 5.5 kg-N ha^{-1} on the day with the lowest values and 48.8 kg-N ha^{-1} at its highest. In the case of PEL, N content in soil ranged from 40.6 to 223.8 kg-N ha^{-1}. For the OF-MSW treatment, N ranged from 27.1 up to 103.9 kg-N ha^{-1}, while for the SMC treatment it was from 16.9 to 155.1 kg-N ha^{-1}. Finally, the NPK treatment

ranged from 77.3 to 180.8 kg-N ha^{-1}. The average soil content of nitrate, ammonium and total amino acids, revealed different tendencies for each N form depending on the treatment. During the sampling period the highest average nitrate content was found for PEL followed by NPK, SMC and OF-MSW, which had a similar content, with the control treatment having the least. NPK showed the highest ammonium content, followed by PEL and OF-MSW, which showed similar values. The lowest average content of ammonium was established for the control and SMC treatments. The highest average total AA content was found for NPK, followed by PEL and OF-MSW, and finally for SMC and the control. At the beginning of the sampling period and until sampling day 15, in general terms, the most important form of nitrogen was ammonium (Fig 2) for the control, PEL and OF-MSW. In the NPK, this effect lasted up to sampling day 21. Afterwards, nitrate concentration was favoured over the other two studied forms. For SMC treatment, the most important form of N for the entire sampling period was nitrate.

Nitrogen mineralization (kg N ha^{-1}) (data not shown) occurred in three major periods. The first stage was from day 1 to day 20, when important increases in mineralization were observed, the most important being those for the soil treated with NPK. The second stage was from day 21 up to day 73, where mineralization stagnated. The final stage was from day 74 to the end of the sampling period, where in all cases the mineral content almost doubled compared with the levels found in the previous period. At

the end of the experiment the net mineralization followed the order: PEL>NPK>SMC>OF-MSW>Control.

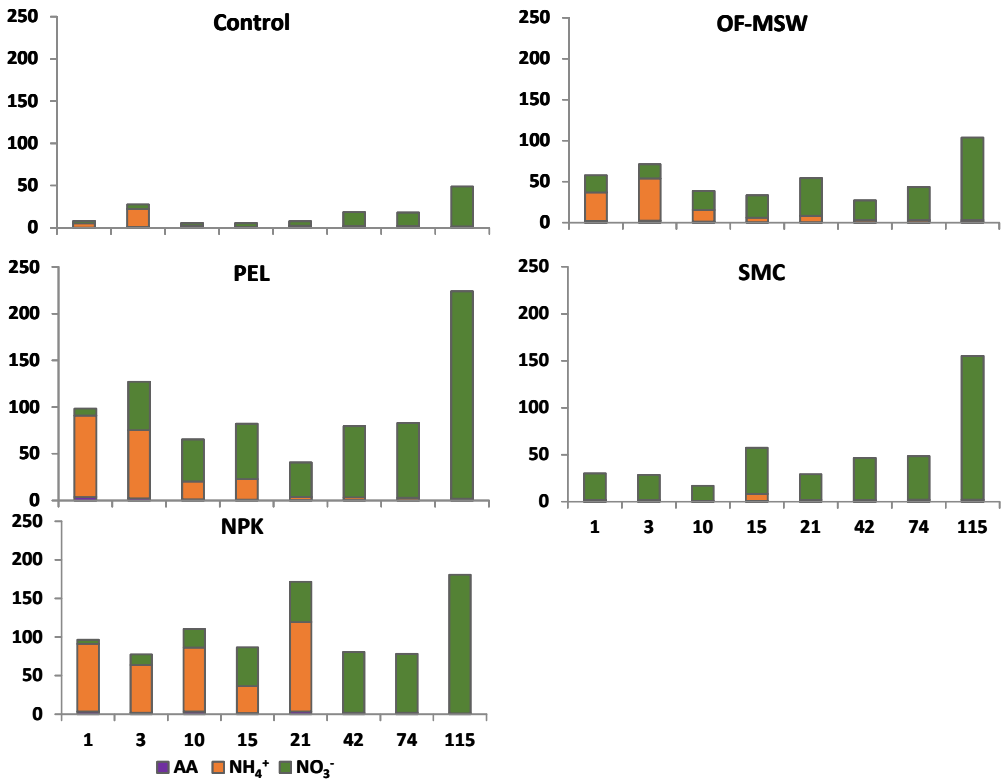

Figure 3. Different N forms (Aminoacid-N kg ha^{-1}, NH_4^+-N kg ha^{-1} and NO_3^--N kg ha^{-1} on each sampling day) and its content in soil, the numbers in the X axis represent the days after fertilization during the sampling period between February and July (2011). Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK).

Enzyme activity significantly increased in soils that received organic amendments. In contrast, enzyme activities decreased in the NPK soil (Table 4). OF-MSW showed the highest and most significant

increases compared to the control (Fig 4) for protease (+ 389%), B-glucosidase (+ 165%), phosphatase (+ 115%) and FDA (+ 174%) hydrolysis. PEL has the most effect on urease (+ 149%) and protease (+ 275%) activity, while it caused no-significant increase in B-glucosidase, phosphatase and FDA hydrolysis. SMC treatment led to an increase in the activity of all of the essayed enzymes, but these increases were not significantly different from the control. NPK inhibited activity for B-glucosidase (- 67%) and FDA hydrolysis (- 26%), while urease, protease and phosphatase values were similar to that of the control. The enzyme pattern only changed in magnitude, as shown in Fig 4. It is worth pointing out that NPK has a slightly different fingerprint, where all the activities decrease but the reduction is more acute for FDA hydrolysis.

Table 4. Enzyme activities in soil, at the Bargota site, 15 days after treatment application 2011

Treatment /Enzyme	***Urease*** *(mg N-NH_4^+ kg dry soil $^{-1}$ hour $^{-1}$)*	***Protease*** *(mg Tyr kg dry soil $^{-1}$ hour $^{-1}$)*	***β-glucosidase*** *(mg ρ-nitrophenol kg dry soil $^{-1}$ hour $^{-1}$)*	***Phosphatase Alkaline*** *(mg ρ-nitrophenol kg dry soil $^{-1}$ hour $^{-1}$)*	***FDA hydrolysis*** *(mg fluorescein salt kg dry soil $^{-1}$ hour $^{-1}$)*	***Overall Enzyme Activity***
CONTROL	22.3 b	15.7 cd	174.5 b	229.0 bc	9.2 b	51.3
PEL	33.8 a	37.2 ab	254.7 ab	290.8 b	14.9 ab	79.5
OF-MSW	29.0 ab	51.9 a	288.1 a	384.5 a	16.0 a	95.9
SMC	27.0 ab	31.2 bc	189.4 b	263.2 b	8.5 b	69.2
NPK	18.4 b	10.5 d	114.8 c	159.7 c	2.3 c	35.2

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK). Different letters within a column indicate Duncan test results between treatments (P<0.1; n=3).

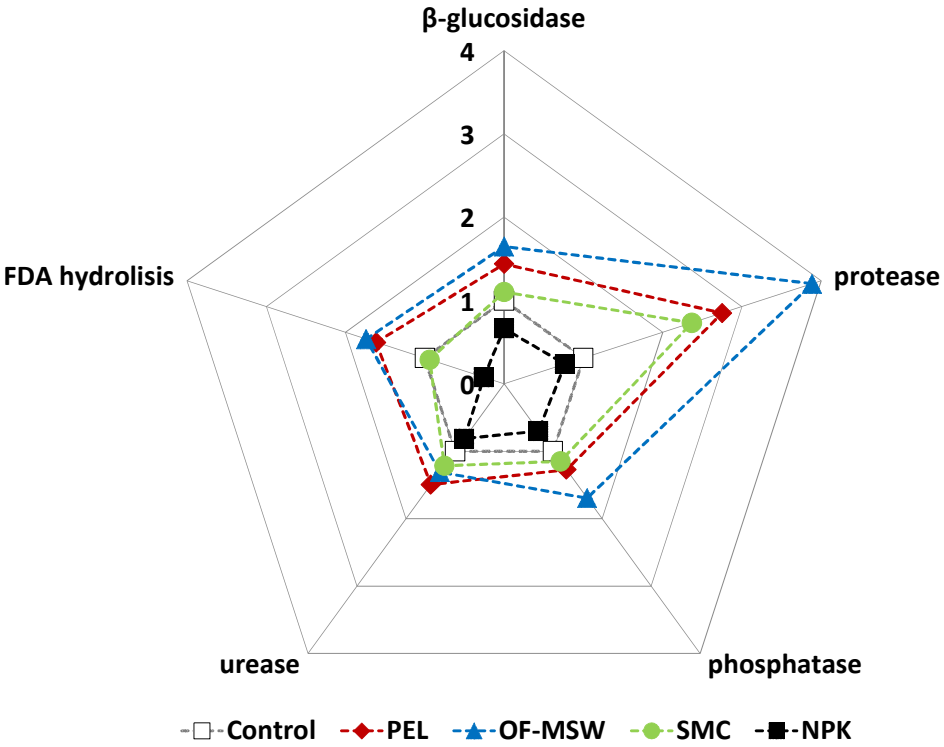

Figure 4. Web plot of the growth/reduction rate of enzyme activities in the different treatments with regard to control treatment at the Bargota experiment (2011). Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), and sheep manure compost (SMC) mineral fertilizer (NPK).

2.4.2 Soil GHG emissions

Daily N_2O emissions from the control treatment ranged between 0.4 and 3.2 g N_2O-N ha^{-1} d^{-1}, while the organic amendments increased fluxes up to 13.6 g N_2O-N ha^{-1} d^{-1} (Figure 5). The NPK and SMC treatments showed emissions close to the control treatment during the entire period. These treatments did not exceed a rate of 4.0 g N_2O-N ha^{-1} day^{-1}, with an average flux of 1.8 g N_2O-N ha^{-1} day^{-1}.

When cumulative losses were calculated (Table 5), no significant differences were observed between the control treatment and the other treatments, with the exception of the PEL treatment. These cumulative losses yielded emission factors of 0.00% for SMC and NPK treatments, and 0.23% and 0.07% for PEL and OF-MSW treatments, respectively.

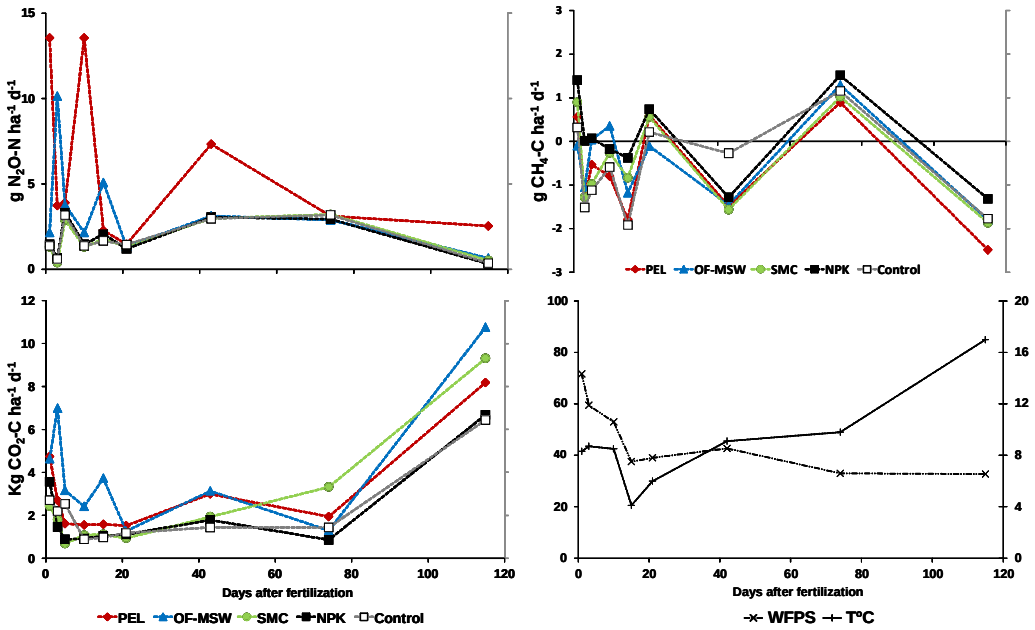

Figure 5. Daily gaseous emissions and soil WFPs and soil temperature during the experiment at the Bargota site (2011).

Daily CO_2 fluxes from the control treatment ranged between 0.9 and 6.4 kg CO_2-C ha^{-1} d^{-1}, in the same range as for the NPK treatment. The application of different organic amendments increased daily fluxes, the maximum rate being 10.7 kg CO_2-C ha^{-1} d^{-1} in OF-MSW. As a result of this increase in daily fluxes, treatments with organic amendments showed higher cumulative losses, although only

those from the OF-MSW treatment proved to be statistically different from the control.

Table 5. Cumulative nitrous oxide, carbon dioxide and methane emissions from the fertilized soils at the Bargota site (2011) over a period of 115 days.

	g N_2O-N ha^{-1}	EF(%)	kg CO_2-C ha^{-1}	g CH_4-C ha^{-1}	Kg CO_2eq ha^{-1}
Control	253 b	-	269 b	-20 n.s.	1104 bc
PEL	463 a	0.23	377 ab	-68 n.s.	1598 ab
OF-MSW	301 ab	0.07	438 a	-37 n.s.	1745 a
SMC	253 b	0.00	402 ab	-46 n.s.	1591 ab
NPK	247 b	0.00	256 b	3 n.s.	1053 c

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK). Different letters within a column indicate Duncan test results between treatments ($P<0.1$; $n=4$).

Daily CH_4 fluxes did not differ between the various treatments. Most of the time the soil acted as a CH_4 sink, with fluxes ranging between -2.5 and 1.5 g CH_4-C ha^{-1} d^{-1}. As a consequence of this, at the end of the assayed period there was net CH_4 uptake for all the organic treatments and the control treatment, with the exception of the NPK treatment, which presented a net CH_4 increase, although differences were not statistically significant.

Organic fertilization induced a higher Global Warming Potential (GWP) than mineral fertilization. The OF-MSW treatment showed the highest GWP, as the only treatment that differed from the control treatment.

Table 6 shows the production and emission of N_2O fifteen days after application of the fertilizers. While production rates ranged between 1.98 and 6.30 g N_2O-N ha^{-1} d^{-1}, the emissions to the atmosphere ranged between 1.67 and 5.08 g N_2O-N ha^{-1} d^{-1}. Application of PEL caused greater N_2O production than the other treatments. In the case of emissions, all fertilized treatments led to higher emissions than for the unfertilized soil.

Table 6. Nitrous oxide production 15 days after fertilization and the daily emission from the corresponding day at the Bargota site (2011).

	g N_2O-N ha^{-1} d^{-1} production		g N_2O-N ha^{-1} d^{-1} emission	
Control	2.17	b	1.67	b
PEL	6.30	a	2.27	a
OF-MSW	5.28	ab	5.08	a
SMC	1.98	b	1.77	a
NPK	3.25	ab	2.06	a

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK). Different letters within a column indicate Duncan test results between treatments ($P<0.1$; n=4).

Nitrification potential is shown in Figure 6. Applying the different fertilizers significantly increased the nitrification potential from 106 µg N kg^{-1} dry soil d^{-1} in the control treatment to 239 µg N kg^{-1} dry soil d^{-1} for the PEL treatment. The other three fertilizer treatments showed lower nitrification potentials of around 180 µg kg^{-1} soil d^{-1}.

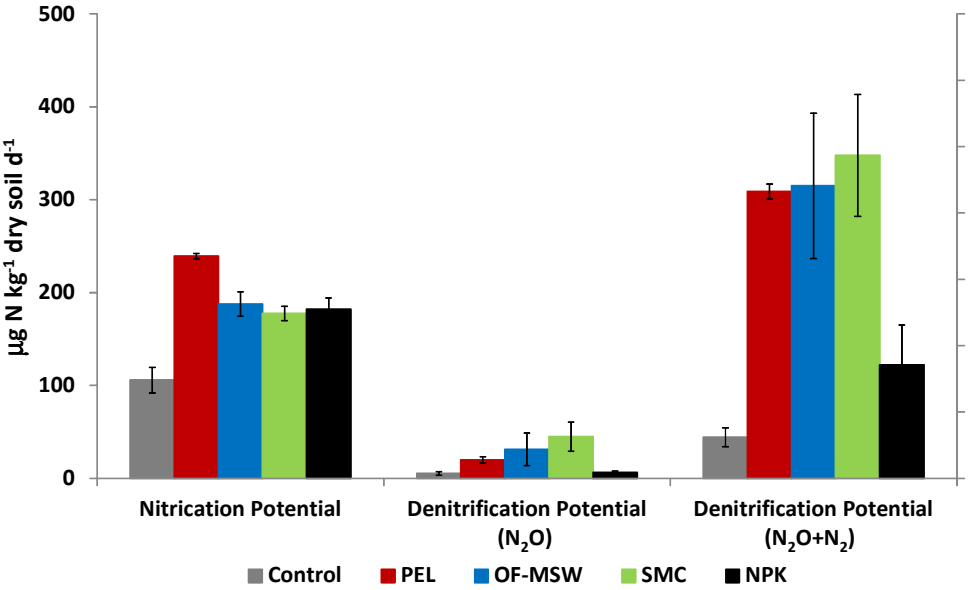

Figure 6. Nitrification and denitrification potential of the different treatments at the Bargota site (2011). Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), and sheep manure compost (SMC) mineral fertilizer (Mineral).

The application of organic amendments significantly increased the denitrification potential up to N_2O in comparison to the control and NPK treatment (Figure 5). While these treatments did not exceed a rate of 6.1 µg N kg^{-1} dry soil d^{-1}, organic treatments varied from 19.7 to 44.7 µg N kg^{-1} dry soil d^{-1}. The potential to reduce N_2O to N_2 was significantly higher for all treatments. In this case, mineral fertilization induced a significant increase with respect to the control treatment, the denitrification potential to N_2 being 121.9 and 44.1 µg N kg^{-1} dry soil d^{-1} respectively. There were no differences between organic amendment treatments, which had potential rates of around 320.0 µg N kg^{-1} dry soil d^{-1}.

2.4.3 Diversity and taxonomic composition of bacterial communities in soil

A total of 111,864 readings were obtained for the five treatments that, after the trimming process, resulted in 76,681 useful sequences, i.e., from 11,528 to 19,570 high quality sequences per sample. Since the number of sequences in the all samples had the same order of magnitude, their number was normalized to 11,528, the lowest amount found, for the OF-MSW treatment. On the basis of a 3% dissimilarity, the number of OTUs varied from 1,482 for NPK treatment, to 1,842 in the SMC sample (Table 7). Chao's index shows a richness of up to 2,801 different OTUs in the soil treated with OF-MSW, very similar to the 2,803 OTUs for the unnnormalised control sample. Shannon's diversity index yielded values of 6.24 for the NPK treatment, up to 6.56 for the SMC sample (Table 7). Prokaryotic diversity coverage was, in all cases, higher than 93% and reached 97% for the NPK treatment. It is therefore a very accurate reflection of soil diversity. The difference between the bacterial communities in the control soil and those in the treated soil was analysed through hierarchical clustering using the Bray-Curtis index, which takes into account all the OTUs and their abundance (Figure 7). The dendrogram of the samples according to this index shows that the OF-MSW treatment was the most diverse with a similarity of less than 56%. The core of the dendrogram was the node of the control and NPK treatments, with similarities close to 70%, and the SMC and PEL samples had similarity values of

between 64% and 68% (Figure 7). Thus the similarity values for all the samples were very coincident.

Table 7. Trimmed and normalized (at the lowest number of sequences of OF-MSW) values of different soil bacterial diversity estimators under different fertilizer treatments at the Bargota site (2011).

