

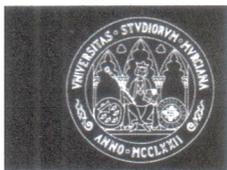


UNIVERSIDAD DE MURCIA
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**Biochar Interaction with N Dynamics Involved
in the Formation and Emission of Nitrous
Oxide in Agriculture**

**Interacción del Biochar con las Dinámicas de N
Involucradas en la Formación y Emisión de
Óxido Nitroso en Agricultura**

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La presentación de la Tesis Doctoral titulada "**Interacción del biochar con las dinámicas de N involucradas en la formación y emisión de óxido nitroso en agricultura**", realizada por D^a. **María Sánchez García**, bajo nuestra inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

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ABBREVIATIONS

| | |
|-----------------------|--|
| Anammox | Anaerobic ammonium oxidation |
| C | Carbon |
| CH₄ | Methane |
| CO₂ | Carbon dioxide |
| DCD | Dicyandiamide |
| DEA | Denitrifying enzyme activity |
| DNRA | Dissimilatory nitrate reduction to ammonia |
| DOC | Dissolved organic carbon |
| DON | Dissolved organic nitrogen |
| GEI | Gases de efecto invernadero |
| GHG | Greenhouse gas |
| GWP | Global warming potential |
| Feammox | Anaerobic ammonium oxidation coupled to iron reduction |
| IPCC | Intergovernmental Panel on Climate Change |
| N | Nitrogen |

| | |
|-----------------------------------|-------------------------------|
| NH₃ | Ammonia |
| NH₄⁺ | Ammonium |
| NO₂⁻ | Nitrite |
| NO₃⁻ | Nitrate |
| N_{org} | Organic nitrogen |
| NO_x | Nitrogen oxides |
| N₂O | Nitrous oxide |
| ppbv | Parts per billion by volume |
| Tg | Teragram (10 ¹² g) |
| TOC | Total organic carbon |
| VOC | Volatile organic compound |
| WFPS | Water filled pore space |
| WHC | Water holding capacity |
| WSC | Water soluble carbon |
| WSN | Water soluble nitrogen |

RESUMEN

La actividad humana a partir de la era industrial ha traído como consecuencia un incremento en la concentración atmosférica de los gases de efecto invernadero (GEI), principales causantes del calentamiento global. El índice de calentamiento global (GWP- acrónimo del inglés Global Warming Potential) es una medida del calor que puede ser retenido por un determinado GEI en comparación con un gas de referencia (normalmente CO₂) en un periodo determinado. De este modo se ha estimado que en un horizonte de 100 años el GWP del CH₄ y del N₂O son, respectivamente, 25 y 298 veces la masa equivalente en CO₂. De esta forma la reducción de las emisiones de estos gases constituye una parte muy importante de las estrategias encaminadas a mitigar el calentamiento global. En concreto, las emisiones derivadas de las actividades relacionadas con la agricultura representan un importante potencial en dichas estrategias, siendo los suelos agrícolas simultáneamente fuente y sumidero de los GEI dióxido de carbono (CO₂), metano (CH₄) y óxido nitroso (N₂O).

El incremento en el uso de fertilizantes nitrogenados, facilitado por el descubrimiento del proceso de Haber-Bosch, ha permitido aumentar la productividad agrícola y de este modo proveer de

alimentos a la creciente población mundial. Sin embargo, ello también ha traído consigo una alteración muy importante en el ciclo biogeoquímico del nitrógeno (N). Se estima que un 60% de las emisiones globales de N_2O derivadas de la actividad antrópica provienen de la agricultura, las cuales representan la principal contribución al cambio registrado en la concentración de este gas en la atmósfera (desde 270 ppbv de la era preindustrial hasta las 324 ppbv actuales). El N_2O , además de ser un importante GEI, es también un compuesto implicado en las reacciones químicas que tienen como consecuencia la destrucción del ozono estratosférico. A pesar de los esfuerzos desarrollados para reducir las emisiones de este gas, sigue existiendo una gran incertidumbre en cuanto a las mejores estrategias a seguir debido a la enorme complejidad del ciclo del N.

Por otra parte, el aumento en el consumo de proteínas animales por parte de los países desarrollados, además de implicar una importante huella de N, lleva consigo la producción de grandes cantidades de residuos orgánicos con altas concentraciones de N derivados de las explotaciones ganaderas. El reciclado de los mismos para su aprovechamiento como fertilizantes se ha perfilado como una opción sostenible desde el punto de vista medioambiental. El compostaje es una tecnología que permite estabilizar e higienizar estos residuos, reducir su volumen y obtener un abono natural de

excelentes características para su utilización en suelos agrícolas. Sin embargo, durante los procesos de compostaje se liberan gases que no sólo acarrearán problemas de tipo medioambiental y de salud pública sino que además implican pérdidas de nitrógeno, principalmente en la forma de los gases nitrogenados NH_3 , N_2O y N_2 , que finalmente devaluarán el compost obtenido. Como consecuencia se hace necesario el desarrollo de estrategias de fertilización y manejo de residuos orgánicos que mejoren la eficiencia en el uso del N.

La reducción de las emisiones de N_2O procedentes tanto de los suelos agrícolas como de los procesos de compostaje es una parte muy importante entre las estrategias para la mitigación del cambio climático. En este sentido, la utilización de biochar, un material rico en carbono (C) generado durante la pirolisis de la biomasa, podría ser efectiva. Se ha demostrado que la incorporación de biochar al suelo afecta los procesos de transformación y retención de C y N, los cuales juegan un papel clave en la reducción de las emisiones o en el incremento de su capacidad de sumidero de GEI. Las interacciones observadas entre el biochar y los componentes del suelo también se prevé que ocurran en las mezclas de compostaje, las cuales están caracterizadas por un elevado contenido en materia

orgánica y masa microbiana. Sin embargo, los estudios acerca de estas últimas han comenzado recientemente.

El objetivo global del presente trabajo de tesis consiste en alcanzar una mejor comprensión sobre cómo el biochar influye en las dinámicas del N implicadas en la formación y emisión de N_2O a partir de dos importantes fuentes agrícolas: los suelos y el manejo de los residuos orgánicos a través del compostaje. Con este propósito, se ha estudiado la influencia del biochar en las emisiones de N_2O en estos dos ambientes. Para alcanzar dicho objetivo global, se han propuesto los siguientes objetivos parciales:

- Analizar la interacción del biochar con los mecanismos específicos que conllevan a la formación y emisión de N_2O en suelos agrícolas a escala de laboratorio bajo condiciones controladas.
- Estudiar el efecto de las enmiendas de biochar, sólo o en combinación con compost, sobre las emisiones de N_2O del suelo y compararlas con las prácticas agrícolas tradicionales en un cultivo ecológico de olivos en condiciones de campo en clima semi-árido.
- Investigar el impacto del biochar durante el compostaje de residuos orgánicos ricos en N, en concreto sobre el

desarrollo del proceso, la liberación de gases y la calidad del producto final.

Para alcanzar dichos objetivos parciales, primero se estudió el impacto del biochar en las emisiones de N_2O desde los suelos, el cual fue evaluado bajo condiciones de laboratorio y en campo, mientras que en segundo lugar se investigó el efecto de la adición de biochar durante el compostaje de residuos orgánicos. Así pues, el trabajo experimental de la tesis se estructuró del siguiente modo:

- **Capítulo 3.** Experimentos de suelo bajo condiciones de laboratorio con medidas de gases ^{15}N e inhibidores de la nitrificación con el objetivo de identificar los principales mecanismos responsables de la formación de N_2O en distintos suelos y cómo el biochar influye sobre los mismos.
- **Capítulo 4.** Experimento de campo de dos años de duración en un suelo calizo bajo cultivo ecológico de olivos en condiciones semi-áridas, en el que se estudió su respuesta a tres enmiendas diferentes: biochar, compost y una mezcla compost/biochar. Durante este tiempo se monitorizó el impacto de estas tres enmiendas sobre las emisiones de N_2O , el almacenamiento de C en el suelo, la disponibilidad

de C y N y la actividad microbiana relacionada con el ciclo del N. También se evaluó el estado nutricional de los olivos.

- **Capítulo 5.** Experimento a escala real del compostaje de gallinaza con el objeto de conocer el impacto de una pequeña adición de biochar en el desarrollo del proceso, la liberación de gases y la calidad del compost obtenido.

A pesar del elevado número de estudios sobre emisiones de N_2O procedentes de suelos enmendados con biochar, se desconoce aún en gran medida el modo en que los mecanismos implicados se ven afectados. Sabemos que los efectos que tiene el biochar sobre las emisiones dependen tanto del tipo de biochar como del tipo de suelo enmendado y que los resultados son tan diversos como las combinaciones posibles. Existe una elevada cantidad de estudios que reflejan una disminución en las emisiones de N_2O de los suelos tras ser enmendados con biochar, pero también los hay que no muestran diferencias respecto a los suelos sin enmendar e incluso los que registran un incremento. Teniendo en cuenta los resultados previos, la primera hipótesis del presente trabajo de tesis fue la de que, aún bajo las mismas condiciones ambientales, distintos suelos

responderían de distinta forma a la adición de biochar en función de los mecanismos predominantes en cada uno de ellos.

En primer lugar, se ensayaron dos tipos de suelos bajo las mismas condiciones de laboratorio cercanas a la saturación de agua. La adición de biochar afectó de forma opuesta a las emisiones de N₂O dependiendo del tipo de suelo enmendado. En el primero de dichos suelos (un suelo de pH ácido), los resultados de los experimentos demostraron que la desnitrificación era el mecanismo responsable de las emisiones, que se vieron reducidas con la adición de biochar. Sin embargo, en el caso del segundo suelo (un suelo calizo mediterráneo), se demostró que el principal mecanismo operante era la nitrificación por parte de los microorganismos nitrificadores y la consiguiente desnitrificación por parte de los mismos (mecanismo conocido como *nitrifier-denitrification*). Aquí la adición de biochar tuvo el efecto opuesto, es decir, provocó un incremento de las emisiones.

El suelo calizo estudiado bajo condiciones de laboratorio fue también evaluado bajo condiciones de campo. El experimento de campo se realizó en una explotación ecológica de olivos con riego deficitario y bajo un clima mediterráneo semiárido. Durante dos años se monitorizaron las emisiones de N₂O tras realizar una enmienda de

compost, de biochar y de una combinación de ambos (compost+biochar). La segunda hipótesis de esta tesis fue la de que el uso de biochar, sólo o en combinación con el compost, reduciría las emisiones de N₂O en comparación con las prácticas habituales de la explotación, consistentes en la adición de compost cada dos años. Sin embargo, las distintas enmiendas realizadas no dieron como resultado un efecto significativo en las emisiones. Las emisiones fueron en general bajas y los picos de N₂O se registraron siempre cuando coexistían condiciones de humedad y temperatura, sin que las adiciones de biochar afectaran dichos flujos de N₂O. Sin embargo, sí se observaron ciertos efectos sinérgicos en los suelos enmendados con la combinación de compost y biochar, los cuales registraron los niveles más altos de actividad enzimática desnitrificante (en inglés: Denitrifying Enzyme Activity, DEA). En cualquier caso, los niveles de DEA fueron bajos, corroborando los resultados obtenidos en laboratorio e indicando que en estos suelos la desnitrificación no es un proceso predominante. Además, en las parcelas enmendadas con la mezcla se registró un incremento en el número de copias del gen *amoA* (gen estructural de la enzima amonio monooxigenasa, implicada en el primer paso de la nitrificación). Estos efectos no se observaron en las parcelas enmendadas sólo con compost o sólo con biochar. Otros parámetros como el carbono

orgánico soluble, N orgánico, amonio (NH_4^+) o NO_3^- no reflejaron variaciones, sugiriendo que las diferencias registradas se debieron a una mayor actividad microbiana en las parcelas que fueron enmendadas con la mezcla.

Aunque las interacciones entre biochar y la materia orgánica durante los procesos de compostaje han sido menos investigadas que en el caso de los suelos, existen evidencias de que la adición de biochar implica efectos positivos. En la última fase de la tesis, se evaluó el compostaje a escala piloto de una mezcla de gallinaza y paja de cebada y se monitorizó todo el proceso con la adición y sin la adición de biochar. La hipótesis de partida para realizar el experimento de compostaje fue que las propiedades físicas del biochar mejorarían la aireación de la mezcla de compostaje y como consecuencia se conseguirían reducir las emisiones de N_2O y las pérdidas totales de N. Durante el experimento se registró una alta variabilidad en las emisiones desde la superficie de las pilas de compostaje, por lo que no se pudieron evaluar diferencias significativas aunque se observaron ciertas tendencias. En contraposición con la hipótesis inicial, los flujos de N_2O durante las fases mesofílica y de maduración fueron más elevadas en las pilas de compostaje que contenían biochar, lo que probablemente estuvo relacionado con procesos de nitrificación. Tampoco se redujeron las

pérdidas de N. En cuanto al contenido en macro y micronutrientes, la adición de biochar no tuvo un efecto distintivo, siendo los parámetros de calidad similares en los dos tipos de composts. No obstante, la adición de un 3% de biochar mejoró el proceso de compostaje en cuanto que se redujo el tiempo requerido en cuatro semanas, con el consiguiente ahorro de los costes de producción.

Finalmente, las conclusiones generales que derivan de estos trabajos son:

1. El biochar afecta a las emisiones procedentes de los suelos de forma diferente en función del mecanismo de formación de N_2O operante en el suelo. Cuando la desnitrificación es el proceso principal, el biochar reduce estas emisiones. No obstante, el biochar favorece la nitrificación y como consecuencia el N_2O derivado de este proceso.
2. El biochar tiene su mayor potencial de mitigación en los procesos de desnitrificación. Así pues, el éxito de las estrategias de mitigación basadas en el uso de biochar dependerán de los principales mecanismos responsables de la formación y liberación de N_2O operando en el suelo objetivo.

3. Las emisiones de N₂O procedentes de suelos calizos agrícolas en condiciones de clima mediterráneo semiárido son bajas y el biochar tiene poco o ningún efecto en la mitigación de N₂O en comparación con las prácticas agrícolas tradicionales.
4. Bajo condiciones agroclimáticas mediterráneas, el uso combinado de biochar y compost promueve los procesos microbianos relacionados con el ciclo del N sin un impacto significativo en las emisiones de N₂O.
5. La adición de biochar en un ratio del 3% (en peso) no afecta significativamente las emisiones de N₂O procedentes de una pila de compostaje con alto contenido en N.
6. La adición de biochar, incluso en pequeñas dosis, a las pilas de compostaje de residuos orgánicos es una herramienta efectiva para reducir el tiempo requerido para su estabilización e higienización con el consiguiente ahorro en los costes.

CHAPTER 1. GENERAL INTRODUCTION



1.1. Agriculture and nitrous oxide

The anthropogenic activity during the industrial era has led to an increase in the atmospheric concentration of greenhouse gases (GHGs), which is the main cause of global warming. Concerns on climate change have resulted in a range of efforts to regulate and reduce GHG emissions. Agricultural soils are simultaneously, source and sink of the GHGs carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O). The global warming potential (GWP) of CH₄ and N₂O are 25 and 298 times greater than the equivalent mass of CO₂ in the atmosphere over a 100-year horizon (IPCC., 2007). The reduction of these emissions thus constitutes an important part of agriculture's mitigation potential concerning climate change.

It has been estimated that the total anthropogenic source of N₂O-N is 6.7 Tg year⁻¹, 42% of which is derived from agriculture. Therefore, it represents the main contribution to the registered change in the mixing ratio of this gas in the atmosphere, from a concentration of 270 parts per billion by volume (ppbv) in 1750 to 324 ppbv in 2013 (Ussiri and Lal, 2013). N₂O is not only a potent greenhouse gas, but also it is involved in the atmospheric chemical reactions resulting in the breakdown of the stratospheric ozone. Despite the efforts, developing strategies to mitigate N₂O emissions represents a difficult challenge, since N has the most

complex biological cycle of all the elements essential for life (Shcherbak, 2013).

Soil N is present in four major forms: organic matter (such as plant material, fungi and humus), soil organisms and microorganisms, ammonium ions (NH_4^+) held by clay minerals and organic matter, and organic and mineral N forms in soil solution (including dissolved organic N, NH_4^+ , NO_3^- and low concentrations of NO_2^-) (Cameron et al., 2013). N is the most limiting factor for plant growth in non-leguminous crops after water availability, which is obtained from soil solution. The rising demand for food and the changing diets (higher meat consumption) require higher N inputs in order to increase crop and animal production. As a consequence, there has been an increase in the use of N fertilisers, facilitated by the discovery of the Haber-Bosch process (the main industrial procedure for the production of ammonia from atmospheric N_2), which has led to the disruption of the global N biogeochemical cycle (Jenkinson, 2001).

Despite the benefits of N-inputs on crop productivity, there are unintended environmental negative consequences related to N losses from agricultural systems: volatilisation of ammonia, eutrophication of water bodies due to NO_3^- leaching and an increase of N_2O release to the atmosphere (Subbarao et al., 2006).

On the other hand, the higher animal protein consumption in developed countries has led to an increase in intensive livestock production, which, apart from having a high N-footprint (Davidson, 2009), produces large amounts of N-rich manures, which are the traditional supply of N and organic matter for agriculture. However the increasing generation of large amounts of N-rich manures can be the source of serious environmental problems, including the release of greenhouse gas emissions during their treatment and stabilisation and after their use in agriculture.

The treatment of animal wastes through composting represents an effective method for recycling nutrients and carbon in agriculture (Kelleher et al., 2002; Larney and Hao, 2007). However, composting is also associated to the release of GHGs and other gaseous emissions such as ammonia (NH₃), hydrogen sulphide (H₂S), volatile organic compounds (VOCs) and carbon monoxide (CO) (Amon et al., 2006; Sánchez et al., 2015). These emissions are not only of environmental and health concerns, but also imply N-losses, mainly through N₂O-N, N₂-N and NH₃-N emissions, which reduce the agronomical value of the end product.

The development of fertilisation and manure management strategies leading to increased N efficiency has become necessary in order to avoid negative environmental impacts. With this objective, the potential benefit

of the use of biochar in the implementation of mitigation GHG strategies is a subject which has evolved increasing interest.

1.2. N cycle

The N cycle involves large natural fluxes from the atmosphere to terrestrial and marine ecosystems and vice versa. The largely unreactive atmospheric N_2 is transformed into reactive N (fixed N) through a series of natural processes. In soils, fresh and marine waters and sediments, this reactive N is subsequently transformed into a wide range of N-compounds, including amino acids and oxidised compounds, and it is eventually returned to the atmosphere. During the fixation steps, different forms of reactive N are generated (NH_3 , NH_4^+ , NO, NO_2 , HNO_3 , N_2O , HONO, peroxyacilnitrates (PAN) and other organic N compounds), which are widely distributed in the atmosphere and cryosphere and play an important role in the biological and ecosystem function in terrestrial and marine ecosystems (Fowler et al., 2013).