		Distance	N	OTUs	Chao1	LCI95	UCI95	H'	varH	E	Coverage
Control	Trimmed	0.03	17987	2037	2803	2662	2975	6.53001	0.00013	0.85704	96.29%
	Normalized	0.03	11528	1667	2393	2255	2564	6.41679	0.00019	0.86494	94.67%
PEL	Trimmed	0.03	15331	1929	2626	2495	2786	6.60478	0.00013	0.87310	95.86%
	Normalized	0.03	11528	1695	2351	2224	2508	6.53528	0.00017	0.87894	94.89%
OF-MSW	Trimmed	0.03	11528	1802	2801	2623	3016	6.4041	0.00023	0.85426	93.55%
	Normalized	0.03	11528	1802	2801	2623	3016	6.4041	0.00023	0.85426	93.55%
SMC	Trimmed	0.03	12265	1891	2705	2559	2883	6.57024	0.00018	0.87082	94.27%
	Normalized	0.03	11528	1842	2669	2519	2851	6.56094	0.00019	0.87263	93.99%
NPK	Trimmed	0.03	19570	1850	2530	2397	2696	6.35924	0.00013	0.84531	97.04%
	Normalized	0.03	11528	1482	2107	1978	2269	6.24345	0.0002	0.85513	95.50%

N = number of sequences; OTUs = total number of detected OTUs at 0.03 distance; Chao1 = Chao1 index of richness; LCI95 and UCI95: Lower and upper confidence intervals of Chao1 index at the 0.05 probability, respectively; H' = Shannon-Weaver diversity index; varH= variance of H'; E = Evenness considering H'; Coverage according to the Good's index.
Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK).

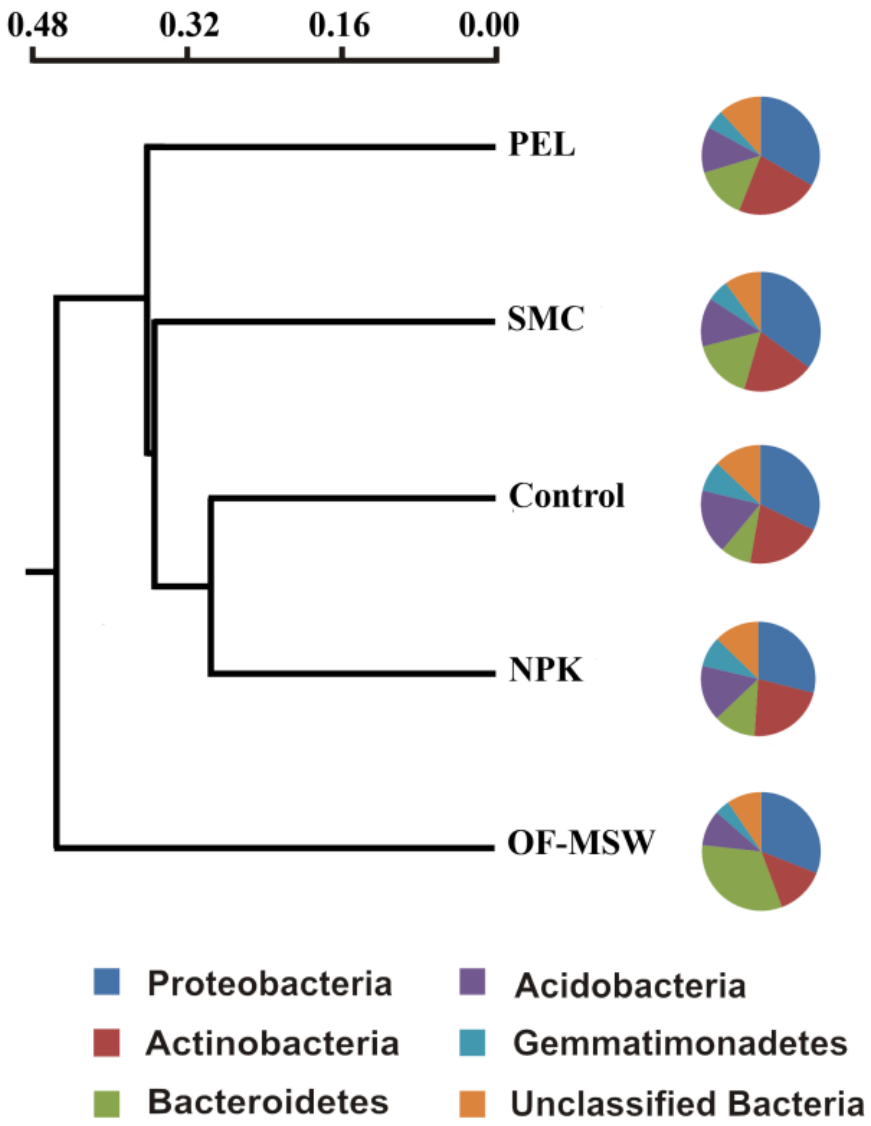

Figure 7. Cladogram of the five different soil bacterial communities based on Jaccard distance (3% dissimilarity). All bacterial communities were studied according to the analyses of molecular variance (AMOVA) and unweighted Unifrac algorithm Pie charts below each branch represent the relative abundance of the different phyla. Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), and sheep manure compost (SMC) mineral fertilizer (Mineral).

Distribution of around 85% of the sequences found corresponded to 5 main phyla: Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria and Gemmatimonadetes, with unclassified genetic material making up an average of 10.9% of the sequences (Figure 7 and Table 8). Proteobacteria was the best-represented phylum comprising an average of 30.7% of the total bacterial population, with the exception of the OF-MSW treatment where the Bacteroidetes phylum was the most abundant (30.8%, Table 8). In this case (OF-MSW) 18.98% of the sequences belong to only three genera *Flavobacterium* (12.05%), *Pedobacter* (4.74%) and *Adhaeribacter* (2.19%, data not shown). The high proportions of these genera indicate that they may be responsible for the pattern observed in the Bray-Curtis analysis. Other phyla found as less than 3% and more than 1% in at least one treatment, were Chloroflexi, Cyanobacteria and Firmicutes. The OD1 phylum was only detected in the soil from the OF-MSW treatment, while the phyla Tenericutes and Spirochaetes were present only in the SMC sample.

Table 8. Percentage of detected phyla in soil samples under different fertilizer treatments at the Bargota site (2011).

	Control	PEL	OF-MSW	SMC	NPK
Proteobacteria	30.52	33.73	32.04	29.48	27.76
Actinobacteria	19.47	18.49	21.64	12.72	21.11
Bacteroidetes	7.88	15.85	13.79	30.81	11.38
Acidobacteria	16.74	12.6	12.03	9.26	15.1
Gemmatimonadetes	7.92	5.63	5.07	3.85	8.21
Chloroflexi	2.44	1.2	1.39	1.48	1.69
Cyanobacteria	1.21	1.12	0.81	0.43	1.55
Firmicutes	0.41	0.93	0.78	1.69	0.23
Verrucomicrobia	0.72	0.41	0.57	0.7	0.33
Nitrospira	0.25	0.16	0.29	0.2	0.31
Planctomycetes	0.04	0.01	0.08	0.19	0.01
BRC1	0.07	0.03	0.05	0.03	0.03
Deinococcus-Thermus	0.01	0.17	0.02	0	0
WS3	0.03	0.04	0.03	0.08	0.03
TM7	0.03	0.03	0.07	0.02	0.02
Fibrobacteres	0	0.01	0	0.01	0.02
Tenericutes	0	0.01	0	0	0
Bacteria_incertae_sedis	0.01	0	0	0	0
OD1	0	0	0	0.01	0
Spirochaetes	0	0.01	0	0	0
Unclassified Bacteria	12.26	9.56	11.34	9.04	12.21

Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK).

The results obtained in the nitrification/denitrification processes necessitated a detailed analysis of the bacterial communities, thus specific genera were looked for in the different treatments (Table 9). Typically important genera in these processes, such as *Nitrobacter, Nitrosococcus, Nitrospina* and *Alcaligenes,* were

not detected in any of the analysed soils, while only one sequence of the genus *Nitrosomonas* was present in the SMC sample. The genera *Pseudomonas* was absent in the control, NPK and SMC treatments and comprised 0.013% of the PEL and 0.043% of OF-SMW, in a similar way *Thiobacillus* was absent in the control, SMC and OF-MSW but comprised 0.013% of PEL and 0.005% of the NPK sample. The genera *Nitrospira* (phylum Nitrospirae) and *Bacillus* (phylum Firmicutes) were represented with a percentage above 0.1% of the total sequences in each treatment, but without statistically significant differences between them. The genera *Rhizobium, Paracoccus* (formerly *Thiosphaera*) and *Nitrosospira* (formerly *Nitrosolobus*) showed statistically significant differences between treatments. The genus *Paracoccus* (□-Proteobacteria) was at least three times more abundant in the PEL soil sample than in the other treatments. The genus *Nitrosospira* (□-Proteobacteria) showed statistically significant differences only in the NPK treatment (0.23% of the sequences) while the samples from the other treated soil samples were similar to that of the control. The best represented genus in all samples was *Rhizobium*, which was more abundant in the sample treated with OF-MSW (0.546% of the sequences), with the other treatments having percentages of around 0.2%, and only 0.111% of the sequences in the control soil belonging to this genus.

Table 9. Percentage of sequences of genera believed to be involved in denitrification, nitrification and ammonification processes. Analysis conducted after the examination of bacterial diversity and denitrification-nitrification potentials in soil at the Bargota site (2011).

	Nitrobacter	Nitrosococcus	Nitrospina	Alcaligenes	Nitrosomonas	Pseudomonas	Thiobacillus	Bacillus	Nitrospira	Nitrosospira/ Nitrosolobus	Paracoccus/ Thiosphaera	Rhizobium
Control	0	0	0	0	0	0	0	0.111	0.245	0.067 a	0.028 ab	0.111 a
PEL	0	0	0	0	0	0.013	0.013	0.163	0.248	0.085 a	0.091 a	0.202 ab
OF-MSW	0	0	0	0	0	0.043	0	0.13	0.2	0.026 a	0.009 b	0.546 c
SMC	0	0	0	0	0.008	0	0	0.106	0.171	0.016 a	0.033 ab	0.277 b
NPK	0	0	0	0	0	0	0.005	0.133	0.312	0.23 b	0.005 b	0.215 ab

Each number is the percentage of sequences respect to the total number of its treatment.
Pelletized organic compost (PEL), municipal solid waste compost (OF-MSW), sheep manure compost (SMC) and mineral fertilizer (NPK).
Letters after number mean statistically significant differences in each lane. Different letters within a column indicate Duncan test results between treatments ($P<0.1$; $n=3$).

2.5 DISCUSSION

This study evaluated the consequences of long-term application of composted organic wastes on soil quality and GHG emissions in a vineyard after 13 years. Also, bacterial communities were observed 15 days after the last application

2.5.1 Soil quality

In the studied area, thirteen years of various soil fertilization treatments were enough to produce consistent differences in the measured soil quality parameters. Nutrient contents in soil are considered good indicators of soil productivity (Dong *et al.*, 2012). Significantly higher organic matter content, total N content, P and K contents in the soil when compared to the control, validate the fact that organic wastes-based fertilizers contribute to enhanced soil fertility. Similar conclusions were drawn by Marinari *et al.*, (2006) and Nautiyal *et al.*, (2010). They argued that these improvements are a consequence of enhancing soil organic matter, which supplies substrates and nutrients for its mineralization. In this study, an observation of the physicochemical properties evidenced the improvements in the soil after continuous application of the treatments, and it appears that the saturation point has not yet been reached. In order to prove this, more studies have to be conducted in the future.

Soil enzyme activities are a sensitive method used in soil biological activity approaches. The examination of soil enzymes is a

potential indication of soil quality, as it integrates chemical, physical and biological characteristics of soil (Dick *et al.*, 1996). Our study supports the assertion that continuously adding organic matter to soil augments microbial activity, as indicated by the increased enzyme activity (OEA). For instance, FDA and protease are measured as enzyme indicators that quantify the overall activity of soil microbial communities. Geisseler and Horwarth (2009) also claimed that enzyme activities yield valuable information about the availability of specific organic compounds and their degradation over time. There was positive correlation between the different enzymes studied. Thus, it is evidenced that the overall nutrient cycling in soil is stimulated by the addition of compost; this implies that neither N nor C are limiting factors for microbial activity. Moreover, protease activity is increased and not inhibited by the presence of high amounts of organic N, as it has been previously claimed (Garcia-Gil *et al.*, 2000). Each assayed enzyme demonstrates a highly stimulated microbial activity with no special inhibitions. The increased biological activity and concomitant correlation between the enzymes is not expected to be a result of the activity of one specific enzyme, such as β-glucosidase which indicates energy releases for microorganism, but more as an intrinsic attribute of the organic matter that was added to the soil. These results agree with other studies (Albiach *et al.*, 2001; García-Ruiz *et al.*, 2008) which demonstrated that compost from wastes stimulates microbial activity.

The observed increase in the metabolic activity, that yields

an increase in nutrient content, can be explained as the result of compost mineralization. Garcia-Gil *et al.* (2000) found similar results in Mediterranean conditions, where carbon availability profoundly affected enzyme activity. So, the addition of compost might activate microbial activity for its mineralization, thus supporting microorganism growth and activation (Gong *et al.*, 2009).

The mechanism that drives the increase in microbial activity could be an apparent priming effect (Kuzyakov *et al.*, 2000). Considering that fresh organic matter inputs in soil activate microorganisms and later enhance the degradation of soil organic matter as a result of activated co-metabolism (Blagodatskaya and Kuzyakov, 2008). Also, the priming effect is defined as an increase in the decomposition rate of native soil organic matter caused by fresh additions of organic residues (Fontaine *et al.*, 2003). However, decomposition rates are not measured in our study, so this reason is not conclusive. The only supporting evidence could be that both microbial activity and CO_2 and N_2O fluxes in soil increased rapidly within 15 days after fertilization, this effect is thereafter lost. The underlying mechanisms can be evaluated by the study of the different C and N pools and their changes in the studied soils, after treatment application.

Additionally, it is relevant to study whether the microbial population of organic amendments can become prominent when applied to soil. For instance, enzyme activity patterns increased in

the soils that were treated with composted residues, however the patterns were no different from that of the control. Thus, the intrinsic microbial activity from the compost surrenders to the activity of the original population in the soil (Stumpe *et al.*, 2012). This is supported by the fact that after 15 days from the application, the only significant shift found on microbial communities is the increase of phylum Bacteriodetes (in OF-MSW) and its absence in the control (see also Lutzow *et al.*, 2006; Gulde *et al.*, 2008). Enzymes are produced by various microorganisms, including fungi; the observed changes in the metabolic activity could also be related to an unregistered increase in fungi and not bacterial changes (Hayano, 1993). Nevertheless, since the mechanisms that drive mineralization are complex and diverse, it is still necessary to explore how organic amendments act as microbial inoculum in soil (Stumpe *et al.*, 2012).

2.5.2 Soil GHG emissions

The daily N_2O fluxes from the unfertilized soil were similar to those described by Steenwerth and Belina (2008) from vineyards under Mediterranean conditions. The larger fluxes observed in PEL and OF-MSW after the application of the amendments were short-lived, returning to the same levels as for the unfertilized soil after two weeks. However, these emission peaks had a maximum magnitude of 13.5 g N_2O-N ha^{-1} day^{-1}, close to the larger emission fluxes (14.1 g N_2O-N ha^{-1} day^{-1}) described by Garland *et al.* (2011) after the application of 5 kg N ha^{-1}, also under Mediterranean

conditions. It has been widely described that N_2O emissions correlate with soil water content (Mosier and Hutchinson, 1981; Davidson, 1991; Dobbie and Smith, 2003). Nevertheless, the correlation in this study was extremely weak (P=0.216, sig=0.006). The high potential of our soil for reducing N_2O to N_2 could be the reason for this lack of relationship (Figure 6). The potential up to N_2 was around 10 times higher than denitrification potential up to N_2O. This agrees with Huérfano *et al.* (2015) who described a denitrification potential up to N_2 7 times higher than up to N_2O under humid Mediterranean conditions is Spain. In fact, the significant differences observed between N_2O production and N_2O emission on day 15, confirm that gas diffusivity was low, leading to N_2O consumption (Slemr and Seiler, 1984; Arah *et al.*, 1991) reducing the N_2O emissions in comparison to N_2O production.

N_2O in soil comes from two microbial processes which are conditioned by O_2 availability. Whereas nitrification requires aerobic conditions, an anaerobic environment is necessary for denitrification (Bremner and Blackmer, 1979). Given that low soil water content (WFPS<60%) was recorded during the experiment, nitrification should be the main process in soil. This meant that PEL showed the highest cumulative losses (Table 5). And, it is verified by the fact that PEL also showed the highest nitrification potential (Figure 6). The other treatments also had nitrification potentials that were higher than the control. Nevertheless, the higher C/N relation in those treatments with respect to the PEL treatment limited N_2O emissions.

In the same way than Klemedtsson *et al.* (2005) described a strong negative relationship between N_2O emissions and soil C:N ratios, we observed a negative correlation between cumulative N_2O emissions and soil C:N ratios (P=-0.538; sig=0.039).

Organic amendments also increased the soil denitrification potential. The addition of carbon sources would have increased the *nos*Z-bearing community abundance (Henderson *et al.*, 2010), resulting in a higher denitrification potential to reduce N_2O to N_2. The effect of organic amendments increasing denitrification potential did not correlate with the percentage of genera sequences involved in denitrification (Table 9). However, other specific relationships that were observed are discussed in the following section. In this sense, Garcia-Plazaola *et al.*, (1993) found that the contribution of *Rhizobium* (the best represented genus in our study) to the total denitrification was virtually negligible compared to other soil microorganisms. Of the other genera detected (Table 9), only the *Bacillus* genera positively correlated with N_2O production (r^2=0.812). Kim *et al.* (2005) described the fact that *Bacillus* strains are simultaneously involved in aerobic nitrification/denitrification.

In contrast to the situation described for N_2O, CO_2 emissions were positively affected by water-filled pore space (WFPS) and soil temperature (CO_2-C=-3.61+ 0.031WFPS+ 0.57TSoil, r^2=0.462, P= 0.000). The influence of WFPS on CO_2 emissions was expected. Several authors have described a positive correlation between soil

WFPS and CO_2 emission rates when WFPS is lower than 60% and a negative correlation when WFPS is higher than 60% (Davidson, 1991; Kiese and Butterbach-Bahl, 2002). Specifically, under humid Mediterranean conditions Huérfano *et al.* (2015) described an inflection point of 55% of WFPS for CO_2 emissions. In this case, we only observed a positive correlation as WFPS remained under 60%, with the exception of the first day. The observation of a negative correlation, as described by the cited authors, may have been hindered by the absence of WFPS over 60% during the assayed period. As the model previously showed, temperature had an important role modulating CO_2 emissions. Carbon dioxide fluxes were positively correlated with the temperature (r^2= 0.873, $p < 0.005$). This positive influence of temperature on CO_2 emissions has been described by Buchmann (2000), especially when the WFPS is low (Beare *et al.*, 2009). CO_2 emissions should not, however, always be expected to increase with temperature. In fact, Menéndez *et al.* (2012) found higher CO_2 emissions at 10ºC than at 20ºC in a lab experiment. As mentioned before, the addition of organic matter stimulated soil enzyme activities as well as soil respiration (Emmerling *et al.*, 2000; Pascual *et al.*, 2002; Kandeler *et al.*, 2006; Henderson *et al.*, 2010). CO_2 fluxes on day 15 correlated with overall enzyme activity (P=0.679; sig=0.005) and organic matter content (P=0.523; sig=0.045).

We did not observe any correlation between CH_4 fluxes and WFPS and or soil temperature. The negative fluxes during the

assayed period are a consequence of the low WFPS which favoured O_2 availability. Molecular oxygen is required by methanotrophs for CH_4 oxidation (Dutaur and Verchot, 2007), resulting in CH_4 consumption.