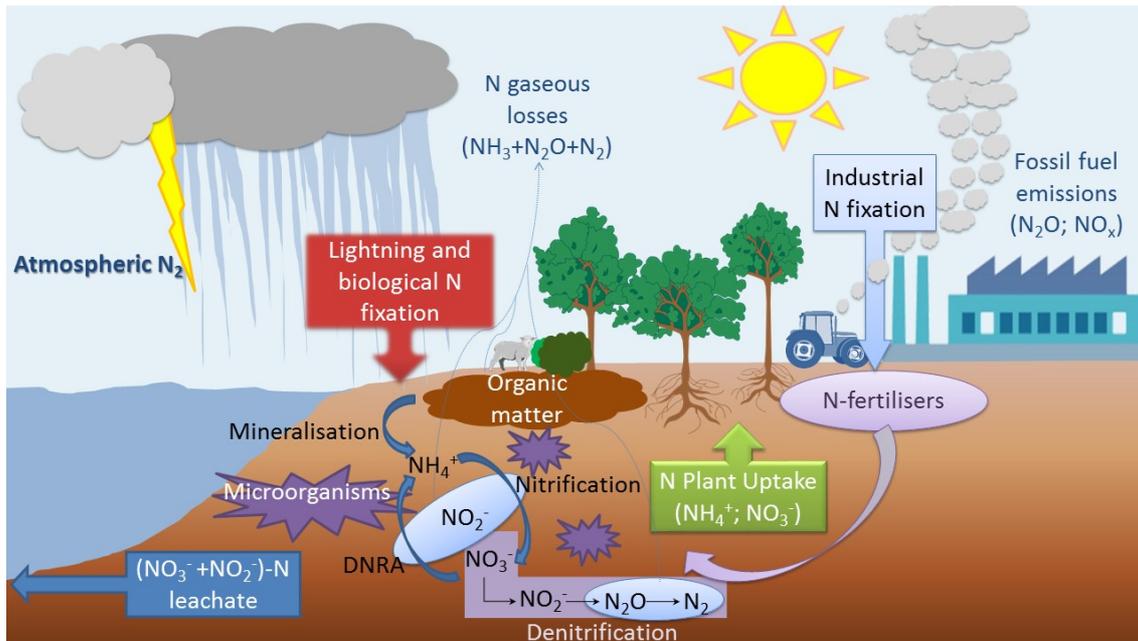


Figure 1. 1. Outline of the processes involved in the N cycle related to agriculture. Adapted from Fowler et al. (2013) and Cameron et al. (2013)

In the absence of human influence, biological nitrogen fixation, lightning and natural fires would be the only sources of new reactive N in the environment. Nevertheless, with the aim of increasing crop yields and providing food to the growing population, there has been an increase in the N supply to agricultural soils. The N applied in agriculture is mainly derived from atmospheric N₂ through the Haber-Bosch process, which also provides reactive N for other industrial applications (Fowler et al., 2013). Industrial activity and combustion of fossil fuels are also anthropogenic sources of reactive N. Overall, reactive N has been doubled in the global cycling of N over the last century due to human activity, with negative costs on biodiversity loss, human health and climate (Fowler et al., 2013).

1.3. Nitrogen processes leading to N₂O formation in soil

N₂O release from soils is almost entirely controlled by microbial activities. Nitrification and denitrification processes have been considered as the main drivers of N₂O release to the atmosphere (Bremner, 1997; Davidson et al., 1991). Nevertheless, current knowledge suggests that there are, at least, five N₂O-genic soil microbial sources which may play a relevant role on its release (Baggs, 2011; Spott et al., 2011; Wrage et al., 2001). These processes are the nitrate and nitrite reduction in denitrification, dissimilatory reduction of nitrate to ammonium (DNRA, also referred to as nitrate ammonification), ammonia oxidation (first step of nitrification), nitrifier-denitrification (which is the ability of ammonia oxidising bacteria to denitrify) and co-denitrification. There are several factors influencing N₂O release, not only the amount of N input but also the fertiliser type, crop type, soil organic carbon content, soil microbial populations, pH and texture and water filled pore space (WFPS) (Baggs et al., 2010; Bateman and Baggs, 2005; Stehfest and Bouwman, 2006). Moreover, although there are environmental factors which can promote a certain N₂O formation pathway, these processes can operate within the same soil at the same time, since there is a strong gradient in biotic and abiotic factors over short distances.

1.3.1. Nitrification

Nitrification is performed by autotrophic bacteria (mainly from *Nitrosomonas* and *Nitrospira* genus). In this process, NH_4^+ is oxidised to NO_3^- in two steps: first NH_4^+ is oxidised to nitrite (NO_2^-) via hydroxylamine (NH_2OH) and secondly NO_2^- is further oxidised to NO_3^- (Schmidt, 1982). These reactions are carried out by two groups of microorganisms: the first part, up to NO_2^- formation, by ammonia oxidisers or primary nitrifiers and the second by nitrite oxidisers or secondary nitrifiers (see Figure 1.2). The first step is regulated by the *AmoA* gene encoding the ammonia monooxygenase enzyme, and the second step is catalysed by the *nxrB* gene encoding the nitrite oxidoreductase (Hu et al., 2015). N_2O can be released during this process as a by-product of ammonia oxidation in the first step. NH_4^+ , CO_2 and O_2 are required to develop this pathway, being NH_4^+ the limiting factor in many cases, since O_2 and CO_2 are normally available in adequate levels in agricultural soils. Other factors limiting nitrification are low pH, low water potential, phosphate (PO_4^-) availability, low temperatures, NO_2^- toxicity and allelopathic compounds (Bremner, 1997; Subbarao et al., 2006). The existence of heterotrophic nitrification has been also demonstrated (De Boer and Kowalchuk, 2001), which is more common among fungi than in bacteria and it is thought to be relevant only under

certain circumstances such as low pH, high oxygen amounts and availability of organic material (Wrage et al., 2001).

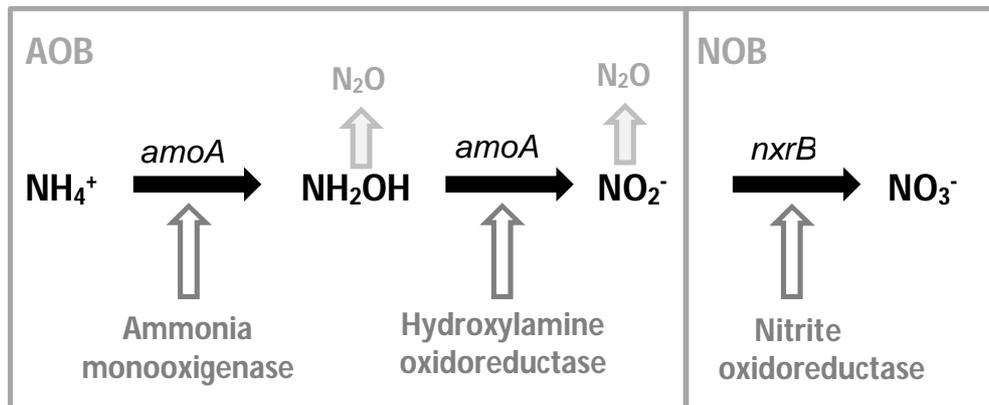


Figure 1. 2. Nitrification: outline of the pathway, enzymes and group of microorganisms involved. AOB: Ammonia Oxidising Bacteria; NOB: Nitrite Oxidising Bacteria. Adapted from Wrage et al. (2001)

1.3.2. Nitrifier-Denitrification

Ritchie and Nicholas (1972) suggested that some nitrifiers could also denitrify, although their contribution to N_2O release remains elusive since a simple method for measuring this pathway does not exist. Nevertheless, there is increasing evidence that, under certain conditions, this mechanism represents a major source of N_2O and contributes to the loss of N from agricultural systems (Kool et al., 2011; Zhu et al., 2013). In this pathway, the oxidation of NH_4^+ to NO_2^- is followed by the reduction of NO_2^- to N_2O and N_2 . This pathway is known as nitrifier-denitrification (Figure 1.3) and must not be mistaken for heterotrophic denitrification coupled with nitrification,

where NO_3^- is formed as an intermediate. Factors enhancing this pathway are NH_4^+ availability and low O_2 concentration (Zhu, 2013), and there is some evidence of high NO_2^- concentrations promoting this process (Baggs, 2011; Stein and Arp, 1998).

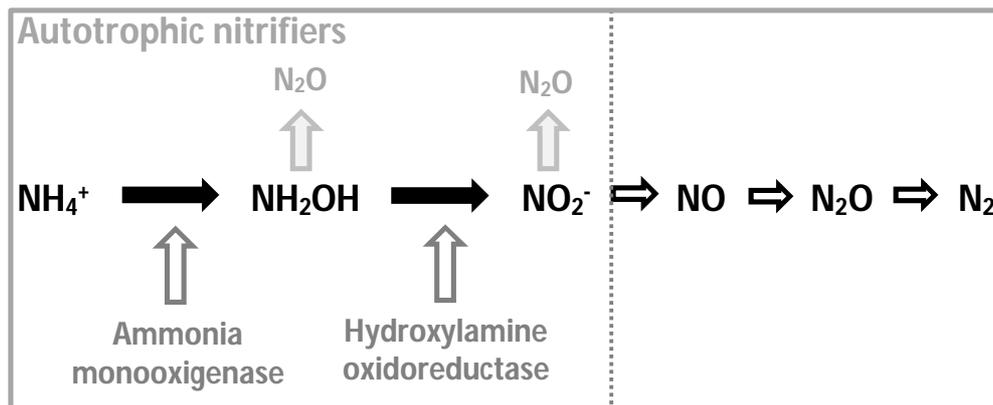


Figure 1. 3. Nitrifier-denitrification pathway. Adapted from Wrage et al. (2001)

1.3.3. Denitrification

There is a wide range of organisms capable of denitrifying, including archaea, bacteria and fungi. Nevertheless, heterotrophic bacteria that use NO_3^- as electron acceptor when O_2 is not available are thought to be the main responsible of the denitrification process. Denitrification is influenced by O_2 availability, water content, pH, temperature, NO_3^- concentration and organic carbon available for denitrifiers (Bremner, 1997). During this process, NO_3^- is reduced to dinitrogen (N_2), being NO and N_2O obligatory

intermediates (see Figure 1.4). This process is sequentially catalysed by the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, which are encoded by the genes *narG*, *nirS/nirK*, *norB* and *nosZ* respectively, and are generally the most used markers to understand the denitrifying community (Giles et al., 2012). The ratio of $N_2O/(N_2O+N_2)$ is highly variable and also dependent on environmental conditions. O_2 concentration reduces denitrification activity, being nitrous oxide reductase the most sensitive of the enzymes involved in the process. Thus, increasing levels of O_2 concentration make difficult to reach the final step of this process because of N_2O reductase inhibition (Knowles, 1982).

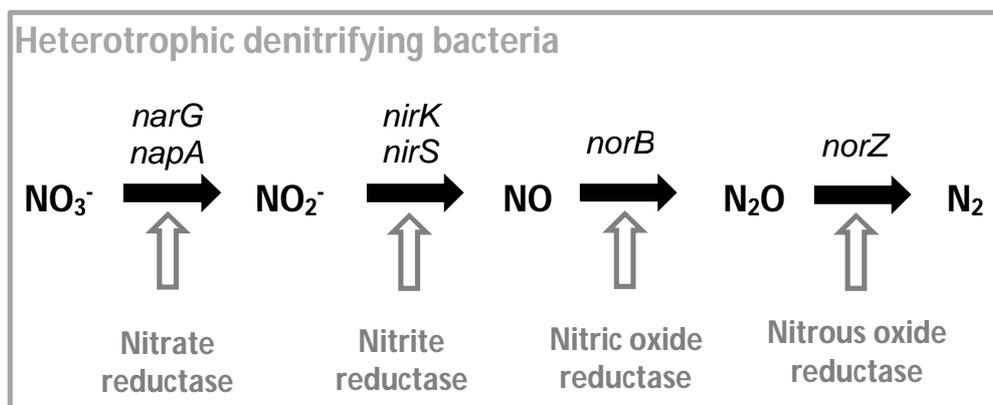


Figure 1. 4. Denitrification: outline of the pathway and enzymes involved. Adapted from Wrage et al. (2001) and Giles et al. (2012)

1.3.4. Codenitrification

Denitrifying organisms have also the potential to utilise co-substrates. This mechanism is called co-denitrification. During this process, one N atom of nitrite or nitric oxide combines via a nitrosyl intermediate with one N atom of another N species. In this pathway two molecules of N_2O can be generated from one molecule of NO_3^- , in contrast to conventional denitrification where only one molecule of N_2O can be produced. It has been reported to be performed by archaea, bacteria and fungi and, although its impact in N cycling remains uncertain, recent findings support evidence of its relevance (Baggs, 2011; Spott et al., 2011).

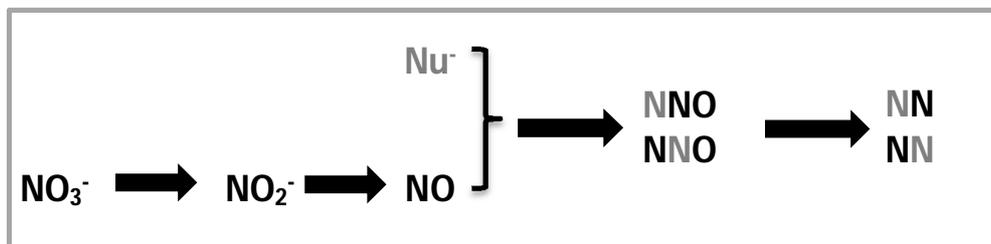


Figure 1. 5. Co-denitrification. Adapted from Baggs (2011). Nu: nucleophile (e.g. $R-NH_2$, NH_4^+ , amino acids or other organic N compounds)

1.3.5. Dissimilatory nitrate reduction to ammonium

During DNRA, NO_3^- is reduced to NO_2^- and NH_4^+ . In this pathway, denitrification to N_2O and N_2 is avoided, and N_2O is produced at the NO_2^- reduction stage to NH_4^+ as a by-product (Schmidt et al., 2011). DNRA is less sensitive than heterotrophic denitrification to O_2 fluctuation and is thought to be favoured in NO_3^- limiting conditions (Giles et al., 2012). Its contribution in soil N cycle seem to be small but it still represents a mechanism which prevent N losses from soils since NO_3^- is transformed to NH_4^+ and can be retained.

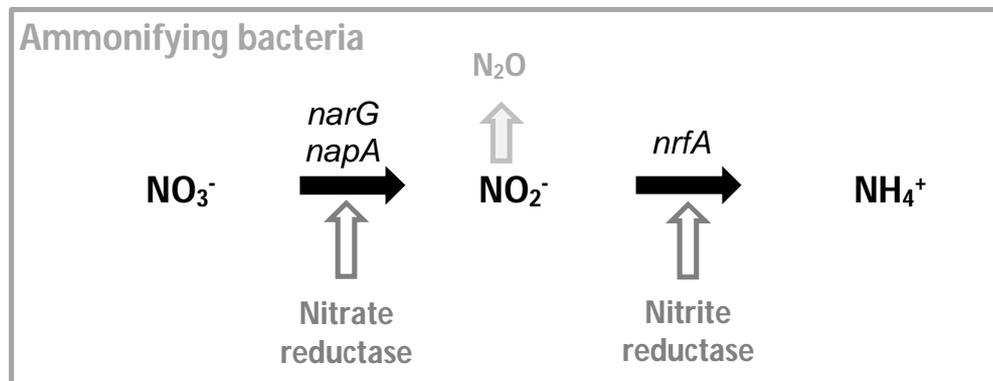
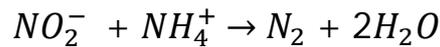


Figure 1. 6. Dissimilatory nitrate reduction to ammonium: outline of the pathway and enzymes involved. Adapted from Giles et al. (2012)

1.3.6. Other processes

The anaerobic ammonium oxidation with nitrite is an important process where N_2 is released from the marine ecosystem, being the reaction that better explains this pathway as follows (Dalsgaard et al., 2005):



Therefore, it represents another way for removing reactive N apart from denitrification. The production of N_2 via the ANAMMOX pathway was discovered in a denitrification bioreactor (Van de Graaf et al., 1995) and it has been extensively reported in marine ecosystems. Recently its occurrence in soils has been also demonstrated (Bai et al., 2015; Humbert et al., 2010). A modification of this reaction takes place when the reduction of ferric ion (Fe^{3+}) is coupled to anaerobic NH_4^+ oxidation to produce N_2 , NO_3^- or NO_2^- (Yang et al., 2012). This process is termed FEAMMOX and has been observed in wet-land soils under iron-reducing conditions. Clément et al. (2005) proposed an hypothesis where NH_4^+ oxidation into NO_2^- is coupled with the reduction of Fe^{3+} into Fe^{2+} along riparian transects as shown in figure 1.7.

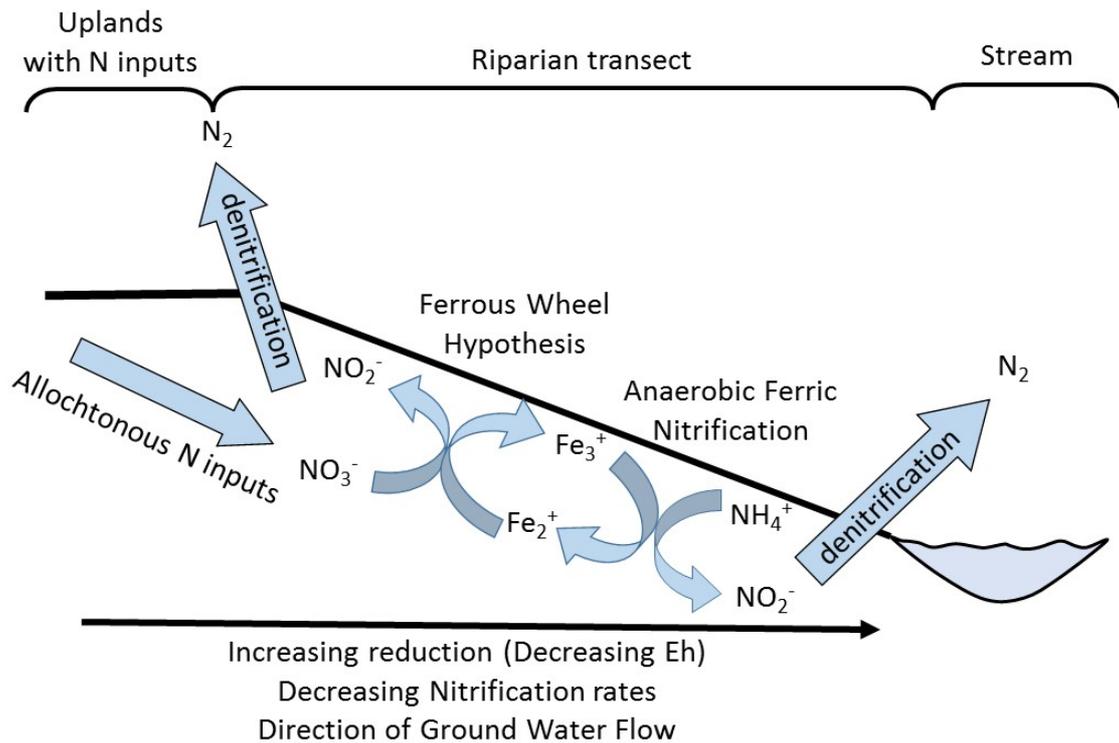


Figure 1. 7. Diagram suggesting the coupling of the ferrous wheel and the anaerobic nitrification along a riparian transect. From Clément et al. (2005)