2.5.3 Soil bacterial community

Our study shows that the soil microbial diversity, measured using the Shannon and Chao1 indices, is not affected by the different amendments applied to the soil (Table 7). The high coverage of the prokaryotic diversity, in all cases higher than 93%, ensures an accurate representation of the soil's microbial diversity. Soil microbial diversity may represent the ability of a soil to cope with perturbations, that is, this diversity is the basis for soil resilience (Hartmann and Widmer, 2006). However, the same authors also claimed that standard anonymous diversity indices (Shannon, Chao1 and ACE and rarefaction) do not detect management-dependent influences on the soil bacterial community. In this sense, these diversity indices are limited and as they do not take into account subtle changes in other dimensions of soil quality. Moreover, recent observations have shown that soil biodiversity loss and the simplification of the soil community composition impair ecosystem functions (Wagg *et al.*, 2014). According to the results recorded for soil quality and GHG emissions, our findings seems to contradict this affirmation, as regardless of the uniformity in the diversity, the ecosystem (agronomical) functions are not impaired and were, on the contrary, enhanced. The advantage of investigating the bacterial

community structure with high throughput sequencing of the V4-V5 hypervariable regions of the 16S rRNA gene is the possibility of finding small changes. Our results for the bacterial communities fifteen days after the application of the amendment during the month of February-March, showed great similarities between treatments, that is to say, the effects of composted organic residues on soil bacterial communities did not vary according to the type of compost, and all treated soils were able to respond uniformly to environmental changes. This response is further supported by the results for GHG emission, where the main driving factors were not only the nutrient input, but also, environmental factors. This result contrasts with some other studies where the application of manure from farming caused pronounced changes in the composition of the bacterial community (Ge *et al.*, 2010; Ding *et al.*, 2014), in that case perhaps due to the presence of the antibiotic sulfadiazine. However, we did not record the presence of other external modifiers. In our case, the minor differences were due to an increase in the phylum Bacteroidetes in all treated soil with respect to the control plot, a slight decrease in the phylum Acidobacteria in the soil treated with organic amendments, and also in the phylum Actinobacteria in the OF-MSW plot. These differences may be due to recent application of treatments, meaning some differences in the community composition could reflect the microbial community of the amendments. In spite of the short period of time since the application, finding the genera *Flavobacterium*, *Pedobacter* and *Adhaeribacter* (phylum Bacteroidetes) demonstrates the presence of

bacteria which typically contribute to enhanced soil quality (Ding *et al.*, 2014).

Since the soil sampling and DNA extraction was carried out 15 days after the application of the treatments, it was possible to expect that the observed bacterial communities partly came from the different manures applied to the vineyard. In this sense, it is clear that the Control and NPK samples must reflect the microbial community present in the soil as they involve no addition microorganisms, but SMC, PEL and OF-MSW treatments could add their own bacterial communities to the soil when used as fertilizers for the vineyard. This may explain the high proportion of the phylum Bacteroidetes found in the soil treated with OF-MSW. However, the differences between the treatments, 15 days after application, are minor compared with those observed after a summer sampling round, 5 months after amendment application (Calleja-Cervantes *et al.* 2015), revealing seasonal variation in the soil bacteria communities rather than a direct variation due to fresh application of treatments. The increase in microbial activity supports a homogenous increase of the native communities of the soil after application, expect for OF-MSW.

The high rates of nitrification, N_2O production and denitrification in the PEL-treated soil could correlate with the presence of the genera *Paracoccus* and *Nitrosospira,* which showed statistically significant differences with respect to the other

treatments. However, it should also be considered that PEL amendment enhanced N_2O emissions in PEL, reflect a less efficient amendment, in terms of losses, compared to the other materials.

2.6 CONCLUSIONS

This study reflects that long-term restoration of soil fertility and other quality parameters, on the one hand, and soil carbon sequestration on the other, require an invaluable in-field examination. It was seen that organic inputs from composted organic wastes exert a direct benefit on soil quality, as microbial activity is augmented. Soil quality and its fertility were consistently enhanced by amendment application over the course of 13 years. According to Powlson *et al.* (2011) the SOC content of soil increases and its potential as an alternative for mitigating climate change depends on what would otherwise have happen to the material that was applied to the soil (the alternative dispose-off). The results show that there seem to be non-significant GHG emissions (in PEL and SMC treatment) with respect to the control, coupled with a modest gain in C sequestration (enhanced organic matter). These results should be further studied to understand the factors controlling them. In addition to this, manure application represents the recycling of already fixed N rather than new fixation (Vitousek *et al.*, 1997). If not used as fertilizers, the three materials studied would have had a less environmentally-friendly fate. Further studies can look into the mechanisms that drive these changes.

Despite the continuous application of treatments, in general terms the GHG emissions were more affected by environmental conditions and seasonal changes than by the application of the different treatments. Mineral and organic fertilizers increased

nitrification potential in a similar way. Nevertheless, organic amendments showed a higher influence on denitrification potential and the N_2O/N_2 ratio. Although these compounds were potentiality induced by organic amendments, they were not reflected by N_2O emissions. However, further studies should be undertaken to understand the reason behind the N_2O emissions.

The changes on soil bacterial activity were not reflected on changes of the gross soil diversity, measured with the Shannon and Chao1 indices. In this case, slight changes on specific genera were recorded, but the expression of soil bacterial communities is underscored by environmental factors. Further driving causes of bacterial communities could be studied at depth as several questions remain to be answered. For instance, to determine possible functions attributable to bacterial communities.

To sum up, the continuous long-term application of organic amendments affects positively soil's quality, however GHG emissions throughout the crop cycle are not particularly different compared to an unfertilized soil. Moreover, GHG emissions are more affected by environmental and seasonal variations than by the application itself. This is reflected in the bacterial community structure of the soil, which seems to remain similar 15 days after amendments application. These three factors considered together, add up for a comprehensive method to understand the link between soil fertility and climate change impacts.

CHAPTER 3

Metales pesados en viña tras doce años de aplicación de distintos tipos de compost: contenido del suelo, baya y biodisponibilidad para *ray grass*.

3

Metales pesados en viña tras doce años de aplicación de distintos tipos de compost: contenido del suelo, baya y biodisponibilidad para *ray grass*

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3.1 RESUMEN

Los compost son ricos en materia orgánica y nutrientes y por tanto pueden mejorar la fertilidad de los suelos. Sin embargo, dependiendo de la composición y origen, éstos pueden dar lugar a una ulterior acumulación, en suelos y plantas, de sustancias inorgánicas o metales pesados que pueden causar efectos nocivos en la salud y en el medio ambiente. En el presente estudio se han comparado los contenidos totales de metales pesados en el suelo y baya de una viña de variedad *Tempranillo* DOC Rioja ubicada en Navarra sujeta a 5 manejos diferentes de la fertilización durante 12 años. Los tratamientos ensayados son: un compost comercial a base de residuos animales, vegetales y lodos de tratamiento de aguas residuales urbanas (PEL), un compost de la fracción orgánica de residuos municipales (con recogida selectiva de materia orgánica) (FORM), un estiércol de ovino compostado (EST), un abono inorgánico convencional (NPK) y un testigo no abonado (Control). De acuerdo al RD 824/2005, la categoría según el contenido de metales pesado para el PEL y el FORM es B, mientras que para el EST es A.

En este trabajo se estudió el contenido total de metales en suelo y su posterior transmisión a las bayas. En el caso de los contenidos en suelo de Cd, Cr, Cu, Ni, Pb y Zn no hay diferencias significativas al final de los 12 años de aplicación. Sin embargo las bayas que fueron abonadas con PEL aumentaron los contenidos de Cr, Cu y Ni. Las bayas abonadas con NPK aumentaron su contenido

en Zn. Las bayas de los tratamientos FORM y EST no presentaron diferencias con el testigo no abonado.

Así mismo, se ha cuantificado la biodisponibilidad residual para *ray grass* (*Lolium perenne* L. var. *Herbus)* de los metales pesados transferidos por estos suelos. Para ello se cultivó durante 4 meses *ray grass* en macetas con suelo procedente de las parcelas experimentales antes mencionadas en una cámara de cultivo en condiciones controladas. Tras la siembra se cuantificó el contenido de metales pesados en la parte aérea del *ray grass* en los días 30, 60 y 120. Los contenidos de Cd y Pb en todos los casos se encontraron por debajo de los 5ppm. A diferencia de los contenidos encontrados en baya, al finalizar el experimento los contenidos de Cr, Cu, Ni y Zn fueron similares en todos los tratamientos; encontrándose algunas diferencias en los primeros cortes atribuibles al estado de crecimiento inicial del *ray grass*.

Se concluye que los compost evaluados en las dosis aplicadas no conllevan un riesgo importante de contaminación de suelos ni de transferencia de metales pesados. La transferencia de los metales pesados varía en función las características de los compost y del cultivo considerado.

3.2 INTRODUCCION

La materia orgánica es una fracción esencial en el suelo, pues ésta no sólo aporta nutrientes al suelo, sino que también interviene en el estado de las propiedades físicas (estructura, porosidad, contenido de humedad, temperatura), químicas (pH, capacidad de intercambio catiónico, salinidad) y biológicas del suelo (García-Gil *et al*., 2000; Hartl y Erhart, 2003). La pérdida de materia orgánica en los suelos de cultivo está estrechamente relacionada con la degradación de la fertilidad del suelo y la erosión, y una forma de intentar restituirla a largo plazo en un suelo, es el aporte paulatino de enmiendas y/o fertilizantes que sean ricos en materia orgánica muy humificada (Fassbender y Bornemisza, 1987; Bertsch, 2003).

Los fertilizantes y/o enmiendas orgánicos (concretamente los compost), al provenir de diferentes orígenes y según su composición, pueden por otro lado, acumular sustancias inorgánicas o metales pesados que pueden causar efectos nocivos en la salud y el ambiente (Zmora-Nahum *et al*., 2007). Se considera que tanto la capacidad de aportar nutrientes, como de acumular sustancias nocivas, no sólo son propiedades de la materia prima y el proceso de fabricación de esas enmiendas, sino también de las condiciones imperantes en el campo (medio ambiente, cultivo, etc.) (Bertsch, 2003; Evanylo *et al.*, 2008). Es preciso destacar que los problemas de metales pesados en el suelo se derivan, además de por los ya mencionados, del comportamiento que éstos puedan tener en el suelo; dependiendo de las características del metal en cuestión y de la cantidad existente, su destino final cambiará (Canet, 2012). Los

metales pesados podrían derivar en destinos distintos: permanecer en la solución del suelo, ya sea como iones libres o complejados; unirse al complejo de cambio, adsorberse por la materia orgánica insoluble, quedar inmovilizados en formas minerales, perderse por lavado o ser absorbidos por la flora circundante. En este sentido, la evaluación de la biodisponibilidad permite tener información de primera mano respecto a los riesgos de transferencia de estos contaminantes y su posible acumulación en la cadena alimenticia. Entendiendo biodisponibilidad como la capacidad de un elemento para ser transferido desde el suelo hasta cualquier organismo vivo (Chopin *et al.*, 2008).

En esta tesitura, los estudios del aporte de materia orgánica a través de compost, son primordiales, no sólo como alternativas a la fertilización química, sino para evaluar su posterior impacto en el medio ambiente. Atendiendo a la situación descrita y considerando que carecemos de estudios en las condiciones agroclimáticas concretas de la DOC Rioja en Navarra, y en especial estudios sobre los posibles riesgos de biodisponibilidad de metales pesados, se plantea el presente trabajo.

En este trabajo el objetivo es evaluar el efecto de la fertilización orgánica e inorgánica a largo plazo en el contenido total de metales pesados en el suelo y su posterior biodisponibilidad en la baya en condiciones DOC “Rioja”.

Complementario al objetivo anterior, se evalúa la capacidad de transferencia residual de los metales pesados presentes en estos suelos determinando su transferencia en *ray grass* cultivado en dichos suelos bajo condiciones controladas en una cámara de cultivo.

3.3 MATERIALES Y METODOS

3.3.1 Ensayo en vid.

3.3.1.1 Localización y diseño del ensayo

El ensayo estaba situado en una parcela de viña en el término municipal de Bargota, localidad de Navarra, dentro del ámbito de producción de bayas de la Denominación de Origen Calificada Rioja. Se trataba de una ladera de acumulación de mínima pendiente. El suelo está clasificado como Typic Calcixerept, de textura franco arcillosa, con un 1.28% de materia orgánica y pH de 7.6. El clima es mediterráneo templado seco según la clasificación de Papadakis (1966). La parcela donde se realizó el ensayo estuvo previamente sembrada de cereal. La viña fue plantada en 1997 con la variedad Tempranillo, porta injerto Richter 110, y con un marco de plantación de 3 x 1.15 metros. La conducción fue en espaldera con el sistema de formación en cordón doble Royat. Los restos de cosecha y poda siempre fueron retirados. El diseño consiste en un bloque compuesto por dos cordones de viña, con tres repeticiones, en cada uno de los cuales se ensayaron cuatro abonos diferentes (Tabla 1) de aplicación anual y un testigo no abonado.

En ensayo quedó constituido por 15 parcelas elementales dispuestas al azar. Cada parcela elemental estaba formada por 15 cepas.

Tabla 1. Propiedades físicas y químicas de los abonos ensayados en Bargota.

	PEL	FORM	EST	NPK
pH	8.8	8.3	9.0	N.A.
E.C. (dS/m)	8.3	8.1	6.6	31.6
Materia seca (% p/p)	86.2	69.8	59.1	98.1
Materia orgánica (% sms)	31.7	56.9	29.1	N.A.
Carbono orgánico oxidable (%sms)	14.9	32.2	14.2	N.A.
Nitrogeno Kjeldahl (%sms)	2.81	2.51	1.39	5
Nitrógeno Orgánico (%sms)	2.48	2.13	1.26	N.A.
C org. /N org.	6.0	15.1	11.3	N.A.
P_2O_5 (% MS)	3.34	2.36	0.62	9.85
K_2O (% MS)	2.48	2.09	3.54	15
CaO (% MS)	13.4	12.9	18.2	5.7
MgO (% MS)	1.1	0.9	1.4	N.A.
Na_2O (% MS)	1.3	0.6	0.8	N.A.
Dosis media (kg ha^{-1} $año^{-1}$PF)	3700	4080	4630	340

Compost comercial peletizado (PEL), Compost de fracción orgánica de residuos municipales (FORM), Compost de estiércol (EST) y mineral (NPK). N.A (No aplicado). PF (Peso Fresco). Sms (sobre materia seca). MS (materia seca)

3.3.1.2 Tratamientos

Los tratamientos ensayados fueron: abonado orgánico comercial pelletizado (PEL), abonado orgánico de compost de la fracción orgánica separada en origen (FORM), abonado orgánico de

estiércol de ovino (EST), un abonado mineral genérico (NPK) y un testigo (Control) al cual no se le aplicó abono alguno.

El fertilizante empleado en el tratamiento PEL es un abono orgánico comercial procedente del compostaje de residuos animales y vegetales y lodos de tratamientos de aguas residuales urbanas, cuya categoría en función de los metales pesados es B. En el tratamiento FORM se aplicó un abono orgánico procedente del compostaje de la fracción orgánica de residuos municipales; por su contenido en metales pesados su categoría es B. El estiércol utilizado en el tratamiento EST es un estiércol de ovino compostado en granja y de maduración muy larga; la categoría a la que corresponde es A. En el tratamiento NPK el abonado mineral que se empleó es un fertilizante de composición, según etiqueta 5-10-15 en NPK.

Se llevó a cabo un abonado de fondo antes de la plantación con dosis medias de 15,000 kg/ha en peso fresco de cada compost. Los doce años posteriores, las dosis fueron ajustadas según la humedad del abono, éstas se han mantenido prácticamente a lo largo de los años de estudio (Tabla 1). La forma de aplicación de los fertilizantes fue manual en superficie. El fertilizante se extendió a ambos lados de la fila control y posteriormente era enterrado con grada vertical (1-20cm). Generalmente los abonos orgánicos se aplicaron en febrero y el abono inorgánico se aplicaba a la salida del invierno.

3.3.1.4 Determinaciones realizadas.

Las muestras para determinación de metales pesados en suelo de cada parcela elemental se tomaron a finales del 2010, en el horizonte 0-20cm, cada muestra estaba formada por dos submuestras, tomadas a cada lado del cordón, con ellas se formó una sola muestra de unos 500 g aproximadamente. Las muestras secas se tamizaron y su composición química fue determinada mediante análisis por ICP-OES (ICAP 6500 DUO/IRIS INTREPID II XDL) tras su digestión.

Para la determinación de metales pesados en baya, se tomó una muestra de 100 bayas de cada tratamiento al momento de la cosecha compuesta por bayas de al menos 4 cepas centrales. Las muestras fueron secadas a 70ºC hasta peso constante, molidas hasta pulverizarlas y su composición química fue determinada mediante análisis por ICP-OES (ICAP 6500 DUO/IRIS INTREPID II XDL), tras su digestión. Los metales pesados determinados tanto en suelo como en baya fueron cadmio (Cd), cromo (Cr), cobre (Cu), níquel (Ni), plomo (Pb), zinc (Zn).

3.3.2 Ensayo de respuesta agronómica en condiciones controladas.

Para evaluar la biodisponibilidad residual de los metales pesados que habían sido acumulados a lo largo de los 12 años en los suelos, se planteó un ensayo en macetas, en condiciones controladas con el suelo proveniente de las parcelas descritas en el punto anterior. El ensayo se realizó con macetas *Mitscherlich* de 10

litros. En este ensayo se estudiaron los mismos tratamientos que en el ensayo previo. La unidad experimental fue la maceta y el ensayo fue en bloques al azar con 4 repeticiones. Se mezcló el suelo original (0-20cm) de forma manual con perlita en una proporción volumétrica 1/1. En cada maceta se sembró 0.6 g de semillas de *ray grass* (*Lolium perenne* L. var. Herbus). Se utilizó *ray grass* porque es un cultivo que crece en todo tipo de suelos, germina y se desarrolla de forma rápida, tiene gran capacidad de tapizado y alta demanda de nitrógeno pero sobre todo porque tiene muchos rebrotes, lo que permitió hacer varios cortes. Las macetas en la cámara de cultivo quedaron bajo condiciones de 20 ºC, 70% de humedad y con una exposición de 15 horas de luz al día. Durante los cuatro meses del experimento se cosechó la producción de *ray grass* en cada maceta los días 30, 60 y 120 tras la siembra. El material vegetal se secó en estufa y posteriormente se molió y tamizó, tras su digestión se determinó el contenido de metales pesados mediante análisis por ICP-OES (ICAP 6500 DUO/IRIS INTREPID II XDL). Los metales pesados determinados fueron cadmio (Cd), cromo (Cr), cobre (Cu), níquel (Ni), plomo (Pb), zinc (Zn).

3.3.3 Análisis estadístico.

Se llevó a cabo un tratamiento estadístico de los datos recopilados de suelo, baya y *ray grass*. Se realizaron ANOVAs de un factor (tratamiento) para cada año con el paquete estadístico SPSS 17.0 (SPSS 17,2010). Posteriormente se realizaron el test de Duncan y la Diferencia Mínima Significativa ($P<0.05$).

3.4 RESULTADOS Y DISCUSION

3.4.1 Resultados de ensayo en vid

La cantidad de metales pesados aportados por cada tratamiento varía en función de la dosis y el contenido intrínseco (Tabla 2). En ninguno de los tratamientos el aporte superó al límite máximo permitido por año, asentado en el Real Decreto 1310/1990. El tratamiento EST presentó siempre los contenidos más bajos para los 6 metales pesados estudiados. Para el caso de Cd, el tratamiento que más aportó fue el PEL con 3 g $ha^{-1}año^{-1}$. Con respecto al Cr, el PEL y el FORM aportaron cantidades similares del orden de 140 g ha^{-1} $año^{-1}$ mientras que el EST sólo aportó 38 g ha^{-1} $año^{-1}$. En el caso del Cu, el FORM resultó el tratamiento con mayores aportes, 378 g ha^{-1} $año^{-1}$. El aporte de Ni también fue mayor en el tratamiento PEL con 61 g ha^{-1} $año^{-1}$. El Pb presentó la mayor tasa de aplicación en el tratamiento FORM con 280 g $ha^{-1}año^{-1}$. Con respecto al Zn, el mayor aporte se encontró también en el FORM con 918 g ha^{-1} $año^{-1}$

3.4.1.1 Resultados en suelo

El límite legal establecido en el Real Decreto 1310/1990 para el contenido de los distintos metales pesados en suelos con pH mayores a 7, como el que se estudió en este trabajo, no fue superado por los valores encontrados en los suelos tras la aplicación durante 12 años de los distintos tratamientos (Tabla 2). Los contenidos totales encontrados no han desvelado diferencias significativas entre los diferentes tratamientos para ningún metal pesado, es decir, los contenidos totales de metales pesados en suelo

son iguales en todas las parcelas. Esta falta de variación en los contenidos totales de metales pesados en el suelo es lógica, debido a las bajas concentraciones iniciales en los compost (Categoría A o B, según RD 826/2005) y a las dosis moderadas en las que fueron aplicados (equivalentes a menos de 80 kg N ha^{-1}). Es así que, las cantidades de metales pesados aplicadas son muy pequeñas en comparación con el contenido del encontrado en el suelo. Por ejemplo, en el mayor de los casos, al cabo de 12 años, se aplicó un total de 11 kg de Zn ha^{-1}, lo que supone sólo un 11.9% del total del Zn en el tratamiento testigo no abonado. Estos resultados se corresponden con los de Korboulewsky (2002) quien al evaluar compost de lodos de aguas residuales comprobó que los contenidos en suelo no eran modificados por el aporte, posiblemente porque los procesos de compostaje reducen el riesgo y la biodisponibilidad de metales pesados presentes en la materia prima usada, a través de la adsorción y el complejamiento de las sustancias húmicas. Los nulos cambios observados en el contenido total de metales en el suelo, también pueden deberse a que los metales pesados se vuelven menos disponibles en suelos con contenidos significativos de arcillas y pH básicos (Miner *et al.*, 1997; Korboulewsky, 2002).