All these processes are inter-linked and can occur simultaneously in different microsites of the same soil. Therefore, it remains a difficult challenge attributing N_2O production to different mechanisms and formulating adequate mitigation strategies. Moreover, there is also a link between C and N cycles, which results in some management practices that may lead to lower CO_2 -C emissions rates with highly variable effect on N_2O -N release, *e.g.* non or reduced-tillage soil management (Kessel et al., 2013). Although our knowledge of N transformation processes has evolved

significantly over the last decades, our understanding of N₂O formation and consumption in soil is still very limited.

1.4. Gaseous emissions and N losses during composting

Manure composting is considered a sustainable option for the recycling of nutrients with agricultural purposes. During this process, an organic material is decomposed in controlled aerobic conditions generating an end product (compost) that is stable and resistant to subsequent decomposition processes. Thus, the use of compost as an agricultural amendment increases the concentration of soil organic matter, representing a way of sequestering C in soils (Serramiá et al., 2012). Nonetheless, the benefits of composting can be counteracted by the emission of GHG during the process. CO₂ is the principal gas released. However, for biodegradable materials whose carbon has been recently captured from the atmosphere (like organic wastes), no global warming potential (GWP) is associated with CO₂ emissions, due to their biogenic origin (Sánchez-Monedero et al., 2011). Instead, CO₂ is used as an indicator of microbial activity in the composting mixture during the process and it is also used as a stability index of the compost (Barrena et al., 2006). CH₄ and

N₂O are released in a lower scale but their GWP is much higher than that of CO₂ and they represent the most climate-relevant gaseous emissions during composting.

N losses during composting cause a devaluation of the final product. They can be due to NO₃⁻ leaching or as a consequence of N-gaseous losses. Management practices that maintain appropriate moisture avoid NO₃⁻ losses through leaching. However, reducing N gaseous losses represents a more difficult approach. Moreover, composting facilities are also a source of other gaseous emissions as well as origin of bad odours. Thus, improved composting processes should minimise gaseous emissions, origin of health and environmental concerns, and also reduce N-losses, which is determinant for the agronomical value of the compost.

1.4.1. Nitrous oxide emissions

N₂O formation and release during composting is a complex biological process that has been mainly associated to nitrification and denitrification processes (Sánchez-Monedero et al., 2010). The mechanisms of N₂O formation during composting have been less well-studied than in soils. For instance, there are no studies evaluating the occurrence of nitrifier

denitrification, co-denitrification or DNRA during composting. In any case, these microbial processes are regulated by the amount of mineral N, available C sources and O₂ concentration, so the amount of N₂O produced is always associated to the nature of the starting composting materials. Therefore, N-rich manures used as composting substrates will favour N₂O emissions compared to other type of wastes with lower or less available N concentrations.

N₂O can be released at different stages of the composting process, but it has been found mainly during the mesophilic and maturation phases (Cayuela et al., 2012). Both nitrification and denitrification mechanisms can coexist during composting, although the changing environmental conditions during the process can promote one mechanism over the other.

Nitrifiers require aerobic conditions, mesophilic temperatures (below 40°C) and pH values above 5. They also require the presence of NH₄⁺, the main precursor of nitrification, which is generated from the degradation of organic matter during the early phases of the process (Sánchez Monedero et al., 2001). Nitrification has been found to significantly contribute to N₂O emissions during the maturation phase (He et al., 2001).

Denitrifiers need anaerobic or low O₂ concentration, sources of available C and the presence of NO₃⁻, NO₂⁻ or NO as electron acceptors. In

the early stages of the process, as previously described, enhanced microbial activity can temporally lead to a decrease in O_2 availability promoting anaerobic conditions favourable for denitrifying microorganisms. In the absence of O_2 , complete reduction of NO_3^- to N_2 is promoted, resulting in no appreciable N_2O production. Nevertheless, N_2O emissions via denitrification are enhanced as O_2 concentration increases. Nitrifier-denitrification can be also promoted in limited O_2 conditions, although this mechanism has not been studied in composting processes. Previous studies have reported a linkage between NO_2^- concentration and N_2O release (Fukumoto and Inubushi, 2009; He et al., 2000). Since denitrifiers are mostly heterotrophs relying on organic carbon, He et al. (2000) suggested nitrifiers being the main microorganisms that reduce NO_2^- to N_2O after the depletion of available organic carbon.

1.4.2. Ammonia emissions

The volatilisation of NH_3 usually represents the major cause of N losses during the composting process and limits NH_4^+ availability for the nitrification process. Besides, its release causes eutrophication and

acidification and thus plays an important role in the decline of biodiversity and dying of forests.

NH₃ is mainly emitted during the thermophilic phase of composting, when organic matter is actively decomposed and the compost mixture reaches temperatures above 60°C (Sánchez-Monedero et al., 2001). The level of NH₃ release is subject to the starting materials composition and varies depending on environmental and management factors. A small part of the organic N contained in the composting mixture, which is mainly associated to the structure of proteins and simple peptides, is mineralised to ammonia via ammonification reactions developed by the microbial community. High temperature and pH displace the NH₄⁺/NH₃ equilibrium to ammonia favouring its volatilisation (Pagans et al., 2006a). The addition of C-rich co-substrates when composting wet manures increases the C/N ratio of the mixtures, factor which can reduce the volatilisation of NH₃ (Bernal et al., 2009).

1.4.3. Methane emissions

CH₄ production during composting is mainly associated with anaerobic processes. Although composting is an aerobic process, the

occurrence of anaerobic sites is possible inside the composting matrix, especially during the early stages, when high quantities of labile organic compounds stimulate an intense growth of the microbial population, leading to a depletion of the oxygen levels (Sánchez et al., 2015). A positive correlation between temperatures, density and size of the composting piles has been reported (Beck Friis et al., 2000), which means that an increase in any of these three variables normally lead to higher CH₄ emissions. The type of management also has a direct influence in the formation of anaerobic niches, being the static piles the option with the highest CH₄ emissions. However, not all CH₄ that is formed inside the pile is emitted to the atmosphere. CH₄ is oxidised to CO₂ from the centre to the surface of the heap of the composting pile, being released only when its concentration is higher than the oxidation rate (Cayuela et al., 2012).

1.4.4. Other gaseous emissions

Nitrogen oxides different to N₂O (NO_x) have low direct GWP (in the order of 5 over a 100 year horizon) but they lead to indirect radiative effects by increasing CH₄ lifetime and elevating concentrations of tropospheric O₃ (IPCC., 2007). From the two gases composing NO_x (NO and NO₂), only NO is

generated during composting through nitrification and denitrification processes.

The emission of carbon monoxide (CO) during composting is generated by abiotic reactions (Hellebrand and Schade, 2008). The maximum CO flux rates have been reported at the beginning of the composting process with decreasing levels during periods of high biological activity (Hellebrand and Kalk, 2001; Hellebrand and Schade, 2008). The indirect effects of CO are the increase of CH₄ lifetime and the elevation of O₃ concentration in the troposphere, whereas its GWP over a 100-year horizon has been estimated to be in a range of 1 to 3 (IPCC., 2007). CO is toxic due to its capacity to inhibit certain electron transport, which was disclosed by Douglas et al. (1912), being its emissions also investigated as a potential health risk to workers in enclosed facilities treating municipal wastes (Phillip et al., 2011).

H₂S release is responsible of bad odours around composting facilities (rotten egg smell) and cause of potential health risk. Its formation is promoted by reducing conditions and availability of sulphates and sulphur-containing organic compounds (Christian et al., 2010). Therefore, its formation and release mainly occur during the thermophilic phase of the composting process, coinciding with the highest microbial activity.

Besides NH_3 and H_2S , organic compounds volatilised during composting are also a major source of bad odours. The term volatile organic compound (VOC) refers to organic compounds which vapour pressure is at least 0.01 kPa and 20 °C and are also characterised by their low water solubility (Pagans et al. 2006b). An extensive list of VOCs is released during the process, where they can be classified in seven families (Krzymien et al., 1999):

- i) Aldehydes, ketones, and ethers
- ii) Acids, alcohols, and esters
- iii) Terpenes and alicyclics
- iv) Aliphatic hydrocarbons
- v) Aromatic hydrocarbons
- vi) Sulphur-containing compounds
- vii) Nitrogen-containing compounds

Some of these compounds are the result of microbial degradation while others are compounds found in the feedstock (Epstein, 1997). Therefore, the evolved VOCs, which are mainly released in the beginning of the composting process, will vary depending on the raw materials of the composting mixture.

1.5. Role of biochar as an amendment in agricultural soils and an additive in composting processes

Biochar is a carbonaceous material produced during the pyrolysis of biomass. During this process, organic carbon structures are modified by exposure to elevated temperatures (>400°C) in a limited or no available oxygen environment, resulting in a product with higher C recalcitrance than the preceding feedstock material. This process is very similar to the industrial charcoal production, but differs in the purpose of the product. The aim of biochar production is its application to soils as a means of improving crop productivity, C storage or filtration of percolating ground water (Lehmann and Joseph, 2015).

Incorporation of biochar to soils is of global interest as a tool for C sequestration as it contains a highly condensed aromatic structure that resists decomposition. There is mounting evidence that biochar application to soils causes changes in their chemical, physical and biological properties. Enhanced nutrient retention and availability due to its high surface area as well as an increased water holding capacity (WHC) have been reported (Basso et al., 2013; Chan, 2007). Higher WHC also benefits nutrient retention because it reduces water percolation and the loss of nutrients

dissolved therein (Glaser et al., 2002). As a result, biochar has been claimed to improve fertiliser use efficiency thus reducing fertilisation requirements. Therefore, biochar incorporation affects C and N transformation and retention processes in soils that play a direct role reducing emissions or increasing the sink capacity for GHGs (Van Zwieten et al., 2015). Focusing on biochar impact on the N cycle in soils, a positive effect on crop yields of the interaction between N-fertiliser and biochar has been reported (Chan, 2007; Jeffery et al., 2011), or an improvement in the N use efficiency (Chan, 2007). However, its effect on N₂O emissions, although extensively studied during the last years, remains controversial. Most studies have shown a decrease in N₂O emissions after biochar application (Taghizadeh Toosi et al., 2011), but several recent field studies have found no changes (Castaldi et al., 2011; Suddick and Six, 2013) or even an increase in N₂O emissions (Verhoeven and Six, 2014).

Investigations about the interactions between biochar and organic matter during composting have begun recently. The reported interactions of biochar with soil's components are also expected to occur within a composting matrix, characterised by a high nutrient and organic matter content and an active microbial biomass (Steiner et al., 2015). Moreover, there is scientific evidence of the impact of biochar addition to composting

mixtures such as improved aeration (Steiner et al., 2010), enhancement of microbial population (Jindo et al., 2012) and changes in nutrients cycling and GHG emissions (Chowdhury et al., 2014). Biochar amendment has also been proposed as a possible low technology solution to lower N₂O formation and release during composting processes (Wang et al., 2013) as well as to prevent N losses due to NH₃ volatilisation (Steiner et al., 2010). However, there is still a lack of understanding of the interactions between organic matter and biochar during composting and the consequent impact on gaseous losses (Wang et al., 2013).

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CHAPTER 2. OBJECTIVES, HYPOTHESIS AND STRUCTURE OF THE THESIS



N₂O is a potent greenhouse gas (GHG) with a high global warming potential (GWP), which also contributes to the depletion of the ozone layer in the stratosphere. Agriculture represents the major anthropogenic source of N₂O emissions to the atmosphere, which is related to the use of synthetic N-fertilisers in agricultural soils and to the management of N-rich manures.

N plays a key role in plant nutrition and it is applied to agricultural soils for maintaining high crop yields and supplying food for the increasing world population. However, the spread use of synthetic N-fertilisers has led to a disruption in the N cycle which has doubled the reactive N in the environment and increased the amounts of N₂O release to the atmosphere. N₂O can also be generated during storage and treatment of manures and after their land application. Composting is a widely accepted technology for manure treatment and sanitisation, which produces a high quality organic fertiliser with improved characteristics for its safe application to agricultural soils. N-gas release from agricultural soils and composting facilities not only contributes to global warming but it also implies a N-loss from soil, decreasing its availability for plant nutrition, and reducing the agronomic value of the compost.

Current research is targeted at developing strategies for reducing N₂O emissions associated to agriculture. Biochar addition could be a useful tool since there is evidence of its effects on C and N dynamics involved in N₂O emissions. However, contrasting results from biochar research have been obtained, with studies reporting a decrease, no effect or even an enhancement of N₂O emissions.

Considering these research requirements, the following **hypotheses** have been subjected to study in this thesis:

- The microbial processes leading to N₂O release (nitrification, nitrifier-denitrification, denitrification, etc.) depend, not only on the environmental conditions, but also on the type of soil. Different soils displaying different N₂O formation pathways will be affected differently by biochar amendment.
- The impact of biochar addition on microbial N transformation processes will reduce soil N₂O emissions compared to common management practices under field conditions.
- Biochar physical properties will enhance aeration when added, even at a low rate, to a composting mixture, resulting in lower N₂O emissions and total N losses during the process.

The global **objective** of this thesis is to improve our understanding of biochar effect on N dynamics in agricultural soils and during organic waste composting, with special emphasis on the processes involved in the formation and release of N₂O. In order to reach this global objective, the following partial research objectives were proposed:

- To unravel the interaction of biochar with the specific mechanisms leading to the formation and release of N₂O in agricultural soils in laboratory scale experiments under controlled conditions.
- To study the effect of biochar amendment, alone or in combination with compost, on soil N₂O emissions and compare it to traditional agricultural practices in an organic olive orchard in semi-arid field conditions.
- To evaluate the impact of biochar during composting of N-rich organic wastes, with focus on the process performance, gas release and quality of the end product.

With the purpose of reaching these partial research aims, the experimental work of this thesis has been structured as follows (Figure 2.1):

- **Chapter 3.** Soil experiments in laboratory conditions with ¹⁵N gas measurements and nitrification inhibitors with the purpose of identifying the main pathways leading to N₂O formation in

contrasting soils and how biochar influences different mechanisms.

- **Chapter 4.** Two-year field experiment in a semi-arid calcareous soil cultivated with olive trees organically managed studying the response to three different amendments: biochar, compost and a mixture compost/biochar. During this experiment, the impact of the three amendments on N₂O release, soil C accrual, C and N availability and microbial activity related to the N cycle were monitored. The nutritional status of the olive trees was also assessed.
- **Chapter 5.** Full scale experiment of poultry manure composting in order to address the impact of a low quantity of biochar addition on process performance, gaseous release and the quality of the end product.