Cabe mencionar que a pesar de no existir diferencias significativas, sí se apreciaron ciertas tendencias. El contenido de Cd tendió a aumentar en el caso del NPK. El Cr tendió a aumentar en todos los casos. El contenido de Cu actuó de modo distinto dependiendo del aporte, el PEL, el EST y el NPK tendieron a

disminuir el contenido, mientras que el aporte del FORM tendió a aumentarlo. El Ni en todos los casos tendió a aumentar por la fertilización, aunque en mayor medida en el caso del NPK. El Pb también tendió ligeramente a aumentar en todos los casos.

Tabla 2. Contenido de metales pesados expresado como mg kg^{-1} (peso seco).

		Límite legal	CONTROL	PEL	FORM	EST	NPK
Cd	Aportado (g $ha^{-1}año^{-1}$)†	150	N.A.	3	2	2	N.A.
	Suelo mg kg^{-1} (MS)†	3	1.2	1.2	1.2	1.2	1.9
	Baya mg kg^{-1} (MS)‡	0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cr	Aportado (g $ha^{-1}año^{-1}$)†	3000	N.A.	144	141	38	N.A.
	Suelo mg kg^{-1} (MS)†	150	38.5	39.8	40.0	39.8	41.3
	Baya mg kg^{-1} (MS)‡	N.A.	16.5 b	53.4 a	20.2 b	18.4 b	7.9 b
Cu	Aportado (g $ha^{-1}año^{-1}$)†	12000	N.A.	245	378	54	N.A.
	Suelo mg kg^{-1} (MS)†	210	23.4	21.1	27.0	22.4	20,0
	Baya mg kg^{-1} (MS)‡	N.A.	4.3 b	5.4 a	4.5 ab	4.6 ab	4.6 ab
Ni	Aportado (g $ha^{-1}año^{-1}$)†	3000	N.A.	61	39	15	N.A.
	Suelo mg kg^{-1} (MS)†	112	15.2	15.9	15.5	16.0	17.1
	Baya mg kg^{-1} (MS)‡	N.A.	6.5 b	12.5 a	6.8 b	6.6 b	3.3 b
Pb	Aportado (g $ha^{-1}año^{-1}$)†	15000	N.A.	146	280	85	N.A.
	Suelo mg kg^{-1} (MS)†	300	9.9	10.3	11.6	10.3	11.1
	Baya mg kg^{-1} (MS)‡	0.2	<0.5	<0,5	<0.5	<0.5	<0.5
Zn	Aportado (g $ha^{-1}año^{-1}$)†	30000	N.A.	461	918	224	N.A.
	Suelo mg kg^{-1} (MS)†	450	39.4	46.2	47.5	41.6	45.8
	Baya mg kg^{-1} (MS)‡	N.A.	5.5 c	13.3 ab	6.4 c	7.9 bc	14.1 a

Compost comercial peletizado (PEL), Compost de fracción orgánica de residuos municipales (FORM), Compost de estiércol (EST) y mineral (NPK). † Límites legales (Real Decreto 1310/1990). ‡ Límites legales (Directiva Europea 2001/22/CE). Las diferencias significativas están nombradas por letras distintas en cada renglón (P<0,05; n=3). N.A (no aplicado). M.S (materia seca)

El contenido de Zn también tendió a aumentar con la fertilización, el FORM es el que más lo aumentó seguido del PEL y el

NPK respectivamente. Estas tendencias detectadas indican que posiblemente a dosis de compost superiores y a mayores periodos de tiempo, podrían aparecer ligeras variaciones significativas del contenido de metales pesados en el suelo.

3.4.1.2 Resultados en baya

Los contenidos de metales pesados en baya se encontraron dentro de los límites fijados para Pb y Cd, en la legislación europea (Directiva 2001/22/CE). La concentración de Cd y Pb en las bayas, en todos los tratamientos fue inferior al límite de detección de la técnica empleada (5 ppm) (Tabla 2). Los contenidos de Zn y de Cu fueron de entre 4,3 y 13,3 mg Zn kg^{-1} MS y de entre 6,5 y 20 mg Cu kg^{-1} respectivamente. Estos valores se sitúan en los mismos rangos que los descritos en bayas por Angelova *et al.* (1999) y Zhu *et al.* (2012). En cambio, comparando los contenidos totales de Cr y Ni en estos mismos estudios con los obtenidos en este ensayo, resulta interesante que las bayas del tratamiento PEL se encuentran por encima de los rangos medios descritos por estos autores.

Tampoco se encontraron concentraciones que pudieran ser fitotóxicas como también se ha constatado en otros trabajos con uso de diferentes materias orgánicas (Pinamonti, 1998). Los contenidos de Cr, Cu, Ni y Zn fueron estadísticamente distintos dependiendo del tratamiento. El tratamiento PEL fue el que más incrementó los contenidos totales en baya de Cr, Cu y Ni. El Cr aumentó en el

tratamiento PEL hasta casi triplicar su contenido en baya respecto del control, coincidiendo con que este tratamiento fue el que más cromo recibió al año. A pesar de que el tratamiento FORM era el que más Cu y Zn aportaba al año, fue en realidad el tratamiento PEL el que aumentó el contenido total en baya un 30% para el caso del Cu y lo duplicó en el caso del Zn, comparándolo con el control. También en el caso del Ni, el PEL aportaba más contenido al año y fue este mismo tratamiento el que duplicó el contenido en baya respecto del control.

La transferencia de los metales pesados estudiados a las bayas es evidente. Destaca la elevada transferencia de los metales estudiados en el tratamiento PEL; por otra parte, tanto el compost de FORM como el de EST no presentaron diferencias con el testigo no abonado, ni con el mineral, excepto para el Zn. La mayor transferencia podría ser explicada en el caso del Ni por haber recibido casi el doble de este elemento que los otros tratamientos. Sin embargo, en el resto de elementos esta explicación no es válida puesto que los otros tratamientos recibieron cantidades iguales o superiores de los elementos. Esto indica una distinta biodisponibilidad para la viña de los metales pesados contenidos en los distintos materiales aportados. La explicación podría estar dada porque la materia orgánica bloquea la disponibilidad de metales pesados para las plantas (Miner *et al.*, 1997; Lema *et al.*, 2003). El hecho de que el tratamiento PEL presente una relación C/N extremadamente baja (Tabla 1), indicaría que la materia orgánica

conforma moléculas lábiles, corroborándose porque el contenido de materia orgánica es bajo y concediéndole un carácter más bien mineral a este compost. Aunque el FORM en muchos casos presenta mayores cantidades metales pesados, su alto contenido de materia orgánica y su relación C/N indican que esta materia orgánica está estabilizada y probablemente reduce la disponibilidad para la baya de los metales pesados que contiene (Antoniadalis y Alloway, 2001). Esto coincide con lo descrito por Planquart *et al.* (1999) quien concluyó que la presencia de sólidos orgánicos solubles, aunque incrementaba la movilidad de Cu en el suelo, también disminuía su disponibilidad para las plantas.

3.4.2 Resultados de ensayo de extracción en ray grass

3.4.2.1 Contenido de metales pesados en ray grass

Las concentraciones totales de metales pesados encontradas en *ray grass* se encontraron dentro de los rangos que han sido estudiados en el pasado por diferentes autores para *ray grass* (Antoniadis y Alloway, 2001; Bidar *et al.*, 2007; Guerra *et al.*, 2007). La concentración de Cd y Pb en el *ray grass* de todos los tratamientos fue inferior al límite de detección de la técnica empleada (5ppm). En la Figura 1 se muestra la evolución en el tiempo del contenido de metales pesados Cr, Cu, Ni y Zn. La cantidad transferida de Cr al ray grass en el primer corte aumentó significativamente por el tratamiento EST, sin embargo esta tendencia no se mantuvo a lo largo del experimento, pues la concentración de Cr no fue distinta para ningún tratamiento en los

posteriores muestreos. Para el caso del Cu, en el primer corte el EST y el CONTROL presentaron mayores concentraciones que los demás tratamientos. En el último corte, el contenido de Cu de todos los tratamientos se igualó, no encontrándose diferencias significativas. En lo que respecta al Ni las diferencias significativas sólo fueron evidentes en el primer corte, siendo el EST el tratamiento en el que más níquel se transfirió, sin embargo, en los demás cortes, los distintos tratamientos no presentaron diferencias.

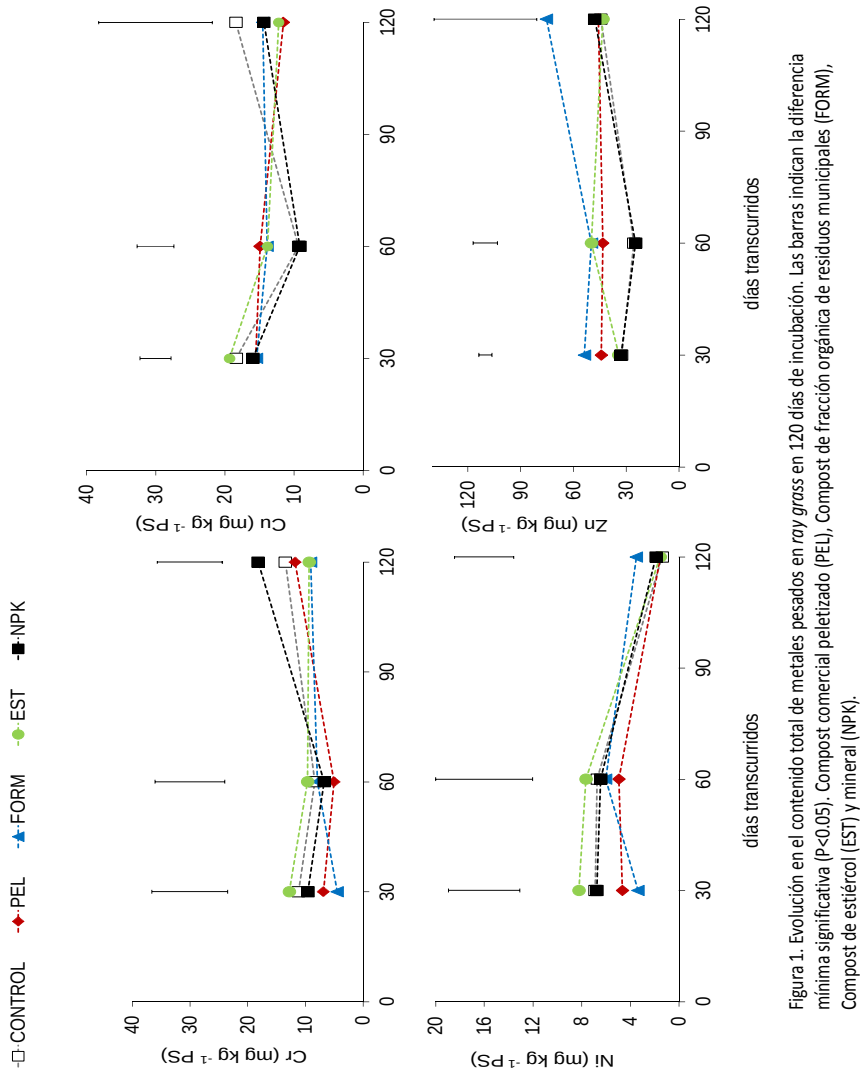


Figura 1. Evolución en el contenido total de metales pesados en *ray grass* en 120 días de incubación. Las barras indican la diferencia mínima significativa (P<0.05). Compost comercial peletizado (PEL), Compost de fracción orgánica de residuos municipales (FORM), Compost de estiércol (EST) y mineral (NPK).

A pesar de esto, se pueden apreciar ligeras tendencias al mantenimiento del tratamiento EST como el que más níquel transfirió al *ray grass*. En lo que respecta al Zn, el FORM fue el tratamiento en el que siempre se transfirió la mayor cantidad de este metal, seguido por el PEL, aunque las concentraciones de Zn detectadas no se encuentran consideradas como tóxicas, al menos para este cultivo.

Se destacan las diferencias respecto a los resultados obtenidos en baya, donde era el PEL el tratamiento que incrementó en mayor medida los contenidos de Cr, Cu y Ni. Y donde ni el FORM, ni el EST figuraron como tratamientos con especial biodisponibilidad para Zn, Cr, Cu y Ni, respectivamente. Es importante notar que se trata de distintos órganos y especies vegetales. En *ray grass* las diferencias encontradas al inicio del experimento pueden ser atribuibles al estado de crecimiento inicial del *ray grass*, al finalizar el ensayo no se encontraron diferencias en la cantidad de metales transferidos y las concentraciones encontradas no son fitotóxicas.

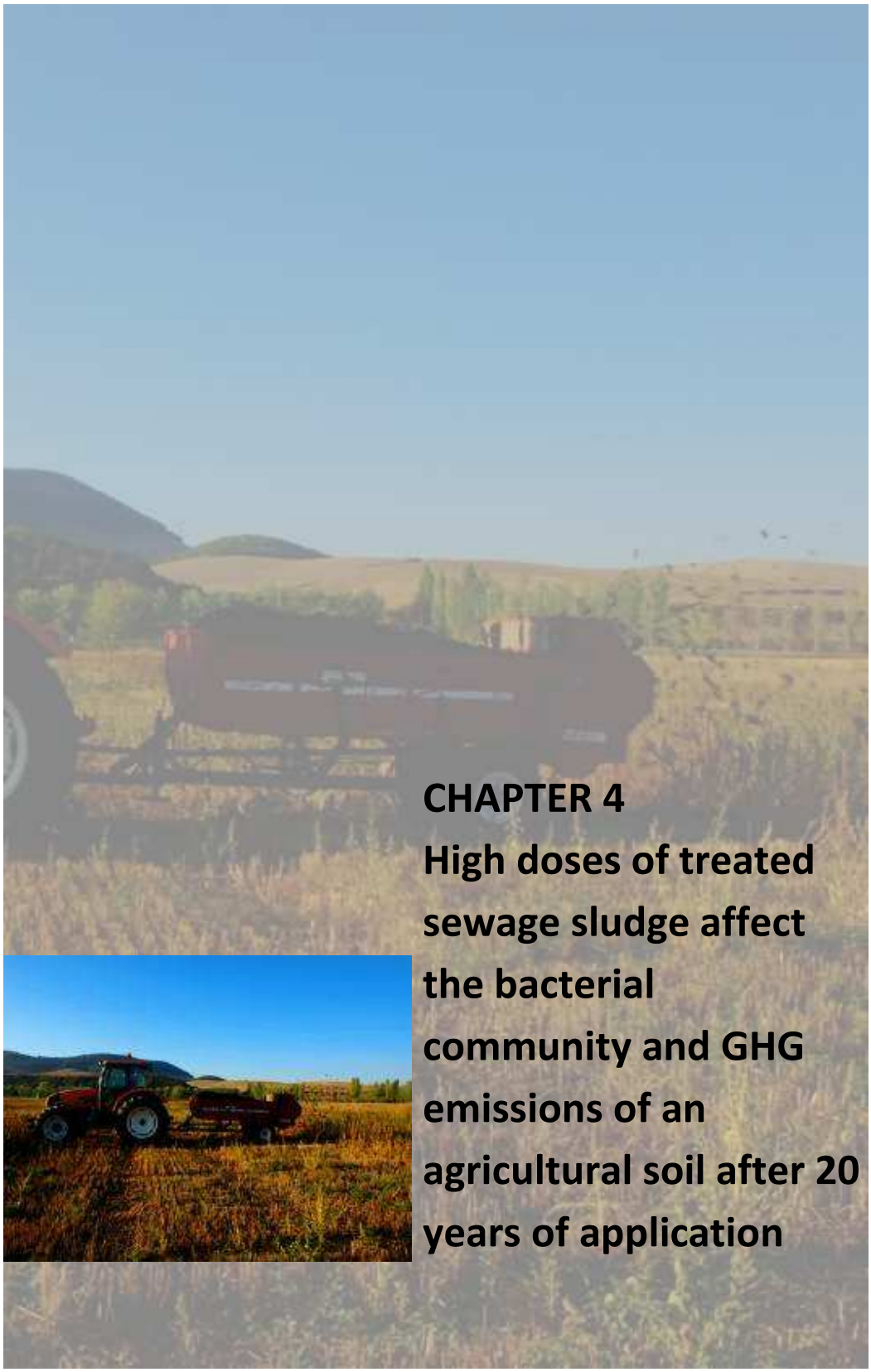
3.5 CONCLUSIONES

Los compost evaluados, tras doce años de ser aplicados a dosis medias anuales de 4 toneladas por hectárea en una viña DOC Rioja, no conllevan un riesgo importante de contaminación de suelos ni de transferencia de metales pesados a las bayas.

La transferencia de los metales pesados varía en función de las características de los compost. En bayas, en las condiciones ensayadas, los metales pesados de los compost con relación C/N extremadamente baja, por debajo de lo que se consideraría materia orgánica humificada, son más biodisponibles.

En *ray grass* las concentraciones de metales pesados se van igualando gradualmente, no encontrándose así un efecto claro de aumento de la biodisponibilidad residual de los metales.

La transferencia de metales pesados, por otro lado, se ve afectada por la especie vegetal y probablemente por el órgano vegetal estudiado. Es necesario realizar estudios detallados sobre trasferencia de metales pesados en distintas especies y órganos vegetales.



CHAPTER 4

High doses of treated sewage sludge affect the bacterial community and GHG emissions of an agricultural soil after 20 years of application

4

High doses of treated sewage sludge affect the bacterial community and GHG emissions of an agricultural soil after 20 years of application

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4.1 ABSTRACT

A common practice around the world is the agricultural application of urban residues to soil, such as sewage sludge (SS) that has been treated with a thermophilic anaerobic digestion. The benefits range from providing abundant organic matter to an effective increase of nitrogen, phosphorus and potassium for crops. Since sewage sludge vary in its composition, it is desirable to obtain an 'in the field' estimate of the effects after continuous application in arable soils. The scope of this study was to examine the behavior of the soil after 20 years of an annual agricultural application of treated sewage sludge (TSS) at different doses. Since 1992, the applied doses have been as follows: 40 t ha^{-1} and 80 t ha^{-1} every year, and 40 t ha^{-1} every 3 years plus an annual mineral fertilization depending on the crop. Along with these treatments there was a control without fertilization or TSS application, and a mineral treatment. In the 20th year of application greenhouse gas emissions, and nitrate and ammonium were measured during the crop's cycle. The bacterial community in the soil was surveyed 15 days after the last annual application and at harvest. TSS application increased microbial activity in these soils at a different rate depending on the dose. The most important increase was registered on the highest dose for O.M content, P_2O_5, and total N. GHG emissions were also affected by the highest dose and significant changes in microbial community composition where also observed. The exaggerated applied doses of TSS in this study are not a common practice and so, the observed changes resulted in the irreversible lodging of the crop

and the concomitant decrease in yield. However, in the 40 t 3y treatment, interesting similarities were found when compared to the control. It is hypothesized that the supplemented mineral fertilization, in years that TSS was not applied, has triggered a beneficial increased microbial activity in soil, coupled to an activation of root metabolism. TSS are soil amendments that exert beneficial outcomes in the studied soils and under this particular climatic region, however application rates must be adjusted to minimize undesirable effects in the yields.

4.2 INTRODUCTION

In Europe, the Sewage Sludge Directive 86/278/EEC encourages SS use in agriculture and it provides regulatory guidelines to prevent irreversible damages to human health and environment. For instance in Spain, approximately 1.2·Tg $yr^{-1}$of dry matter of SS are produced from wastewater treatment, about 4% of this material is destroyed by incineration; 8% is dumped on nearby landfills, and almost 80% is applied to soil as an organic amendment, the rest is recycled in other types of soil usage (MAGRAMA, 2013).