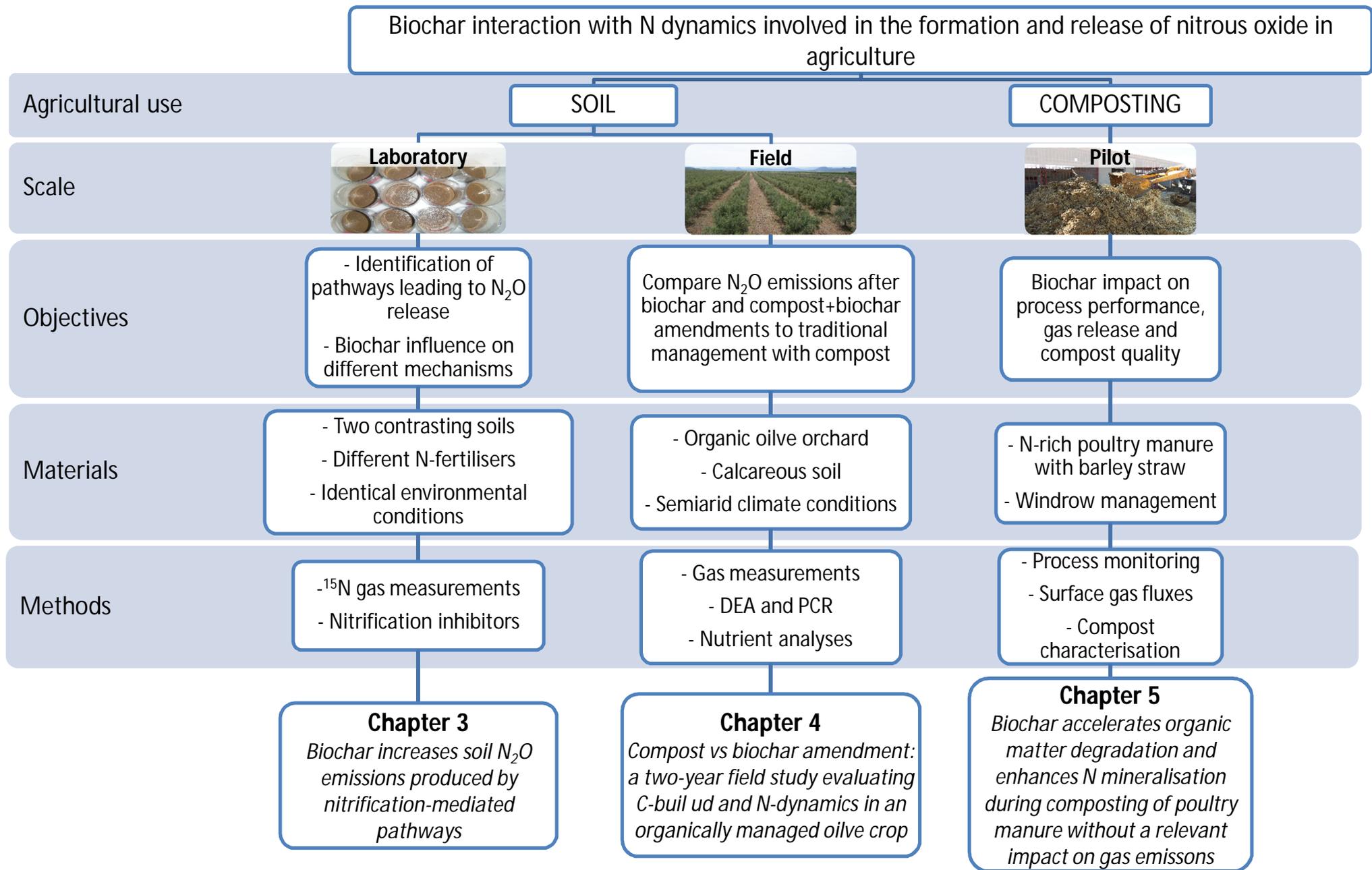


Figure 2. 1. Structure of the experimental work

CHAPTER 3. BIOCHAR INCREASES SOIL N₂O EMISSIONS PRODUCED BY NITRIFICATION-MEDIATED PATHWAYS



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3.1. Abstract

In spite of the numerous studies reporting a decrease in soil nitrous oxide (N₂O) emissions after biochar amendment, there is still a lack of understanding of the processes involved. Hence the subject remains controversial, with a number of studies showing no changes or even an increase in N₂O emissions after biochar soil application. Unravelling the exact causes of these changes, and in which circumstances biochar decreases or increases emissions, is vital to developing and applying successful mitigation strategies. With this objective, we studied two soils (Haplic Phaeozem (HP) and Haplic Calcisol (HC)), which showed opposed responses to biochar amendment. Under the same experimental conditions, the addition of biochar to soil HP decreased N₂O emissions by 76%; whereas it increased emissions by 54% in soil HC. We combined microcosm experiments adding different nitrogen fertilisers, stable isotope techniques and the use of a nitrification inhibitor (dicyciandiamide) with the aim of improving our understanding of the mechanisms involved in the formation of N₂O in these two soils. Evidence suggests that denitrification is the main pathway leading to N₂O emissions in soil HP, and ammonia oxidation and nitrifier-denitrification being the major processes generating N₂O in soil HC. Biochar systematically stimulated nitrification in soil HC,

which was probably the cause of the increased N₂O emissions. Here we demonstrate that the effectiveness of using biochar for reducing N₂O emissions from a particular soil is linked to its dominant N₂O formation pathway.

3.2. Introduction

Biochar, a carbonaceous material produced during the pyrolysis of biomass, has been found to decrease N₂O emissions from soils (Cayuela et al., 2010; Spokas and Reikosky, 2009; Van Zwieten et al., 2010). A recent meta-analysis of 30 papers (published from 2007 to 2013) revealed a statistically significant reduction of 54% in N₂O emissions when soils were amended with biochar (Cayuela et al., 2014). However, a substantial number of studies contradict this result, they reporting no difference or even an increase in soil N₂O emissions after biochar application (Clough et al. 2010; Saarnio et al., 2013; Suddick and Six, 2013). A remarkable finding was that the same biochar could lead to opposite effects (increasing or decreasing N₂O emissions) depending on the soil to which the biochar was applied (Malghani et al., 2013; Yoo and Kang, 2012).

Soils are a major source of N₂O, which is a potent greenhouse gas and contributor to ozone layer destruction. N₂O is produced during several soil processes and its release to the atmosphere is almost entirely controlled by microbial activities. Current knowledge suggests five N₂O-genic soil microbial sources (Baggs, 2011; Spott et al., 2011). These are the nitrate or nitrite reducing processes of denitrification and dissimilatory nitrate reduction to ammonium (DNRA), and ammonia oxidation (the first step in nitrification, facilitated by ammonia oxidising bacteria). Nitrifier denitrification, the ability of ammonia oxidising bacteria to denitrify, is often also seen as a separate process. Finally, codenitrification has also been identified as a relevant N₂O formation pathway in soils (Spott et al., 2011). Understanding the mechanisms of the interactions of biochar with soil N₂O formation pathways represents a difficult challenge. No evidence has been reported that would serve to unambiguously define the cause for the observed variations (increase or decline) in soil N₂O fluxes. This is due to the extremely complex set of reactions leading to N₂O formation and consumption in soils and also to the fact that the number of studies which analyse how biochar influences specific N₂O formation pathways is still very limited.

In a recent study using the ^{15}N gas flux method, Cayuela et al. (2013) observed a consistent decrease in the $\text{N}_2\text{O}/\text{N}_2$ ratio after biochar amendment in 15 agricultural soils, pointing to denitrification as the N_2O formation pathway that biochar might be altering. According to this, biochar would enhance the last step of denitrification (*i.e.* the reduction of N_2O to N_2). Subsequently, Harter et al. (2014) found that soil biochar amendment increased the relative gene and transcript copy numbers of the *nosZ*-encoded bacterial N_2O reductase, a result which could explain the previous mechanistic findings. Nevertheless, Cayuela et al. (2013) also found contrasting results for the flux of total denitrified N ($\text{N}_2\text{O} + \text{N}_2$), which was significantly reduced in the majority of soils (10 out of 15), but highly amplified in others. No conclusive explanation was found for this paradoxical finding.

In this study we aimed to look more closely at the reasons for these contrasting results. Our hypothesis was that, besides denitrification, other microbial processes (*e.g.* nitrifier-denitrification, dissimilatory nitrate reduction to ammonia, codenitrification) could have led to N_2O and N_2 formation in these soils, mechanisms that had not been addressed in previous studies. Hence, we studied two soils that, under identical experimental conditions, showed opposite responses to biochar

amendment, *i.e.* whereas biochar addition decreased N₂O emissions in one soil, it increased emissions in the other. The main objective was to investigate by ¹⁵N gas measurements and the use of nitrification inhibitors, the main pathways leading to N₂O formation in these two soils, with the aim of understanding why biochar might be influencing N₂O emissions differently.

3.3. Materials and methods

3.3.1. Soils and biochar selected for the experiments

Two agricultural soils were selected for the experiments (Table 3.1). Soil HP was used as a reference soil, since it had been previously used in numerous studies that proved that denitrification was the major process responsible for N₂O emissions (Čuhel et al., 2010). Soil HC was selected from a series of agricultural soils because it was the only one where (under identical optimal denitrifying conditions) the addition of greenwaste biochar increased N₂O emissions. The soils were sampled from a depth of 0-0.25 m, air-dried and sieved (< 2mm).

We used a biochar produced by continuous slow pyrolysis of greenwaste at 550 °C provided by Pacific Pyrolysis Pty. Ltd. (Australia) (Table 3.1). Herbaceous and woody biochars have been found to be the most promising for mitigating N₂O emissions from soil (Cayuela et al., 2014). Therefore, this biochar was selected for its mitigation potential and as a representative standard biochar commonly used in other studies. The biochar was ground to a particle size <1mm before soil application.

Table 3. 1. Physical and chemical characteristics of soil and biochar samples used in the experiments.

| | SOIL HP | SOIL HC | BIOCHAR |
|---|-------------------|---------------------------------|----------------|
| Management | Pasture | Olive orchard (organic farm) | -- |
| Location | 48°52' N 14°13' E | 38°23' N 1°22' W | -- |
| Classification (WRB) | Haplic Phaeozem | Haplic Calcisol | |
| Texture | Loamy Sand | Sandy loam | -- |
| Sand (%) | 78 | 57 | -- |
| Clay (%) | 6 | 16 | -- |
| Volatile matter (%) | -- | -- | 26.8 |
| Ash (%) | -- | -- | 7.0 |
| H:C _{Org} | -- | -- | 0.534 |
| pH (in water, 1:20 w:w 25°C) | 6.89 | 8.01 | 7.87 |
| EC (μS cm ⁻¹) | 140 | 518 | 166 |
| Ca CO ₃ (%) | -- | 30 | -- |
| TOC (g kg ⁻¹) | 11.6 | 16.8 | 701.7 |
| Total N (g kg ⁻¹) | 2.0 | 2.4 | 2.7 |
| WSC (mg kg ⁻¹) | 439.5 | 694.0 | 285.1 |
| DOC (mg kg ⁻¹) | 315.7 | 356.9 | 113.2 |
| WSN (mg kg ⁻¹) | 34.7 | 74.0 | 8.6 |
| DON (mg kg ⁻¹) | 10.2 | 35.9 | 7.1 |
| NH ₄ ⁺ - N (mg kg ⁻¹) | 19.3 | 5.0 | 1.3 |
| NO ₂ ⁻ -N (mg kg ⁻¹) | <0.2 | 16.2 | <0.2 |
| NO ₃ ⁻ -N (mg kg ⁻¹) | 5.3 | 16.9 | <0.2 |

TOC: total organic carbon; WSN: water soluble nitrogen; DON: dissolved organic nitrogen; WSC: water soluble carbon, DOC: dissolved organic carbon.

3.3.2. Microcosms experiments

The incubation experiments were performed in 250 ml polypropylene jars at optimum conditions for denitrification: 25 °C and moisture content of 90% water filled pore space (WFPS). The control treatments consisted of 100 g dry soil and the biochar treatments of 98 g dry soil and 2 g biochar (2% w:w). The biochar was thoroughly mixed with the dry soil to obtain a completely homogeneous mixture. Subsequently deionized water (or a solution containing the appropriate concentration of N fertiliser) was added to reach 90% WFPS (and the required N concentration in the fertilised treatments). The jars were incubated aerobically, covered with a polyethylene sheet that allows gas exchange but minimises evaporation. Moisture was gravimetrically adjusted every other day with the addition of deionised water for each individual jar. The experiments were laid out as randomised block designs with four replicates per treatment.