Technological developments have enabled important rates of sanitization in waste water treatment, but collateral residues or by-products are still generated. One of such by-products is sewage sludge. Supplementary treatments, such as thermophilic anaerobic digestion, ensure that potential biological hazards are minimized or removed from SS. The thermophilic anaerobic digestion is an adequate way to remove unpleasant smells, sanitize SS, and to a less extent stabilize the generated organic matter. Considering that TSS contains high proportions of organic matter and plant nutrients, agricultural application as a soil amendment or fertilizer is a common practice (Wang, 1997). Physical, chemical and biological properties of soils are positively modified after TSS application. Plants are provided with essential nutrients and waste recycling is promoted (Singh and Agrawal, 2008). However, regional assessments after long-term applications and on extreme applications rates should be conducted (Paramasivam *et al.*, 2008)

to explore the limitations of its application to soil, as important risks to the environment are associated (Roig *et al.*, 2012).

An important and increasing number of studies focus their efforts on the evaluation of heavy metal evolution, accumulation and translocation in soil (Lake *et al.*, 1984; Smith, 2009). In Spain a similar tendency is observed. However, only a few studies asses the environmental performance of TSS application in terms of greenhouse gas emissions (GHG). Arguably, agricultural application of TSS would induce important N imbalances in soil. Nitrification and denitrification processes could theoretically be exacerbated, increasing N_2O emissions and triggering other nutrient losses (Sheppard *et al.*, 2005; Pezzolla *et al.*, 2012). Moreover, bacterial community shifts remain understudied after continued applications to soil. Undoubtedly, soil biota plays a key role in the functions that control gas losses and nutrient cycling. Alterations at community level have been recorded by Dennis and Fresquez (1989), Pascual *et al.* (2008) and Mattana *et al.* (2014) after TSS application. Their findings stress the importance of evaluating each individual case in detail, as the composition of the TSS has an important degree of variation, beginning with the treatment to stabilize and sanitize, and finishing with the dose of application (Zaman *et al.*, 2004).

After 20 years of continued treatment application to soil (treated sewage sludge; mineral and control) chemical, environmental and biological performance of these practices were

assessed. The scope was to reveal fertility differences between the doses, and to compare GHG emissions during the assayed period. Additionally, the bacterial community was survey to discover their status after treatment application and at harvest.

4.3 MATERIALS AND METHODS

4.3.1 Site description

This research was carried out at a long-term experimental site established in 1992 in Arazuri, Navarra, Spain (42º 48'N-1º 43'W). The area is localized in the experimental station of Arazuri, adjacent to the wastewater treatment plant of the Federation of Municipalities of Pamplona (*Mancomunidad de la Comarca de Pamplona*). The experiment is managed by the *Instituto Navarro de Tecnologias e Infraestructuras Agroalimentarias* (INTIA, Spanish Acronym). According to the agro-climatic classification system of Papadakis (1961), the climate is Humid-template-Mediterranean with an annual rainfall of 760 mm and a mean temperature of 12.4°C. Figure 1 shows the precipitation levels and air temperatures recorded during the assayed period. The soil is classified as Cambisol calcaric (FAO) or Calcixerollic Xerochrept with a silty-clay loam texture. The characteristics of the control soil in the year 2011 are found on Table 1.

Table 1. Chemical properties of the soil at the Arazuri site (2011-2012) after 20 years of TSS application.

	Total C (% w/w)	O.M (% w/w)	Total N (% w/w)	P_2O_5 (mg/l)	Total P (% w/w)	K_2O (mg/l)	Total K (% w/w)
Control	3.08 c	2.04 c	0.15 c	38 c	0.07 c	169 a	1.05 a
40 t ha^{-1} y^{-1}	3.41 ab	2.23 c	0.16 ab	91 b	0.10 b	149 a	1.06 a
80 t ha^{-1} y^{-1}	3.51 a	2.89 a	0.18 a	117a	0.14 a	154 a	1.08 a
40 t ha^{-1} 3y^{-1}	3.43 ab	2.55 b	0.19 a	98 b	0.11 b	135 a	1.07 a
Mineral	3.16 bc	1.96 c	0.15 c	42 c	0.07 c	169 a	1.09 a

Treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{-1} 3y^{-1}. Different letters within a column indicate Duncan test results between treatments ($P<0.05$; n=3).

Ever since the beginning of the experiment a series of crop rotations had taken place following the sequence of cereal/cereal/non-cereal. Crop rotation is a common practice in this region aimed to manage soil fertility and to control crop's diseases. Also, nutrient requirements differ between crops, so nutrient exhaustion is avoided. In 2011 the cultivated crop was oats (*Avena sativa* L. var Aintree) which was sowed at a density of 100 kg ha^{-1}. No weed control was carried out and no supplementary irrigation systems were used. The agricultural management did not differ among treatments.

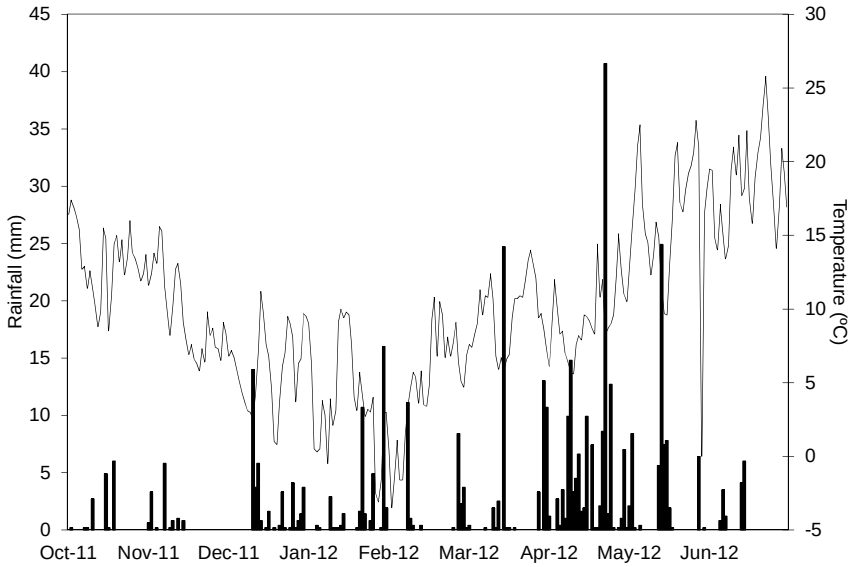


Figure 1. Daily precipitation (bars) and mean air temperature (line) for 261 days of study at the Arazuri site (2011-2012)

4.3.2 Treatments and experimental design

This study involved the application of three doses of TSS, along with an unfertilized control and a mineral fertilizer. The application of the treatments began in 1992. The TSS were applied to the soil surface and incorporated into the soil at a depth of 30 cm around October every year, corresponding to the sowing. TSS characteristics are found on Table 2. The doses were: 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1} and 40 t ha^{-1} $3y^{-1}$, this latter received mineral fertilization every year, and a mineral fertilizer: urea (60 kg N-Urea ha^{-1} split in two applications). These doses correspond to 834 kg N ha^{-1} y^{-1} (80 t), 418 kg N ha^{-1} y^{-1} (40 t), and 139 kg N ha^{-1} y^{-1} (40 t 3y). The every-3-year dose was supplied with 120 kg N as urea (splitted application). In the year 2011, treatments were applied in October the 23rd, oats were sowed right after. In the case of mineral application, as it was usually split in two, the first application was on January 17th corresponding to oats' tillering. The second application was on March 12th, coinciding with the stem elongation. For each treatment an experimental plot of 35 m^2 was identified. The experiment followed a randomized complete block design with four blocks.

Table 2. Physical and chemical properties of the treated sewage sludge during applied at the Arazuri site (2011-2012).

	pH	E.C. (µS/m)	Dry matter (% w/w)	TOC (% w/w)	Nitrogen Kjeldahl (% w/w)	C/ N	P_2O_5 (mg/l)	K_2O (mg/l)	CaO (% Ca/DW)	MgO (% Mg/DW)	$N\text{-}NH_4^+$ (% N/DW)
TSS	8.2	1706	14.1	36.75	7.4	4.97	6.12	0.43	5.15	0.89	1.02

Treated sewage sludge (TSS), D.W. Dry weight.

4.3.3 Soil sampling for general soil properties analysis

Before treatment application a bulked soil sample, composed of four soil samples, was collected on each experimental plot from the 0-30 cm layer. Since each experimental plot had its own bulk sample, a total of 4 independent samples were analysed per treatment. The samples were then air-dried at room temperature and stored at 4°C. The physical and chemical properties of the soil were determined after samples had been sieved through 2 mm mesh. Soil pH was measured in suspension with deionized water (w/w) ratio of 1:2.5. Soil organic matter was determined by the Walkley–Black organic C method. Total Nitrogen was measured trough ICP-OES after a microwave digestion with HNO_3 and H_2O_2. Phosphorus trough the Olsen method. Available K content was determined colorimetric after extraction with a 0.1 M ammonium acetate solution.

4.3.3.1 **Soil nitrate and ammonium contents**

Bulked soil samples were taken from all plots to a depth of 30 cm and analysed for ammonium and nitrate content on days 1, 3, 7, 11, 13, 24, 58, 107, 112, 130, 154, 164, 169, 176, 197, 212, after treatment application. Fresh samples of 100 g were extracted with 200 mL KCl (2M). The extracts were filtered and stored at -20ºC until analysis. The method used was the Gries-Illosvay colorimetric method modified by Barnes and Folkard (1951) by means of a Bran & Luebbe II AutoAnalyzer. An extra soil sample was used to determine gravimetric water content and expressed as the

percentage of water filled pore space (WFPS) with the method described by Menéndez *et al.* (2009).

4.3.3.2 Soil enzyme activity

In order to evaluate enzyme activities in soil, 15 days after TSS application, and then again 15 days after the mineral fertilizer had been applied, soil samples were obtained from three random spots within each plot. Samples were sieved through 2 mm and stored at 4º C and to await for enzyme activity assays in the following days. Protease, urease, phosphomonoesterase, β-glucosidase and also the hydrolysis of fluorescein diacetate were assayed.

Protease activity was assayed by determining the tyrosine released after a 1-hour incubation of 0.4 g of soil with 1 ml of 200 mM THAM buffer (pH 8.0) and 1 ml of 2% Na-caseinate at 50ºC for 2 h. The remaining substrate was precipitated with 0.92 M trichloroacetic acid and measured colorimetrically using Folin–Ciocalteu reagent at 700 nm (Geisseler and Horwath, 2009). Urease activity was measured with 1 g of soil with 1.75 ml of 100 mM Borate buffer (pH 10.0) and 0.25 ml of 820mM urea solution at 37ºC for 1 h. Excess urea was extracted with KCl solution and estimated colorimetrically at 670nm (Kandeler *et al.*, 1999). Phosphomonoesterase (acid phosphatase) activity was assayed using 1 g of soil, 1.6 ml of 20 mM modified universal buffer (pH 6.5), and 0.25 ml of 50 mM p-nitrophenyl phosphate. After incubation

the enzyme reaction was stopped and centrifuged. Following, absorbance was measured in the supernatant at 410 nm (Taylor *et al.*, 2002). β-glucosidase activity was determined using p-nitrophenyl-b-D-glucopyranoside (PNG, 0.05 M) as substrate. The assay is based on the release and detection of p-nitrophenol (PNP). The amount of PNP was determined using a spectrophotometer at 410 nm (Tabatabai, 1982). The hydrolysis of fluoresceín diacetate [3',6'-diacetylfluorescein (FDA)] was determined with 2 g of soil with 50 ml of 60 mM sodium phosphate buffer (pH 7.6) and 0.50 ml of 4.9 mM FDA lipase substrate solution was added. After mixing, samples were incubated, the reaction was stopped and samples were filtered. The absorbance was measured on a spectrophotometer at a wavelength of 490 nm based on Shawy and Burns (2005).

The geometric mean of the values of all enzyme activities (Overall Enzyme Activity, OEA) was calculated according to formula [1]:

$$[1]\ OEA = \sqrt[5]{\beta glucosidase \times protease \times alkaline\ phosphatase \times urease \times FDA}$$

This indicator has been previously applied as an overall indicator of microbial activity on soil quality (García-Ruiz *et al.*, 2008).

4.3.3.2 Potential of nitrification.

Fifteen days after fertilization, potential nitrification of soil was measured following Norton and Stark (2011). Briefly, 15 g of soil

were incubated at 24 ºC for 24 h in a solution of 0.2 *M* KH_2PO_4, 0.2 *M* K_2HPO_4 and 0.05 *M* NH_4SO_4. The solution was sampled up to eight times during the incubation. Afterwards, the samples were analyzed for NO_3^- and the rate of NO_3^- production was calculated by linear regression of solution concentration over time.

4.3.4 Gaseous emission sampling

On days 1, 3, 7, 11, 13, 18, 24, 42, 48, 77, 100, 102, 105, 107, 112, 120, 133, 144, 154, 159, 167, 175, 188, 206, 219, 242, 248, and 261 after applying the treatment (during the productive period of the crop) N_2O, CO_2 and CH_4 were measured. Gaseous emissions were determined using the closed chamber technique. Diurnal variations were minimized by a constant measurement in the morning (from 10 to 12 a.m.) (Baggs and Blum, 2004). Air temperature and soil temperature (10 cm depth) were measured just before starting gaseous emissions sampling. Emission rates and cumulative emissions were calculated, taking into account the concentration increase with time (Menéndez *et al.*, 2008). The samples were analysed by gas chromatography (GC) (Agilent, 7890A) with an electron capture detector (ECD) for N_2O detection and a flame ionization detector (FID) for CH_4. For the determination of CO_2, the GC was equipped with a methanizer to reduce CO_2 to CH_4. A capillary column (IA KRCIAES 6017: 240ºC, 30 m x 320 mm) was used. The column temperature ramped from 40ºC to 80ºC and ECD's temperature was 350ºC; a 5% mixture of Ar, with CH_4 was used as the carrier with N_2 as make up (15 mL min^{-1}). A headspace

autosampler (Teledyne Tekmar HT3) was connected to the gas chromatograph. Standards were stored and analysed at the same time than field samples. The cumulative gas production during the experiment was estimated by averaging the fluxes of two successive determinations, multiplying that average flux by the length of the period between the measurements, and adding that amount to the previous cumulative total.

4.3.4.1 Nitrous oxide production

The production of N_2O was determined 15 days after TSS application, and then again, 15 days after the first mineral application. Three soil cores per experimental plot (2.5 cm diameter×30 cm depth) were incubated in tightly closed one-litre glass bottle. The bottles were then incubated at ambient temperature of the soil, in a hole dug adjacent to the experimental plots. Samples from the air headspace were taken at the beginning of the incubation and after 24 h and analyzed by gas chromatography, checking that the accumulation of N_2O was linear during this time.

4.3.4.2 Potential of denitrification.

Denitrification potential was measured as the denitrifying enzyme activity (Phase 1) described by Smith and Tiedje (1979), following the method described by Tiedje *et al.* (1989). 25 g of fresh soil and 25 mL of a solution containing glucose 1 M, KNO_3 1 mM and 1 gL^{-1} chloramphenicol were put into 125 mL flasks. The flasks were

sealed with a rubber stopper and repeatedly flushed with N_2 for 10 min to create anaerobic conditions. The flasks were divided in two groups (with or without addition of acetylene 5%) to determine denitrification potential up to N_2O or N_2O+N_2 by difference (Estavillo *et al.*, 2002).

The flasks were incubated in an orbital shaker at 20º C and 1mL of the headspace air was sampled for N_2O determination at 1 and 3 hours incubation time, with the increase in N_2O concentration checked for linearity. N_2O was analyzed by gas chromatography.

4.3.5 Soil bacterial community survey

In order to survey the bacterial communities, soil samples for DNA extraction were collected (i)15 days after TSS application on 2011, and (ii) after harvest on 2012, from the 0-30 cm layer at three random spots on each experimental plot. Bulked samples were stored at 4°C and sieved through 2 mm, before DNA extraction within 24 h from sampling. Soil DNA was extracted from each individual soil sample using the PowerSoil™ DNA Isolation kit (MoBio, Laboratories Inc., CA) as recommended by the manufacturer. Briefly, DNA extraction begins with the chemical lysis of microbial cells with gentle bead-beating; released DNA was purified by filtration through a silica spin filter and recovered with elution buffer. DNA yields and quality were checked after electrophoresis in 0.8% (w/v) agarose gel stained with RedGel under UV light.

Partial prokaryotic 16S rRNA gene sequences were obtained from the analysis of each individual sample using the coded-primer approach to multiplex pyrosequencing (Binladen *et al.*, 2007). PCR amplification of the hypervariable V3-V5 regions of the 16S rRNA gene was performed for each individual soil DNA extraction using universal primers U341F and U926R (Baker *et al.*, 2003) with an 8 bp bar-coded sequence (Calleja-Cervantes *et al.*, 2015). The PCR mixtures (25 µl) contained 25 pmol of each primer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 1 X of the corresponding Taq buffer, 5 µl of Taq Master PCR Enhancer, 1 U of Taq Master (5 Prime, USA) and 10 ng of the DNA template. The PCR program involved an initial denaturation step at 94ºC for 4 min, 25 cycles of denaturation at 94ºC for 15 sec, primer annealing at 55ºC for 45 sec and extension at 72ºC for 1 min, followed by a final step of heating at 72ºC for 10 min. For each sample, amplicons were generated in several replicate PCRs. Amplicons of the same treatment were pooled to reduce per-PCR variability and purified using the Ultracentrifugal Filters Ultracel-100 K membranes from Amicon (Cork, Ireland) according to the instructions of the manufacturer. After quantification with QuantiFluor dsDNA System (Promega), the samples were combined in equimolar amounts and subjected to pyrosequencing with the Genome Sequencer Titanium GS-FLX system (454 Life Sciences, Branford, CT, USA) at LifeSequencing S.L. (Valencia, Spain). Sequences files were submitted to the MG-RAST (http://metagenomics.anl.gov/linkin.cgi?project=12480) and are

available with the accession numbers that range from 4617168.3 to 4617197.3.

For the taxonomic assignment of sequence reads, raw sequences were processed through the Mothur 454 SOP (Schloss *et al.*, 2009) using the average quality scores. Those sequences that met any of the following criteria were left out of the analysis: 1. If the bar-code contained more than two errors; 2. If the forward primer sequences contained more than three errors; 3. If the sequences had 1 or more Ns; 4. If the quality index, according to the .qual file generated during the pyrosequencing process, was less than 20; 5. If the sequences were identified as chimeras using chimera.uchime as the detection method and silva.bacteria as reference; and 6. If one sequence appeared once in a unique sample (virtual OTU) using split.abund command with cutoff=1. Eligible sequences were clustered into Operational Taxonomic Units (OTUs), based on a distance of 3%, by complete linkage clustering. The phyla were assigned using an 80 % confidence threshold (Wang *et al.*, 2007). Sequences that could not be classified to a phylum at this level of confidence were excluded from subsequent phylum composition analyses. The resulting clusters were utilized to calculate the Shannon, Shannoneven and Invsimpson indices, Chao 1 estimator and rarefaction curves at the level of 3% dissimilarity, being approximate to species level.

4.3.6 Plant and Soil $\delta^{15}N$ isotope analysis

At tillering and stem elongation, in each experimental plot, three different spots with a surface of 0.5 m^2 were chosen for stem sampling. The same surface per plot was sampled at harvest. The sampled material was bulked to obtain one composed sample for each experimental plot. The grain was detached from the straw to determine grain yield and it was adjusted to a 12 % moisture content. After harvest, stem, straw and grains were dried and grounded to await for further analysis. The $\delta^{15}N$ isotope was determined in grounded samples of stem, straw, grain and soil samples (1g dry weight) that were sieved to pass through a 2 mm mesh. The samples were analysed via a mass spectrometer (Delta Plus, Thermoquest, Finnigan) coupled to an NC 2500 elemental analyser (CE Instruments, Milan).

4.3.7 Statistical Analysis

The data presented in this work are the mean values of, at least, four independent samples per treatment. Each independent sample was composed of a bulked sampled that contained a different amount of sub-samples depending on the analysis, as stated above. In the case of statistical analyses of TSS, one-way ANOVA tests were used. To compare differences between treatments, Duncan test were performed with a significance level $P > 0.05$ or $P > 0.01$ using the SPSS software, version 21.0 (IBM Corp., Released 2012). Correlations were analysed among variables where appropriate, and the Pearson value was also calculated.

Comparison of bacterial communities was made using the cluster file format conversion and the corresponding matrix was obtained in table form. The distance matrix was completed with the Bray-Curtis index in the Ginkgo software (De Cáceres *et al.*, 2007). The software was used to produce an illustration of the clustering using the unweighted pair group method with arithmetic mean (UPGMA). Finally, a hierarchical clustering was obtained that took into account the abundance of each OTU. Moreover, the five different libraries generated from each soil treatment were pair-wise compared with the STAMP software (Parks *et al.*, 2014) using the parameters recommended by the developers (the statistic test was the two-sided type Fisher's exact test, with a confidence interval of 95% evaluated using the differential Newcombe-Wilson proportions and the FDR multiple test correction of Storey).

4.4 RESULTS

As TSS are unique in their composition; the evaluation of the chemical, environmental and biological performance of a continued application of TSS is relevant. The results of this study report on those performance aspects corresponding to the 20th year of treatment application.

4.4.1 Effects on soil chemical properties.

Soil chemical characteristics after 20 years of TSS application are shown on Table 1, the nutrient content in soil was increased by TSS application. Total C and O.M. recorded an increase of 14% and 41% respectively with the 80 t ha^{-1} y^{-1} treatment. No significant changes were observed in total C by the other two doses of TSS. The every-2-year application recorded a significant increase following that of the of 80 t dose. TSS application resulted in higher levels of total N and P_2O_5 compared to the control. The most significant changes were recorded in the 80 t ha^{-1} y^{-1} treatment where total N had 20% more and doubled the amount of P compared to the control. Extractable K (K_2O) content remained unaffected by treatment application.

4.4.1.1 Status of different forms of N in soil (ammonium, nitrate).

The total N-mineral content (kg-N ha^{-1}) in soil and the percentage of different N forms in soil, such as NH_4^+ and NO_3^- differed among treatments (Fig 2). At the beginning of the experiment right after TSS application the nitrate content in soil

followed the order Control<Mineral<40 t 3y <40 t y< 80, that is, 15.7 kg kg NO_3^--N ha^{-1}, 18.9 kg NO_3^--N ha^{-1}, 31.9 kg NO_3^--N ha^{-1}, 36.3 kg NO_3^--N ha^{-1}, and 50.3 kg NO_3^--N ha^{-1} respectively. At the end of the experiment nitrate content in all treatments had decrease significantly compared to the beginning. The control and both 40 t treatments had similar amounts, ranging from 1.56 kg NO_3^--N ha^{-1} to 1.79 kg NO_3^--N ha^{-1}; the NPK was the lowest with 0.85 kg NO_3^--N ha^{-1}; and the 80 t treatment had the highest with 4.54 kg NO_3^--N ha^{-1}. For control and mineral treatments, the highest nitrate content was found around November and decreased thereafter. In contrast, nitrate content in TSS treatments was kept over 20 kg NO_3^--N $ha^{-1}$for a period of almost two months. Resulting the 80 t treatment with the highest observed content 92.1 kg NO_3^--N ha^{-1} that doubled the content in the other two TSS.

Ammonium contents in soil at the beginning compared to the end varied less. At the beginning, TSS and mineral application stimulated ammonium in soil, then this effect was rapidly lost in time. At the beginning the highest contents were observed in 40 t and 80 t, with 16.74 kg NH_4^+-N ha^{-1} and 9.25 kg NH_4^+-N ha^{-1} respectively; at the same time, ammonium in the other treatments was significantly lower ranging from 2.07 kg NH_4^+-N ha^{-1} (control) to 3.76 kg NH_4^+-N ha^{-1} (NPK). An important variation in nitrate and ammonium amounts was observed right after the application of the mineral treatment.

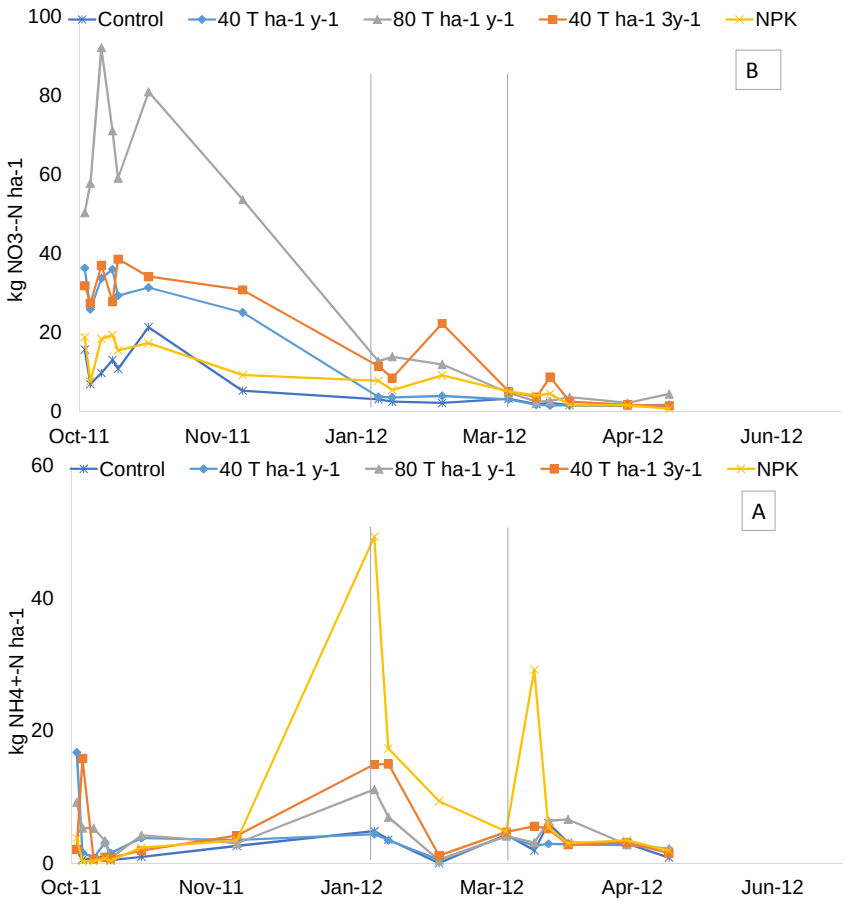


Figure 2. Soil ammonium content (A) and soil nitrate content (B) in the studied treatments: treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} $3y^{-1}$, control and mineral. Vertical lines show fertilizer application.

4.4.1.2 Soil enzyme activities assay

Enzyme activities significantly increased in soils that received TSS (Table 3). Urease and protease activities increased in February compared to October. B-glucosidase and Phosphatase decreased their activity in February. This is an indication of a seasonal variation, however the pattern of increase remained consistent between both

dates. That is to say, it was observed that 80 t significantly increased enzymes in the following percentages: urease 30% (Oct) and 42% (Feb), protease only in Feb (96%), β-glucosidase 43% (Oct) and 8% (Feb), phosphatase around 28% in both dates, and FDA hydrolysis 96% compared to the control. The 80 t treatment increased the overall enzyme activity followed by 40 t 3y and then by 40 t.

Table 3. Enzyme activities in soil 15 days after TSS application* (OCT 2011) and 15 days after Mineral application (FEB 2012) at the Arazuri site.

Treatment /Enzyme	*Urease (mg N-NH^{4+} kg dry soil $^{-1}$ hour $^{-1}$)*		*Protease (mg Tyr kg dry soil $^{-1}$ hour $^{-1}$)*		*β-glucosidase (mg ρ-nitrophenol kg dry soil $^{-1}$ hour $^{-1}$)*		*Phosphatase (mg ρ-nitrophenol kg dry soil $^{-1}$ hour $^{-1}$)*		*FDA hydrolysis (mg fluorescein salt kg dry soil $^{-1}$ hour^{-1})*	*Overall Enzyme Activity*	
	2011	2012	2011	2012	2011	2012	2011	2012	2012	2011	2012
Control	23.3 c	25.2 b	18.3 a	34.1 c	301.2 c	136.0 bc	158.4 c	136.3 b	46.8 bc	91.4 b	87.1 bc
40 t ha^{-1} y^{-1}	27.2 b	23.6 b	21.6 a	42.3 bc	392.3 ab	113.7 bc	190.4 a	162.8 b	83.3 bc	112.1 a	91.3 ab
80 t ha^{-1} y^{-1}	31.2 a	35.9 a	20.6 a	67.1 a	431.3 a	147.8 a	199.7 a	177.4 a	90.9 a	120.6 a	121.0 a
40 t ha^{-1} 3y^{-1}	27.8 b	32.8 ab	21.6 a	52.0 bc	414.4 a	137.3 bc	183.9 ab	148.7 b	89.6 ab	113.9 a	102.0 a
Mineral	25.9 bc	30.8 ab	16.2 a	26.5 c	339.8 bc	100.4 c	166.2 bc	135.6 b	30.4 b	95.8 b	79.1 c

Control, *40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha1 3y^{-1}. Different letters within a column indicate Duncan test results between treatments (P<0.05; n=3).

4.4.2 Effects on soil GHG emissions: N_2O, CO_2 and CH_4

Daily N_2O emissions from control treatment ranged between 0.2 and 8.6 g N_2O-N ha^{-1} d^{-1}, while 80 t increased fluxes up to 39.2 g N_2O-N ha^{-1} d^{-1} (Figure 3). The mineral treatment showed emissions close to the control treatment during the whole period, with a maximum rate of 15.5 g N_2O-N ha^{-1} d^{-1}. Treatment application increased cumulative losses (Table 4) of N_2O compared to the control. The mineral fertilizer doubled the control losses, while the different TSS increased them between 3 and 6 times, being that 80 t showed the highest cumulative loss (3.5 kg N_2O-N ha^{-1}). The doses 40 t and 40 t 3y resulted to have slightly lower cumulative losses. The calculated emission factors for all the treatments ranged from 0.25% to 0.46%.

Daily CO_2 fluxes from the control treatment ranged between 1.7 and 62.7 kg CO_2-C ha^{-1} d^{-1}, in the same range as for the mineral fluxes. The maximum rate of 128.2 kg CO_2-C ha^{-1} d^{-1} was observed in the mineral and 40 t y around March. TSS application increased daily fluxes compared to the control, and a concomitant effect was observed TSS application increased cumulative losses (Table 4)

Daily CH_4 fluxes did not differ between treatments. Most of the time the soil acted as a CH_4 sink, with fluxes ranging between -9.3 and 22.5 g CH_4-C ha^{-1} d^{-1} (Figure 3). Nevertheless, when soil acted as a source (for various periods) for CH_4, fluxes increased in all treatments. As consequence of this, at the end of the assayed period

only the control resulted in a net CH_4 uptake (Table 4). The rest of the treatments presented net CH_4 increase and differences between them were not statistically significant.In terms of Global Warming Potential (GWP) expressed as CO_2 equivalents the treatments with TSS presented a higher GWP compared to the mineral and control treatments.

Table 4. Cumulative nitrous oxide, carbon dioxide and methane emissions during 261 days at the Arazuri site (2011-2012)

Treatment	kg N_2O-N ha^{-1}	EF(%)	kg CO_2-C ha^{-1}	g CH_4-C ha^{-1}	T CO_2eq ha^{-1}
Control	0.58 d	-	7172 c	-147 b	26.8 c
40 t ha^{-1} y^{-1}	2.41 b	0.46	9629 ab	50 a	39.7 a
80 t ha^{-1} y^{-1}	3.49 a	0.36	10512 a	96 a	41.8 a
40 t ha^{-1} $3y^{-1}$	1.92 b	0.25	9315 ab	162 a	36.0 ab
Mineral	0.91 c	0.27	8212 bc	16 ab	31.0 bc

Different letters within a column indicate Duncan test results between treatments ($P<0.1$; n=4).

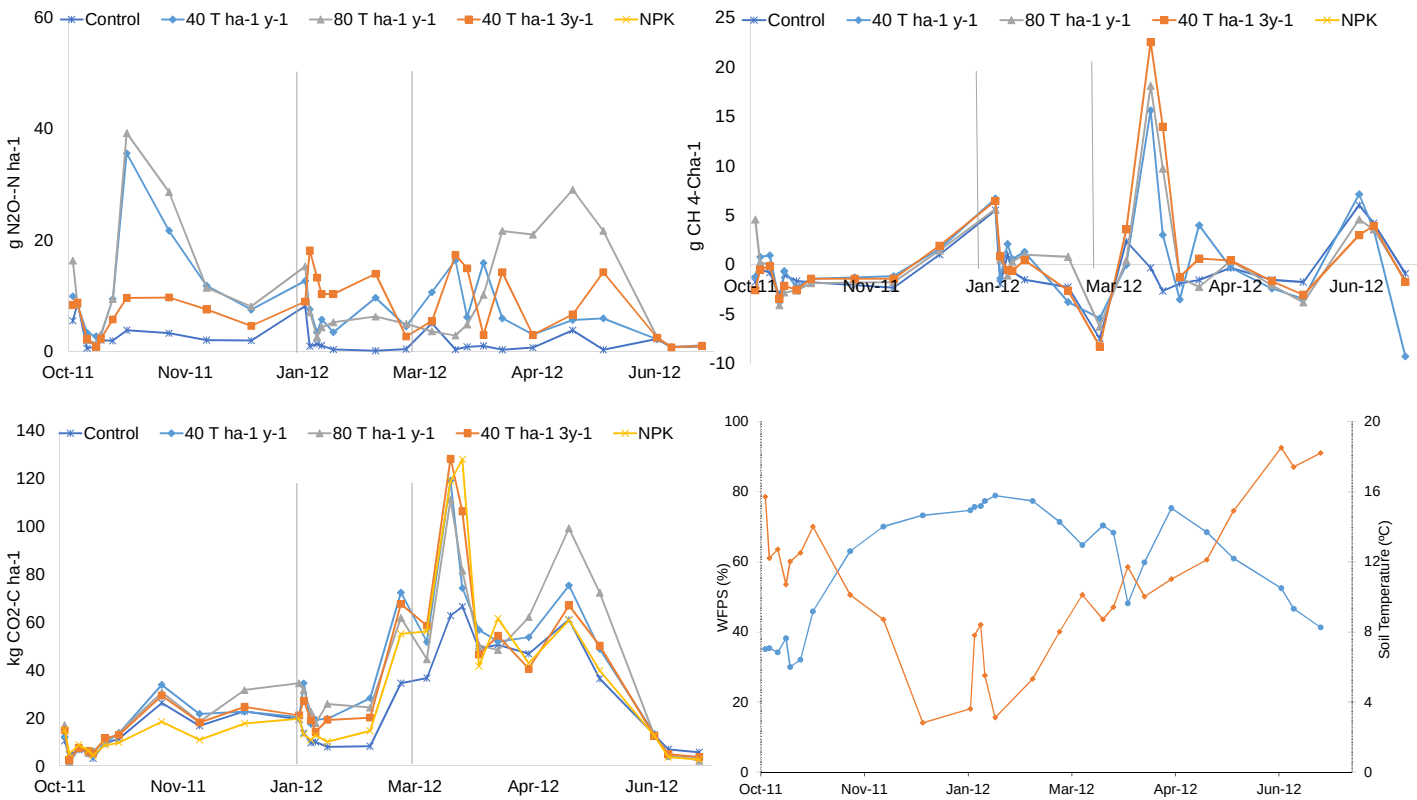


Figure 3. N_2O (A), CO_2 (B), CH_4 (C) emission rates and WFPS [p1] and soil temperature (D) at 20 cm depth in the studied treatments: treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} $3y^{-1}$, control and mineral. Vertical lines show fertilizer application.

4.4.2.1 Production and emission of N_2O after treatment application

Table 5 shows the production and emission of N_2O fifteen days after organic amendments application at sowing; and then 15 days after the first mineral application at tillering. In October production rates ranged between 2.01 and 3.59 g N_2O-N ha^{-1} d^{-1} while emissions ranged between 2.07 and 3.16 g N_2O-N ha^{-1} d^{-1}. At that moment, only the 80 t ha^{-1} y^{-1} treatment resulted statistically different from control treatment. In January, production rates increased, ranging between 5.22 and 29.96 g N_2O-N ha^{-1} d^{-1} while the emissions to the atmosphere ranged between 0.39 and 10.35 g N_2O-N ha^{-1} d^{-1}. By that moment, only the 40 t ha^{-1} $3y^{-1}$ treatment was statistically different from the other treatments in terms of N_2O production and N_2O emission.

Table 5. Nitrous oxide production in soil and emission to atmosphere (g N_2O-N ha^{-1} d^{-1}) 15 days after TSS application (OCT 2011) and 15 days after Mineral application (FEB 2012).

	OCT 2011		FEB 2012	
Treatment	**Production**	**Emission**	**Production**	**Emission**
Control	2.01 b	2.07 b	5.22 b	0.39 b
40 t ha^{-1} y^{-1}	2.94 ab	2.87 ab	5.75 b	3.49 b
80 t ha^{-1} y^{-1}	3.59 a	3.16 a	10.19 b	5.31 ab
40 t ha^{-1} $3y^{-1}$	2.94 ab	2.31 ab	29.96 a	10.35 a
Mineral	2.98 ab	2.81 ab	8.35 b	2.30 b

Different letters within a column indicate Duncan test results between treatments ($P<0.1$; n=4). OCT 2011 corresponds to 15 days after TSS application. FEB 2012 corresponds to 15 days after mineral application.

4.4.2.2 Potential of nitrification and denitrification

Nitrification potential is shown in Table 6. The control treatment and mineral showed values of 228 μg N kg^{-1} dry soil d^{-1}. The addition of 40 t ha^{-1} annually or every 3 years increased the nitrification potential to 260 μg N kg^{-1} dry soil d^{-1} in both treatments. The extreme application rate of 80 t ha^{-1} y^{-1} amplified significantly the nitrification potential to 350 μg N kg^{-1} dry soil d^{-1}. There were not significant differences among treatments for denitrification potential up to N_2O. Values ranged from 245 g N kg^{-1} dry soil d^{-1} to 319 μg N kg^{-1} dry soil d^{-1}. The potential to reduce N_2O to N_2 was significantly higher in all treatments, especially in treatments with TSS, which rates range between 1901 μg N kg^{-1} dry soil d^{-1} and 2576 μg N kg^{-1} dry soil d^{-1}. Although, the maximum dose (80 t ha^{-1} y^{-1}) was the only statistically higher compared to control and mineral treatments.

Table 6. Denitrification potential up to N_2O or up to N_2O+N_2 at the Arazuri site (2011-2012)

Treatment	Nitrification Potential (μg N kg^{-1} dry soil d^{-1})	Denitrification Potential (N_2O) (μg N kg^{-1} dry soil d^{-1})	Denitrification Potential (N_2O+N_2) (μg N kg^{-1} dry soil d^{-1})
Control	228 c	319 a	1901 b
40 t ha^{-1} y^{-1}	260 b	245 a	2271 ab
80 t ha^{-1} y^{-1}	350 a	249 a	2576 a
40 t ha^{-1} $3y^{-1}$	263 b	307 a	2263 ab
Mineral	227 c	245 a	1956 b

Different letters within a column indicate Duncan test results between treatments ($P<0.05$; n=4).

4.4.3 Diversity and taxonomic composition of bacterial communities

A total of 282,422 reads were obtained for the 5 treatments and their 3 replicates, with an average of 9,415 reads per replicate. Prokaryotic diversity coverage was around 87% for all the replicates, except in 40 t ha^{-1} $3y^{-1}$ where the coverage was only of 81% (2,111 reads) (Table 7). On the basis of a 3% dissimilarity, the number of OTUs varied from 807 to 1762 with a total of 42,368 OTUs from all the replicates (Table 7). Chao1's index calculated with the MOTHUR software, indicated a richness variation from 1220 up to 2845 OTUs per replicate. Shannon's diversity index yielded values ranging from 5.94 to 6.56, that is to say, the samples were diverse. The difference between the bacterial communities composition was analysed through the hierarchical agglomerative clustering, which considers the presence or absence of OTUs (Jaccard Analysis), yielded that samples are grouped into two main statistically different clusters (Figure 4). In one of the clusters, all the replicates from the 80 t ha^{-1} y^{-1} treatment (from both Oct 2011 and July 2012) are grouped together. In the second cluster, the mineral, control and the 40 t samples are grouped found. Thus, meaning that the differences are from the 80 t ha^{-1} y^{-1} treatment. Although, it should be noted that in the 80 t cluster, two replicates from the control and one from the 40 t ha^{-1} $3y^{-1}$ appear. Similar results were obtained when the hierarchical agglomerative clustering was performed using MOTHUR software (data not shown), taking into account the each OTU abundance (Thetayc Analysis; see also Yue and Clayton (2012)).

Table 7. Trimmed and normalized (at the lowest number of sequences in Oct for the 40 t ha^{-1} $3y^{-1}$ treatment) values of different soil bacterial diversity estimators at the Arazuri site (2011-2012)

		Read N	Sequences	OTUs	Chao 1	Invsimpson	Shannon	Pielou	Coverage
OCT 2011									
Control	Trimmed	11649	**7911**	**1762**	2845	264	6.52	0.87	**89.69%**
R1	Normalized		**2111**	**867**	1925	280	6.20	0.92	**74.37%**
Control	Trimmed	10848	**7812**	**1724**	2792	205	6.42	0.86	**89.59%**
R2	Normalized		**2111**	**823**	1757	192	6.07	0.90	**76.36%**
Control	Trimmed	11148	**4915**	**1384**	1896	280	6.52	0.90	**88.20%**
R3	Normalized		**2111**	**904**	1602	269	6.28	0.92	**75.13%**
40 t ha^{-1} y^{-1}	Trimmed	9143	**6846**	**1422**	2024	202	6.32	0.87	**91.48%**
R1	Normalized		**2111**	**797**	1516	214	6.05	0.91	**77.83%**
40 t ha^{-1} y^{-1}	Trimmed	10944	**8266**	**1584**	2267	207	6.35	0.86	**92.18%**
R2	Normalized		**2111**	**810**	1581	213	6.07	0.91	**77.12%**
40 t ha^{-1} y^{-1}	Trimmed	6095	**4433**	**1105**	1588	201	6.18	0.88	**88.97%**
R3	Normalized		**2111**	**772**	1431	189	6.01	0.90	**79.49%**
80 t ha^{-1} y^{-1}	Trimmed	7739	**3705**	**1047**	1473	216	6.26	0.90	**87.85%**
R1	Normalized		**2111**	**816**	1356	228	6.15	0.92	**79.44%**
80 t ha^{-1} y^{-1}	Trimmed	8817	**3857**	**1152**	1516	310	6.45	0.91	**87.87%**
R2	Normalized		**2111**	**881**	1508	327	6.31	0.93	**76.98%**
80 t ha^{-1} y^{-1}	Trimmed	10840	**5349**	**1221**	1392	202	6.39	0.90	**93.42%**
R3	Normalized		**2111**	**822**	1312	212	6.19	0.92	**79.87%**
40 t ha^{-1} $3y^{-1}$	Trimmed	8531	**6303**	**1368**	1855	207	6.33	0.88	**91.56%**
R1	Normalized		**2111**	**797**	1446	183	6.03	0.90	**78.73%**
40 t ha^{-1} $3y^{-1}$	Trimmed	8996	**7172**	**1532**	2147	256	6.46	0.88	**91.34%**
R2	Normalized		**2111**	**847**	1643	266	6.19	0.92	**76.36%**
40 t ha^{-1} $3y^{-1}$	Trimmed	5071	**2111**	**807**	1220	325	6.23	0.93	**81.00%**
R3	Normalized		**2111**	**807**	1220	325	6.23	0.93	**81.00%**
Mineral	Trimmed	10133	**6975**	**1698**	2792	268	6.54	0.88	**88.24%**
R1	Normalized		**2111**	**888**	1816	286	6.25	0.92	**74.33%**
Mineral	Trimmed	9061	**6110**	**1528**	2545	205	6.35	0.87	**87.32%**
R2	Normalized		**2111**	**831**	1717	207	6.07	0.90	**75.89%**
Mineral	Trimmed	9492	**5933**	**1418**	2309	150	6.20	0.85	**88.17%**
R3	Normalized		**2111**	**800**	1636	143	5.94	0.89	**76.65%**

Control, *40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha1 $3y^{-1}$. OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest. R1 (1^{st} replicate), R2 (2^{nd} replicate), R3 (3^{rd} replicate)

Table 7 continuation. Trimmed and normalized (at the lowest number of sequences in Oct for the 40 t ha^{-1} $3y^{-1}$ treatment) values of different soil bacterial diversity estimators at the Arazuri site (2011-2012)

		Read N	Sequences	OTUs	Chao 1	Invsimpson	Shannon	Pielou	Coverage
JUL 2012									
Control	Trimmed	9511	**6361**	**1528**	2514	219	6.37	0.87	**88.21%**
R1	Normalized		**2111**	**838**	1783	212	6.10	0.91	**75.60%**
Control	Trimmed	11148	**7291**	**1694**	2577	279	6.52	0.88	**89.29%**
R2	Normalized		**2111**	**895**	2220	261	6.20	0.91	**72.38%**
Control	Trimmed	12133	**5258**	**1425**	1995	296	6.56	0.90	**88.65%**
R3	Normalized		**2111**	**901**	1628	302	6.30	0.93	**75.27%**
40 t ha^{-1} y^{-1}	Trimmed	9860	**7455**	**1480**	2031	218	6.37	0.87	**92.22%**
R1	Normalized		**2111**	**796**	1417	216	6.09	0.91	**78.92%**
40 t ha^{-1} y^{-1}	Trimmed	7465	**5596**	**1300**	1933	237	6.33	0.88	**89.74%**
R2	Normalized		**2111**	**799**	1550	264	6.13	0.92	**78.49%**
40 t ha^{-1} y^{-1}	Trimmed	8881	**6887**	**1478**	2194	234	6.41	0.88	**90.90%**
R3	Normalized		**2111**	**807**	1529	225	6.12	0.91	**78.30%**
80 t ha^{-1} y^{-1}	Trimmed	10180	**4423**	**1304**	1781	287	6.52	0.91	**87.63%**
R1	Normalized		**2111**	**936**	1782	273	6.33	0.93	**73.61%**
80 t ha^{-1} y^{-1}	Trimmed	7419	**3719**	**1019**	1186	347	6.40	0.92	**91.42%**
R2	Normalized		**2111**	**800**	1150	361	6.27	0.94	**82.38%**
80 t ha^{-1} y^{-1}	Trimmed	9884	**4615**	**1234**	1702	195	6.33	0.89	**88.80%**
R3	Normalized		**2111**	**851**	1560	195	6.14	0.91	**76.79%**
40 t ha^{-1} $3y^{-1}$	Trimmed	10638	**8185**	**1641**	2276	233	6.46	0.87	**92.12%**
R1	Normalized		**2111**	**836**	1639	230	6.13	0.91	**76.65%**
40 t ha^{-1} $3y^{-1}$	Trimmed	8584	**6518**	**1413**	1929	232	6.38	0.88	**91.27%**
R2	Normalized		**2111**	**806**	1598	226	6.09	0.91	**77.83%**
40 t ha^{-1} $3y^{-1}$	Trimmed	8325	**3810**	**1191**	1569	328	6.51	0.92	**87.11%**
R3	Normalized		**2111**	**897**	1427	336	6.35	0.93	**77.31%**
Mineral	Trimmed	9912	**6537**	**1658**	2569	243	6.50	0.88	**87.91%**
R1	Normalized		**2111**	**875**	1835	244	6.19	0.91	**74.47%**
Mineral	Trimmed	10835	**7267**	**1690**	2585	225	6.45	0.87	**89.10%**
R2	Normalized		**2111**	**873**	1976	202	6.11	0.90	**73.28%**
Mineral	Trimmed	9160	**6214**	**1561**	2425	220	6.44	0.88	**88.16%**
R3	Normalized		**2111**	**872**	1782	224	6.16	0.91	**74.66%**

Control, *40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha1 $3y^{-1}$. OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest. R1 (1^{st} replicate), R2 (2^{nd} replicate), R3 (3^{rd} replicate)

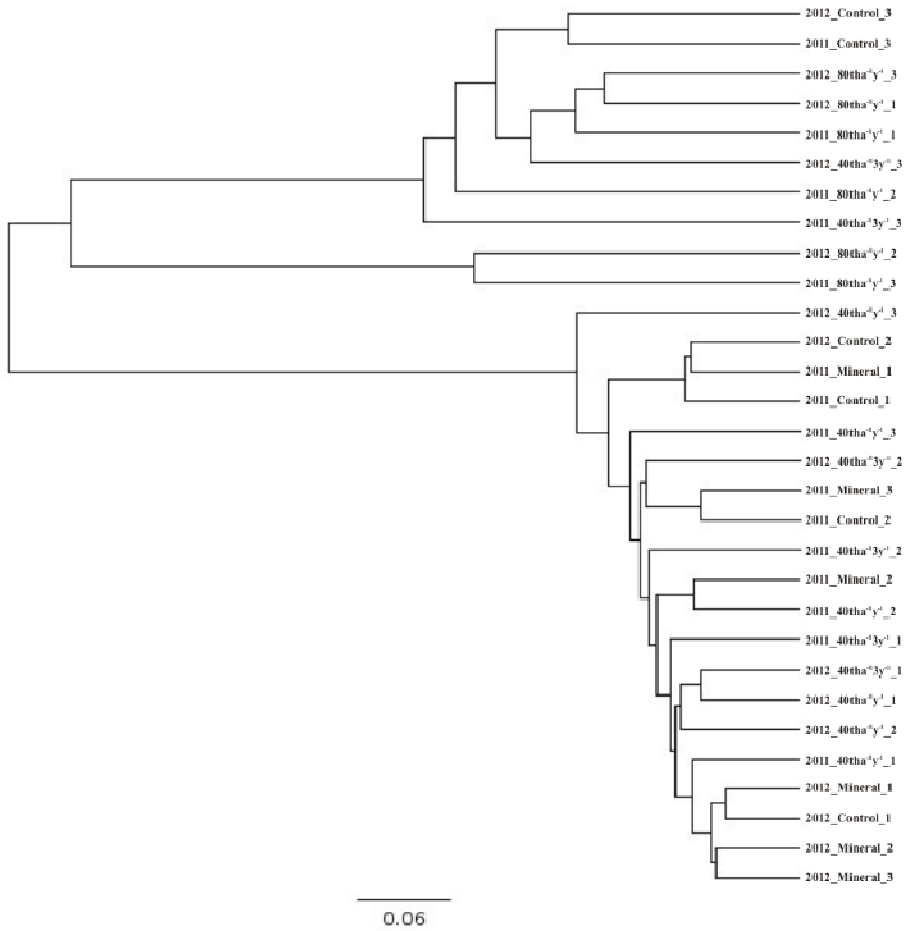


Figure 4. Cladogram of the different soil bacterial communities based on Jaccard distance (3% dissimilarity). Treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} $3y^{-1}$, control and mineral. OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest. R1 (1st replicate), R2 (2nd replicate), R3 (3rd replicate)

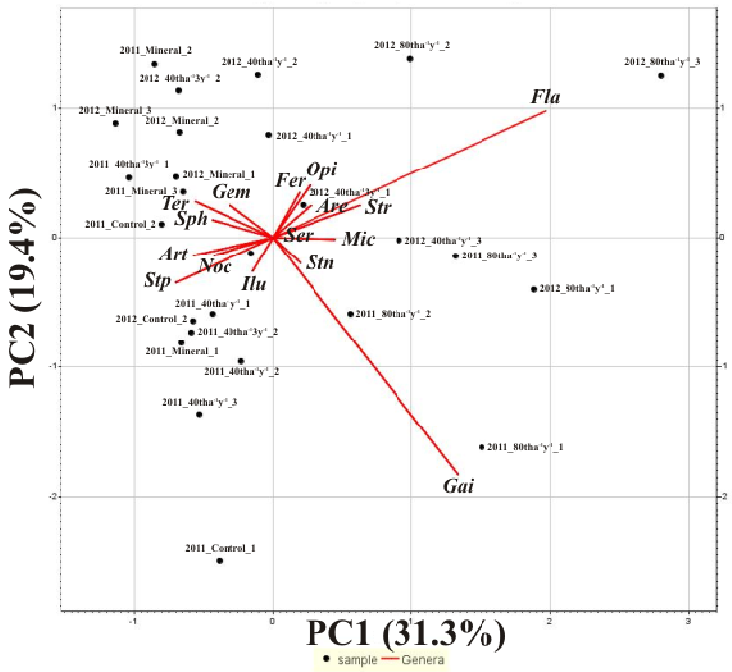


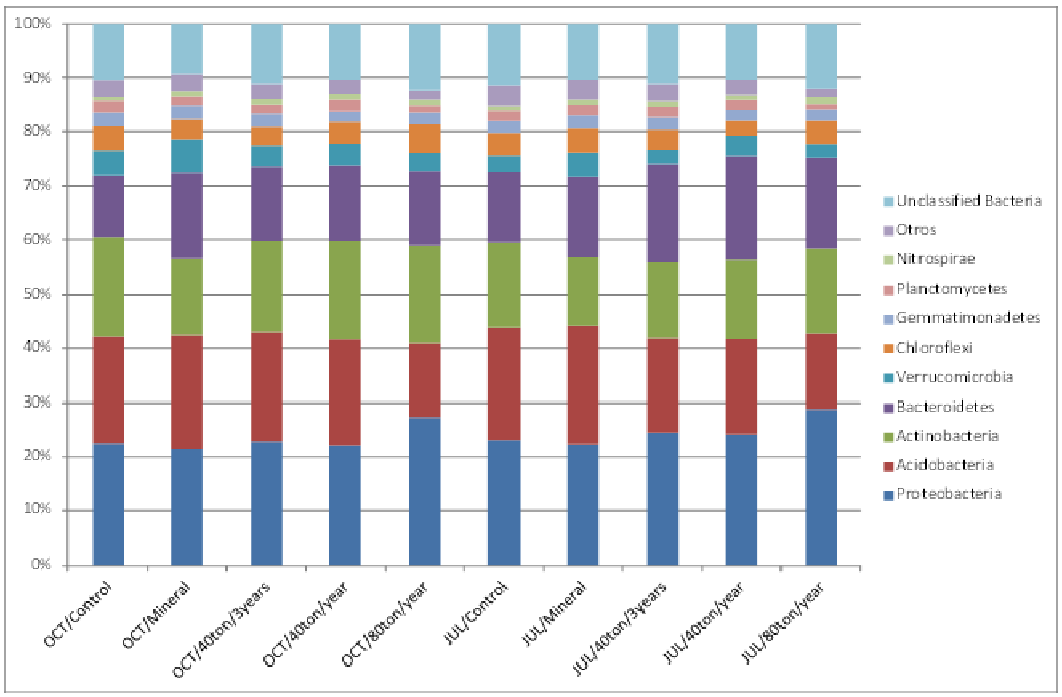
Figure 5. Plot representing the eigenvectors for the (PCA) of the significant genera. Treated sewage sludge (TSS), 40 t ha-1 y-1, 80 t ha-1 y-1, 40 t ha1 3y-1, control and mineral. OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest. R1 (1st replicate), R2 (2nd replicate), R3 (3rd replicate). Fla (Flavobacterium), Str (Steroidobacter), Opi (Opitutus), Fer (Ferruginibacter), Are (Arenimonas), Mic (Microlunatus), Ser (Serpens), Stn (Stenotrophomonas), Ilu (Ilumatobacter), Gem (Gemmatimonas), Ter (Terrimonas), Sph (Sphingomonas), Art (Arthrobacter), Noc (Nocardiodes), Stp(Streptomyces), Gai (Gaiella).

The same results were observed when PCA was performed with the STAMP software (Parks and Beiko, 2010) and shown in Figure 5, using the total sequences, and the normalized number (to the lowest amount found) of each replicate.

Phyla distribution of microbial communities is shown in Figure 6 and Table 8. 80% of the sequences found corresponded to 6 main phyla: (from highest to lowest) Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia and Chloroflexi, with unclassified genetic material making up an average of 11% of the sequences. Phylum Protebacteria was increased by 80 t treatment, from an observed abundance of 22.58% in the control to a 27.33% in October, to an abundance of 28.84% at harvest. On the contrary, in the phylum Acidobacteria the opposite trend was found. While the control treatment registered the abundance of Acidobacteria as 19.95%, it decreased to 13.67% in October and stayed at 13.93% at harvest in the 80 t treatment. No other significant differences were observed among treatments. However, an increase in the phylum Bacteroidetes was common to all treatments in both sampled dates, but these differences were not statistically significant. The mineral treatment resulted in an increase of the phylum Verrucomicrobia compared to the other treatments and the control without being statistically significant.

At a lower taxonomic level, only about 25% of the sequences could be allocated at genus level. The most notable difference is

that, apparently, the 80 t ha^{-1} y^{-1} treatment suppresses the genera: *Streptomyces*, *Arthrobacter* and *Microbacterium* (phylum Actinobacteria), *Rhizobium*, *Haliangium*, *Microvirga* and



Bradyrhizobium (phylum Proteobacteria) and *Bacillus* (phylum Firmicutes) from the bacterial community (Figure 5). In the rest of the samples, the mentioned genera had a presence ranging from 0.14% to 0.59%. There were 38 more genera with a lower presence that disappear in the 80 t treatment.

Figure 6. Percentage distribution of the detected phyla between the different treatments in soil. Treated sewage sludge (TSS), 40 t ha-1 y-1, 80 t ha-1 y-1, 40 t ha1 3y-1, control and mineral. OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest.

Table 8. Distribution of phylum between the bacterial communities at the Arazuri site (2011-2012).

	Proteobacteria	Acidobacteria	Actinobacteria	Bacteroidetes	Verrucomicrobia	Chloroflexi	Gemmatimonadetes	Planctomycetes	Nitrospirae	Otros	Unclassified Bacteria
OCT 2011											
Control	22.58	19.59	18.32	11.53	4.64	4.24	2.59	2.12	0.72	3.20	10.49
40 t ha^{-1} y^{-1}	22.20	19.48	18.02	14.25	4.03	3.78	1.93	2.17	1.08	2.70	10.37
80 t ha^{-1} y^{-1}	27.33	13.67	17.90	13.90	3.35	5.07	2.30	1.22	1.15	1.69	12.42
40 t ha^{-1} 3y^{-1}	22.84	20.27	16.59	14.02	3.87	3.27	2.40	1.68	1.07	2.89	11.11
Mineral	21.38	21.13	14.00	16.00	6.16	3.64	2.40	1.78	0.95	3.24	9.32
JUL 2012											
Control	23.14	20.81	15.54	13.09	3.18	3.94	2.23	1.91	0.81	3.93	11.42
40 t ha^{-1} y^{-1}	24.15	17.57	14.60	19.39	3.41	2.83	1.99	1.88	0.99	2.82	10.38
80 t ha^{-1} y^{-1}	28.84	13.93	15.57	16.98	2.58	4.05	2.15	0.95	1.24	1.62	12.09
40 t ha^{-1} 3y^{-1}	24.48	17.43	13.95	18.37	2.54	3.46	2.36	1.93	1.06	3.29	11.13
Mineral	22.39	21.86	12.46	15.03	4.49	4.32	2.44	1.87	1.00	3.75	10.40

Control, *40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha1 3y^{-1}. OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest.

The observed differences in the phylum Proteobacteria in the 80 t treatment could be due to an increase in genera such as *Arenimonas, Hyphomicrobium, Naxibacter, Serpens, Stenotrophomonas o Steroidobacter* with a significant increase ($p < 0.05$) of 1% of the total sequences up to 1.46% (data not shown).In the 80 t treatment a significant increase ($p < 0.05$) in *Flavobacterium,* phylum Bacteroidetes (1.26% on average in all treatments to 2.38%) and *Gaiella*, phylum Actinobacteria (from 3.62% to 4.44%) was seen (Figure 5).

4.4.3 Plant and soil effects on $\delta^{15}N$ enrichment. (TSS, soil, stem, straw and grain).

The total $\delta^{15}N$ enrichment was significantly influenced by treatment application (Figure 7). TSS and mineral application affected the total $\delta^{15}N$ in soil and the grain enrichment. $\delta^{15}N$ enrichment in soil was similar for all the treatments. However, it was lost during the evolution of crop, while only the 80 t and 40 t persisted through the crop's stages. The 40 t 3y treatment affected differently the straw and grain's enrichment.

The exaggerated applied doses of TSS in this study are not a common practice. For instance, in the case of oats, the excessive amount of nitrogen applied to soil lead to the lodging of the crop, causing an irreversible decay in the yield. It is acknowledge that the reported grain yield, corresponds to the harvested yield. The unfertilized treatment showed a grain production of 3,288 kg ha^{-1}

(Table 9). TSS application improved significantly the grain yield compared to the control in a range between 4,600-4,800 kg ha^{-1} when 40 t were applied. Nevertheless, the dose of 80 t ha^{-1} y^{-1} decreased the yield to 3,701 kg ha^{-1} which did not differ from the control. The highest yield production was observed in the urea treatment with a value of 6,057 kg ha^{-1}. This latter performance is in the average yield of 5,451 kg ha^{-1} expected in alike climatic conditions of Spain (Genvce, 2010).

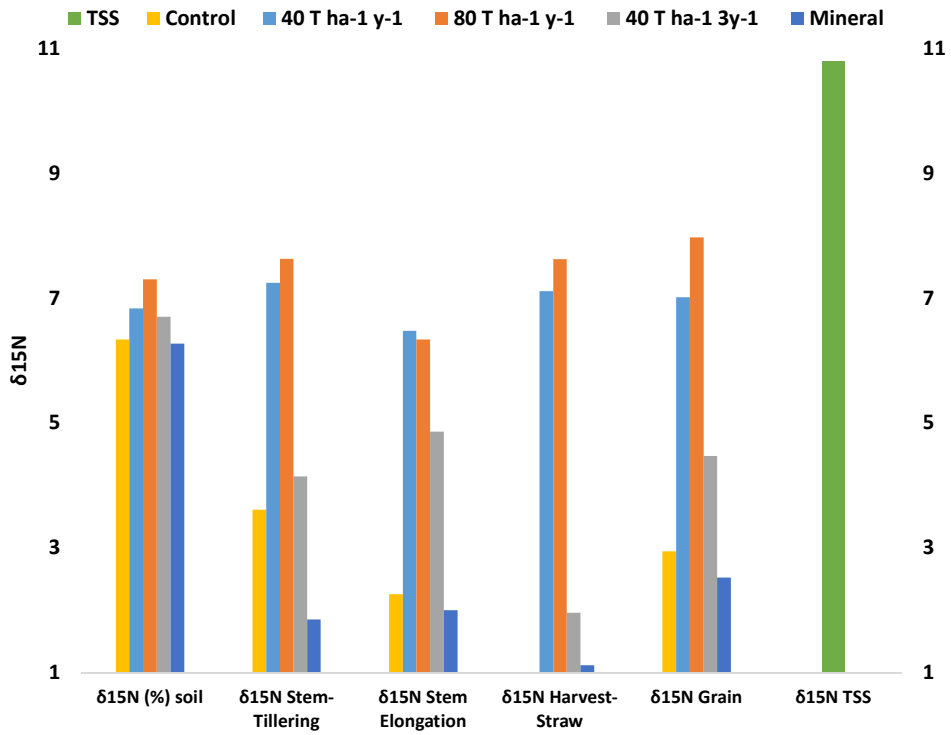


Figure 7. $\delta^{15}N$ enrichment on soil in the studied treatments: treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} $3y^{-1}$, control and mineral; stem (at tillering and stem elongation) and straw at harvest, grain and TSS.

Table 9. Yield production of oats at the Arazuri site (2011-2012)

Treatment	Yield (kg ha^{-1})	GHGI (kg CO_2eq ha^{-1}/ Kg yield ha^{-1})	Grain N yield scaled Emissions (g N_2O-N ha^{-1}/kg N ha^{-1})
Control	3288 c	7.1 b	8.6 c
40 t ha^{-1} y^{-1}	4856 b	6.8 b	24.3 b
80 t ha^{-1} y^{-1}	3701 c	9.6 a	46.1 a
40 t ha^{-1} $3y^{-1}$	4604 b	6.7 b	20.3 b
Mineral	6057 a	4.4 c	7.3 c

Different letters within a column indicate Duncan test results between treatments ($P<0.05$; n=4).
TSS caused plant lodging, hence the decrease in yields.

4.5 DISCUSSION

The studied doses were designed to evaluate the influence of an extremely high application intensity over different soil parameters. This exaggeration, by increasing the quantity of applied material, reduces an important amount of residues in a one-off effort.

An appropriate agricultural application of TSS as a soil amendment could prevent an unsafe dispose of this material as it induces desirable agronomic effects in soil (Singh and Agrawal, 2008). In the case of this study, nutrient availability in soil was increased due to TSS application and the increase is closely related to the dose and frequency of application. Similar nutrient improvements have already been reported by Parat *et al.* (2005) in an evaluation of a similar dose. The changes in the 40 ton 3y treatment represent a novel result. It is hypothesized that the supplemented mineral fertilization on years that TSS was not applied have triggered an increased metabolism in soil, coupled to an activation of root metabolism that induce microbial activity (Garcia-Gil *et al.*, 2004).

The metabolic activity of soils, measured by active enzymes in the soil is known to be a potential indicator of the effects of a soil management in the soil fertility. Some studies have demonstrated that TSS consistently increases microbial activity (Antolin *et al.*, 2005; Fernandez *et al.*, 2009). In the same way we also observed an

increase of enzyme activities, especially when 80t per hectare were applied. However, some other studies argue that heavy metal presence in TSS reduces and inhibits enzyme expression. Paz-Ferreiro *et al.* (2012) reported that an increase in the amount of SS, decreases OEA. Moreover, other studies conclude that fertilization management does not affect enzymes Marschner *et al.* (2003). None of these assertions have been confirmed by this study. It is important to note that regardless of the annual amount applied of TSS, enzyme activities were not specially increased between the plots that received TSS. The difference is found based on frequency and mineral application. In the case of Chu *et al.* (2007) they reported that balanced fertilization increases the metabolic activity of soils.

In the case of urease activity, the increase must be due to the presence of high amounts of enzymatic substrates in the sewage sludge (Garcia *et al.*, 1993). Urease requires the presence of urea and Ni which is usually present in sewage sludge (Antonious, 2009), so it is not a limiting factor. As expected the higher inputs of phosphate with the organic amendments induced phosphatase activity, possibly due to the action of the roots as reported by Johansson *et al.* (1999). However, further studies have to be conducted to confirm the relation between plant root excretion of this enzyme and microbial action. Protease and FDA hydrolysis establish a noticeable dominance over the other assayed enzymes in the TSS treatments. This may have been because C and N were

increased by TSS application, given by the provision of organic matter. Also, a repeated exogenous application of soil enzymes could also enrich the soil matrix with enzymes.

Even so, the contradictory reported results in literature on TSS application may be due to other reasons such as treatment processes, incorporation methods, and crop interactions with soil and differentiated climatic conditions.

Gaseous emissions

Bøckman and Olfs (1998) have described that the addition of N in a mineral form or from an organic source such as sewage sludge (in which ammonium is released by mineralisation) to agricultural soils are recognised as major drivers of N_2O emissions. N_2O emissions were increased by treatment application in our study. As expected the highest application dose of 80 t ha^{-1} y^{-1} induced the highest losses. In spite of the application rate, maximum losses did not exceed the maximum fluxes of 48 g N_2O-N ha^{-1} d^{-1} described by Huérfano *et al.* (2015) from a winter crop fertilized with mineral fertilizer under humid Mediterranean conditions similar to the present study. In a temperate humid marine climate N_2O fluxes from an oats crop fertilized with mineral N did not exceed 20 g N_2O-N ha^{-1} d^{-1} (Beheydt *et al.*, 2008). The increase induced by the application of sewage sludge was much lower than that described by Scott *et al.* (2000) after sewage sludge application in grassland. The significant input of exogenous C to the soil should have favoured denitrification

process (Bowman and Focht, 1974; Weier *et al.*, 1993). Nevertheless the denitrification potential to N_2O was not increased by the addition sewage sludge, although it enlarged the denitrification potential up to N_2 (Table 6). This would explain why observed losses were lower than the described in the literature as previously discussed. In fact, the N_2O production was lower than the N_2O emissions (Table 5) suggesting that N_2O was reduced to N_2 while diffused to the atmosphere. However, the N_2O production rates in our experiment are of the same magnitude that those described by Zaman *et al.* (2004).

Carbon dioxide (CO_2) emissions were regulated by soil water content which is known to modulate O_2 availability in soil. It has been widely described that CO_2 emissions increase when WFPS is between 55%-60% and decrease when WFPS is higher (Davidson *et al.*, 1998; Huérfano *et al.*, 2015). Nevertheless, we observed a positive correlation between CO_2 fluxes and soil moisture (r^2=0.841; p<0.000) when WFPS was lower than 70% and a negative correlation (r^2=-0.809; p<0.005) when the WFPS was higher than 70%. Although it could be expected that global warming enhanced CO_2 emissions (Kirschbaum, 2000); Xu and Qi (2001) have described a negative correlation between soil temperature and soil respiration. In our study we observed a negative correlation (r^2=-0.501; p<0.20) when the soil moisture was under 70%.

The incorporation of TSS to soil increased significantly CO_2 cumulative losses. The unfertilized control showed similar values to those described by Huérfano *et al.* (2015) from an unfertilized wheat crop. However, these values are higher than the 40 kg CO_2-C ha^{-1} d^{-1} described by Bortolotto et al. (2015) from an oats crop. Scott *et al.* (2000) observed daily CO_2 fluxes of 84 kg CO_2-C ha^{-1} d^{-1} after sewage sludge application in the UK. However, under Mediterranean conditions Quemada and Menacho (2001) describe losses up to 384 kg CO_2-C ha^{-1} d^{-1} 1 year after the application of 80 t ha^{-1} y^{-1} of TSS. The continuous application of TSS during 20 years resulted in daily fluxes that did not exceed 128 kg CO_2-C ha^{-1} d^{-1} (Figure 3). This reduction of CO_2 fluxes could be explained by the fact that a single addition of C induced more priming effect than repeated and continuous inputs, and therefore higher CO_2 release (Qiao *et al.*, 2014).

Generally, soils act as CH_4 sinks, except soils with a very high water table that are sources of CH_4 emission. In the present study, both mineral fertilization and organic fertilization increased CH_4 emissions, resulting in a net source of CH_4. The high NH_4^+ content in soil as a result of the fertilization could inhibit CH_4 oxidation probably due to competitive inhibition of the methane monooxyegnase by ammonium (Conrad, 1996). The anaerobic decomposition of soil organic matter C input with TSS provided the C substrates for methanogens controlling methanogenic activity (Segers, 1998). Evidence in this study is that the maximum CH_4 fluxes

took place in spring corresponding with the maximum CO_2 fluxes (Figure 3) when soil water content and temperature favoured the organic matter decomposition providing C substrates for methanogenesis. Maximum CH_4 daily fluxes were two times higher than the fluxes reported by Ambus *et al.* (2001) after sewage sludge application. Nonetheless, the cumulative CH_4 losses after 11 months (76 g CH_4-C ha^{-1}) presented by these authors had the same magnitude than the cumulative losses in the present study (Table 4).

TSS application clearly increases $\delta^{15}N$ compared to mineral fertilization (Hogberg and Johannisson, 1993; Hogberg *et al.*, 2014) argued that N processes, like the ones involved in decomposition of organic matter, discriminates against ^{14}N, thus enriching ^{15}N. Also, ammonia volatilization and other processes related to the treatment of organic wastes are closely related to this enrichment. The mechanisms and processes that drive the links of further plant/grain enrichment are not yet described as physiological routes, but by the reported results a clear enrichment occurs during the treatment of SS, and the enrichment is definitely transferred into the soil and the plant/grain.

The addition of urea increased significantly grain yield (Table 9). It is well known that N fertilization is the best way to maximize the production of crops. Sewage sludge are a significant source of organic N that has to be mineralized be used by crops (Antolin *et al.*, 2005). Though, the high organic N inputs with the assayed organic

resulted in excess of mineral N after mineralization causing plant lodging and the decline of grain yield. Fernández *et al.* (2009) also observed a reduction of grain yield after sewage sludge application as consequence of an excessive dose. For this reason, the reduction of grain yield and the high N_2O emissions induced by sewage sludge resulted in a significant increase of the N yield-scaled emissions (Table 6). However, the mineral fertilized did not affect it with respect to the control treatment. According to this, several authors (Venterea *et al.*, 2011; Huérfano *et al.*, 2015) have described the same or even lower N yield-scaled emissions than control treatment when mineral fertilizer is applied.

In terms of Global Warming Potential (GWP) expressed as CO_2 equivalents, the different doses of sewage sludge induced an increment of the CO_2eq losses. If the GWP from each treatment is related to its grain yield and expressed as Green House Gas Intensity (GHGI) (Mosier *et al.*, 2006), the dose of 80t ha^{-1} y^{-1} increased significantly the GHGI while the mineral fertilizer reduced it. This reduction with respect to control treatment was also described by Huérfano *et al.* (2015) after applying mineral fertilizer in humid Mediterranean conditions like us. However, the exaggerated applied doses of TSS in this study are not a common practice and a balanced fertilization has to be adjusted for further studies and in the field recommendations.

This study shows that the microbial diversity of soil (measured with the Shannon and Simpson's inverse indices) and richness (measured with Chao1 index) were not significantly affected by the different amounts of TSS applied to the soil. The high coverage of the analysis, more than 87 %, assures an accurate representation of the soil diversity. However, recent observations demonstrated that biodiversity and its community composition determine the ecosystem functionality (Wagg *et al.*, 2014) and not its richness or diversity. Therefore, we investigated the microbial community structure by in-depth-sequencing of the V4-V5 hypervariable regions of the 16S rRNA gene. The results indicate that after fertilization, and also after the harvest, there was a statistically significant difference between the TSS at the highest dose compared to the rest of treatments. That is to say, the application of TSS at 80 t per year, during 20 years, results in a different structure of the bacterial community if we consider the presence/absence of OTUs, and also it is consider the specific abundance of each OTUs (Figure 4 and 5). This result is in agreement to some reports where the application of manure from farming caused pronounced changes in bacterial community composition (Ge *et al.*, 2010; Ding *et al.*, 2014). However, in these works the difference may be due to the presence of antibiotic sulfadiazine, but this is not our case since in the TSS applied there are not xenobiotics or other contaminants. The observed difference in the structure of the microbial community is due to the dose of the treatment and to its persistence in time, since a similar community is detected previously to sowing and after

harvest. Moreover, it can be concluded that there is not a seasonal effect since the sampling was done in October and July. The observed changes in the community range from the level of phylum to the genus level. The application of the highest dose resulted in a lower abundance of the phylum Acidobacteria, and in an increase of the phylum Proteobacteria, and within this phyla there is an increase of the genera *Arenimonas, Hyphomicrobium, Naxibacter, Serpens, Stenotrophomonas* o *Steroidobacter*. Interestingly, some of these genera have been identified in microbiome's changes correlated with the growth stages of crops (Li *et al.*, 2014), drough resistance (Zolla *et al.*, 2013) and other root-soil interactions that favor disease suppression in arable crops (Yin *et al.*, 2013). Likewise, the genera *Streptomyces, Arthrobacter*, *Microbacterium, Rhizobium, Haliangium, Microvirga, Bradyrhizobium* and *Bacillus* were not detected (Fig 5) after the application of 80 Tm per year, and some of these genera have been described as bacteria which typically contribute to high soil quality (Ding *et al.*, 2014) and to the promotion of plant growth. Therefore, it is confirmed that this dose is excessive for cereal fertilization that could have negative effect over the microbial community. Additionally, the absence of differences between the rest of treatments (TSS application versus control or mineral) could represent an indication of benefits to the crops without alteration of structure of the bacterial communities.

4.6 CONCLUSIONS

The observed increases in nutrients, enzyme activities and GHG emissions are the evidence of an increased metabolic activity after TSS application to soil. These changes are not consistently beneficial if the yield is considered. Further research must be done in order to optimize and adjust doses and frequency.

This work stresses the importance of developing a broader approach for the environmental evaluation of agricultural applications of recycled materials in soil. The role of waste recycling cannot be fully understood if soil fertility is underestimated within the climate change framework. Further research on possible consequences that the joint action of TSS + Mineral fertilization would have on soil properties, and to the environment should be thoroughly examined / monitored. Also, influence of the rotation and root exudates should further revised to determine possible links between nutrient cycling and bacterial community composition.

Final considerations-
Síntesis Final

En las últimas décadas se ha producido un aumento del volumen de los residuos agrícolas, ganaderos, industriales y urbanos que se generan. Dependiendo del manejo que se les aplique estos residuos pueden suponer un importante problema medioambiental o por el contrario ser una fuente importante de nutrientes y materia orgánica para la agricultura. Sin embargo, los aspectos negativos de su reciclaje como fertilizantes son similares a aquellos que tiene la fertilización mineral tanto para la salud como para el medio ambiente. La salud humana se puede ver afectada por el aumento de los nitratos y nitritos en las aguas de consumo. En el aspecto ambiental el uso excesivo de fertilizantes puede provocar un aumento de las emisiones gaseosas nitrogenadas, de dióxido de carbono y metano. Estos gases están implicados en los procesos de calentamiento global (efecto invernadero), destrucción de la capa de ozono, lluvia ácida y eutrofización de ecosistemas. A estos, se añade la posibilidad de encontrarse altos contenidos de metales pesados y otros contaminantes. Si se realiza un uso responsable y racional de los residuos orgánicos, su aplicación conlleva numerosos beneficios.

Los beneficios de la aplicación de materia orgánica son múltiples y en distintas escalas. La degradación del suelo, puede verse mitigada físicamente, por el aporte de materia orgánica, también se ve transformada la matriz biológica del suelo y al mismo tiempo se contribuye a la nutrición vegetal. Otro beneficio consecuencia del aporte de materia orgánica como fertilizante, es que se puede favorecer el secuestro de carbono en el suelo. Este secuestro no resulta nada desdeñable si se considera globalmente el problema tan acentuado que supone la eliminación residuos orgánicos y purines/lodos, el intensivo uso de nutrientes para agricultura y la

problemática medioambiental como consecuencia de las emisiones de CO_2.

Es por esto, que esta tesis tuvo como objetivo general el estudio del uso de residuos orgánicos en suelos agrícolas tras aplicaciones a largo plazo. Se centra en aspectos clave de la fertilización que son relevantes para el medio ambiente. El objetivo concreto ha sido el de describir el efecto de estas aplicaciones sobre la fertilidad del suelo y diversas características químicas del suelo; el metabolismo de los microbios del suelo, a través del estudio del estado de las enzimas tras la aplicación de los residuos; la diversidad bacteriana del suelo y las emisiones de gases de efecto invernadero en cada cultivo y a lo largo de un ciclo productivo

La memoria de esta tesis quedó estructurada en cuatro capítulos centrales que reportan los resultados de los experimentos

Como consecuencia de los distintos manejos de la fertilización, la cantidad de materia orgánica en el suelo aumentó con respeto al testigo. En Bargota, en un 58% aplicando 4.6 ton anuales por hectárea de compost de oveja. En un 41% en Arazuri aplicando 80 toneladas anuales por hectárea de lodo de EDAR. En el caso de Arazuri, todas las dosis aumentaron significativamente el carbono y nitrógeno total en el suelo, el incremento más alto se registró en la dosis de 40 toneladas cada tres años por hectárea con un incremento de 13% y 26% respectivamente.

La actividad metabólica del suelo se ve aumentada por las aplicaciones, reflejándose en la actividad enzimática total, donde los incrementos registrados son de hasta el 45% en Bargota y del 24% en Arazuri con respecto del control.

La dinámica de las comunidades bacterianas del suelo, en el caso de Bargota parece estar influenciada directamente por el tipo de residuo cinco meses tras la fertilización. Aunque esta situación se

revierte si el compost ha sido aplicado recientemente. El estiércol compostado de oveja registró la mayor diversidad entre las comunidades encontradas. En Bargota, la comunidad bacteriana presenta variaciones estacionales. En el caso de Arazuri, la comunidad bacteriana que presenta las mayores diferencias es la encontrada en los suelos a los que se aportó 80 toneladas por hectárea anuales.

Los anteriores incrementos en el metabolismo coinciden con un aumento de las emisiones de N_2O y CO_2 a la atmósfera en ambos casos. Y un aumento en la capacidad de secuestro de CH_4 en Bargota y una disminución de esta capacidad en Arazuri. Es así que la emisión de gases de efecto invernadero en forma de CO_2 equivalentes se ve incrementada en Bargota entre un 40 y 60% y en Arazuri entre un 35 a 55%.

Por todo ello es interesante fomentar estas prácticas si bien sería necesario abordar en otros estudios los mecanismos de interacción entre las raíces del suelo y la actividad microbiana, o el estudio de los mecanismos que regulan la mineralización de la materia orgánica en el suelo o bien, la exploración de posibles funciones de las comunidades bacterianas, entre otros.

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