Experiment 1. Impact of biochar on soil N₂O emissions and mineral N after the addition of different N fertilisers.

A set of 48 jars (2 soils (HP/HC) x 2 management treatments (biochar/control) x 3 fertilisation treatments (no fertiliser /KNO₃ / CO(NH₂)₂) x 4 replicates) was set up for the first experiment. The fertilisers were homogeneously distributed in the soil at a rate of 200 kg N Ha⁻¹ (corresponding to 55 mg N kg⁻¹ based on a plough layer of 25 cm). N₂O samples were taken twice a day during the first two days decreasing subsequently to daily measurements, then every other day, then three times per week, etc. (see Figure 3.1). At the end of the incubation (14 days) mineral N (NH₄⁺, NO₃⁻ and NO₂⁻) was extracted and determined in all jars.

Experiment 2. Isotopic composition of N₂O and N₂ emitted after application of labelled ¹⁵N fertilisers.

The following ¹⁵N-tracer experiments were performed:

- (i) Soil HP + ¹⁵NO₃⁻, vs soil HP + ¹⁵NO₃⁻ + biochar,
- (ii) Soil HC + ¹⁵NO₃⁻ vs soil HC + ¹⁵NO₃⁻ + biochar,
- (iii) Soil HC + CO(¹⁵NH₂)₂ vs Soil HC + CO(¹⁵NH₂)₂ + biochar

Moisture was adjusted to 90% WFPS in each jar by adding the required volume of a solution containing $K^{15}NO_3$ or $CO(^{15}NH_2)_2$ (>99% ^{15}N enrichment) at the appropriate concentration to obtain 90% WFPS and exactly 5.5 mg of ^{15}N -per jar. Rewetting the soils in this way guaranteed a homogenous ^{15}N pool. Gas samples for isotopic analysis were taken daily during the first three days and on day 10. For each treatment, two gas samples were collected using a 12-ml syringe and needle: one immediately after the screw cap was fitted to the jar ($t=0$) and the second after 60 min ($t = 60$). The gas samples were transferred to 12-ml vials (Labco) previously purged with He and evacuated. Selected samples (a total of 192 samples) were analysed for the isotope ratios of N_2 (29/28 (29R) and 30/28 (30R)) and N_2O (45/44 (45R) and 46/44 (46R)) by automated isotope ratio mass spectroscopy (ThermoFinnigan GasBench & PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany)).

Experiment 3. N₂O emissions, mineral N and N₂O isotopic composition after addition of NO₂⁻ in soil HC.

Experiments 1 and 2 were reproduced in soil HC with a different source of nitrogen: NaNO₂ was added to a set of 8 jars (4 replicates x 2 management treatments (biochar/control)) and homogeneously distributed in the soil at a rate of 200 kg N Ha⁻¹. N₂O and final concentrations of mineral N were determined as for experiment 1 (see Figure 3.5).

Subsequently, the following ¹⁵N tracer experiment was performed: Soil HC + Na¹⁵NO₂ vs Soil HC + Na¹⁵NO₂ + biochar (as for experiment 2).

Moisture was adjusted to 90% WFPS in each jar by adding the required volume of a solution containing NaNO₂ (>98% ¹⁵N enrichment) at the appropriate concentration to obtain 90% WFPS and exactly 5.5 mg of ¹⁵N-per jar. Gas samples for isotopic analysis were taken daily during the first three days and on the 10th day of incubation in the same way as in experiment 2. A total of 64 gas samples (2 management treatments (biochar/control) x 4 replicates x 4 days (1/2/3/10) x 2 times per day (t=0/t=60)) were analysed.

Experiment 4. N₂O emissions and mineral N after addition of dicyandiamide to soil HC.

The nitrification inhibitor dicyandiamide (DCD) was applied in combination with N fertilisers in soil HC. DCD inhibits the first stage of nitrification, the oxidation of NH₄⁺ to NH₂OH, by rendering the enzyme ammonia monooxygenase (AMO) ineffective. It is not a bactericide, and does not affect other heterotrophs responsible of the soil biological activity (Zacherl and Amberger, 1990).

A set of 24 jars (2 management treatments (biochar/control) x 3 fertilisation treatments (no fertiliser /KNO₃ /CO(NH₂)₂) x 4 replicates) was set up for the experiment. DCD was applied at a rate of 30 mg kg⁻¹ soil to ensure its persistence over the entire incubation period (Rajbanshi et al., 1992). The fertilisers were homogeneously distributed in the soil at the same rate as in the previous experiments (200 mg N Ha⁻¹) in the solution including the DCD. N₂O samples were taken following the same intervals as in experiment 1. Mineral N (NH₄⁺, NO₃⁻ and NO₂⁻) was also extracted and determined in all jars at the end of the incubation period.

3.3.3. N₂O sampling and measurements.

For N₂O sampling each unit was sealed with gas-tight polypropylene screw caps for an accumulation period of 60 min. The headspace gas was then sampled directly with a membrane air pump (Optimal 250, Schego, Offenbach am Main, Germany), attached to a gas chromatograph (VARIAN CP-4900 Micro-GC, Palo Alto, CA, USA) (Mondini et al., 2010).

N₂O fluxes were calculated assuming a linear increase during the accumulation (closed) period, an approach which was verified prior to the experiments. Cumulative N₂O was calculated assuming linear changes in fluxes between adjacent measurement points (Velthof et al., 2003).

3.3.4. Chemical-physical analyses of biochar and soils

3.3.4.1. Biochar

Proximate analysis was conducted using ASTM D1762-84 Chemical Analysis of Wood Charcoal. Total N and C were analysed by automatic elemental analysis (FlashEA 1112 Series, Thermo scientific, Madrid, Spain). Water soluble C and N were determined in 1:10 (w/v) water extracts using a Photometer Nanocolor 500D MACHEREY-NAGEL. Electrical conductivity

(EC) and pH were determined in a 1:10 (w/v) water-soluble extract. NH_4^+ was extracted with 1.0 M KCl at 1:10 (w/v) and determined by a colorimetric method based on Berthelot's reaction. NO_3^- and NO_2^- were extracted with water at 1:10 (w/v) and determined by ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland).

3.3.4.2. Soil

Soil texture was determined using the pipette method according to Kettler et al. (2001). Soils were extracted by shaking four replicates of moist soil (1/10, w/v dry weight basis) with 2.0M KCl (for NH_4^+) or water (for NO_3^- and NO_2^-) for two hours. Extracts were centrifuged (2509 G) and filtered (0.45 mm) before analysis. NH_4^+ was determined by a colorimetric method based on Berthelot's reaction. NO_3^- and NO_2^- were determined by ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland).

3.3.5. ^{15}N calculations

The ^{15}N atomic fraction in N_2O was calculated from the 45/44 and 46/44 ratios of N_2O . The ^{15}N gas-flux method (Mulvaney and Boast, 1986; Stevens and Laughlin, 2001; Stevens et al., 1993) was used to quantify N_2O

and N₂ emissions from denitrification in soil HP. The molar fraction of ¹⁵N-NO₃⁻ (¹⁵X_N) in the soil pool was calculated from D45R and D46R according to Stevens and Laughlin (2001). The flux of N₂ and N₂O was then calculated by the equations given by Mulvaney and Boast (Mulvaney and Boast, 1986). The presence of hybrid nitrous oxide (⁴⁵N₂O) co-metabolically introduced into the reaction pathway of denitrification was tested by the model developed by Spott and Florian Stange (2011). This model considers two different N sources, where each source generates non-hybrid N₂O (⁴⁶N₂O and ⁴⁴N₂O) and, simultaneously, both N sources can be combined to form hybrid N₂O (⁴⁵N₂O). According to this model, the contribution of each pathway to the total N₂O formation can be calculated from the mass distribution of the released N₂O and the ¹⁵N mole fraction of the labeled N source (Spott and Florian Stange, 2011).

3.3.6. Statistical Analysis

Univariate analysis of variance was used to investigate the significant differences in N₂O emissions and mineral N concentrations between biochar and control treatments with IBM SPSS Statistics 21, Sommers, USA.

3.4. Results

Experiment 1. Cumulative N₂O emissions and mineral N in soils HP and HC

Soil HP emitted N₂O when NO₃⁻ was added but not in the absence of fertiliser or after the addition of urea. In this soil, biochar significantly reduced N₂O emissions, by an average of 76% (Figure 3.1 (A1), (A2), (A3)).

Soil HC emitted N₂O in all treatments: without N fertilisation, after the addition of NO₃⁻ and urea. In this soil, biochar consistently increased total cumulative N₂O emissions and the average increase was larger in the non-fertilised (95%) and urea (129%) treatments than in the NO₃⁻ treatment (54%), (Figure 3.1 (B1), (B2), (B3)).

Comparing treatments without biochar, the addition of NO₃⁻ increased total N₂O emissions in soil HP (from 54 to 11580 mg N₂O-N kg⁻¹ soil), whereas it increased N₂O emissions slightly in soil HC (from 3443 to 4546 mg N₂O-N kg⁻¹ soil). The addition of urea had no impact on soil HP, and increased emissions in soil HC (from 3443 to 5799 mg N₂O-N kg⁻¹ soil).

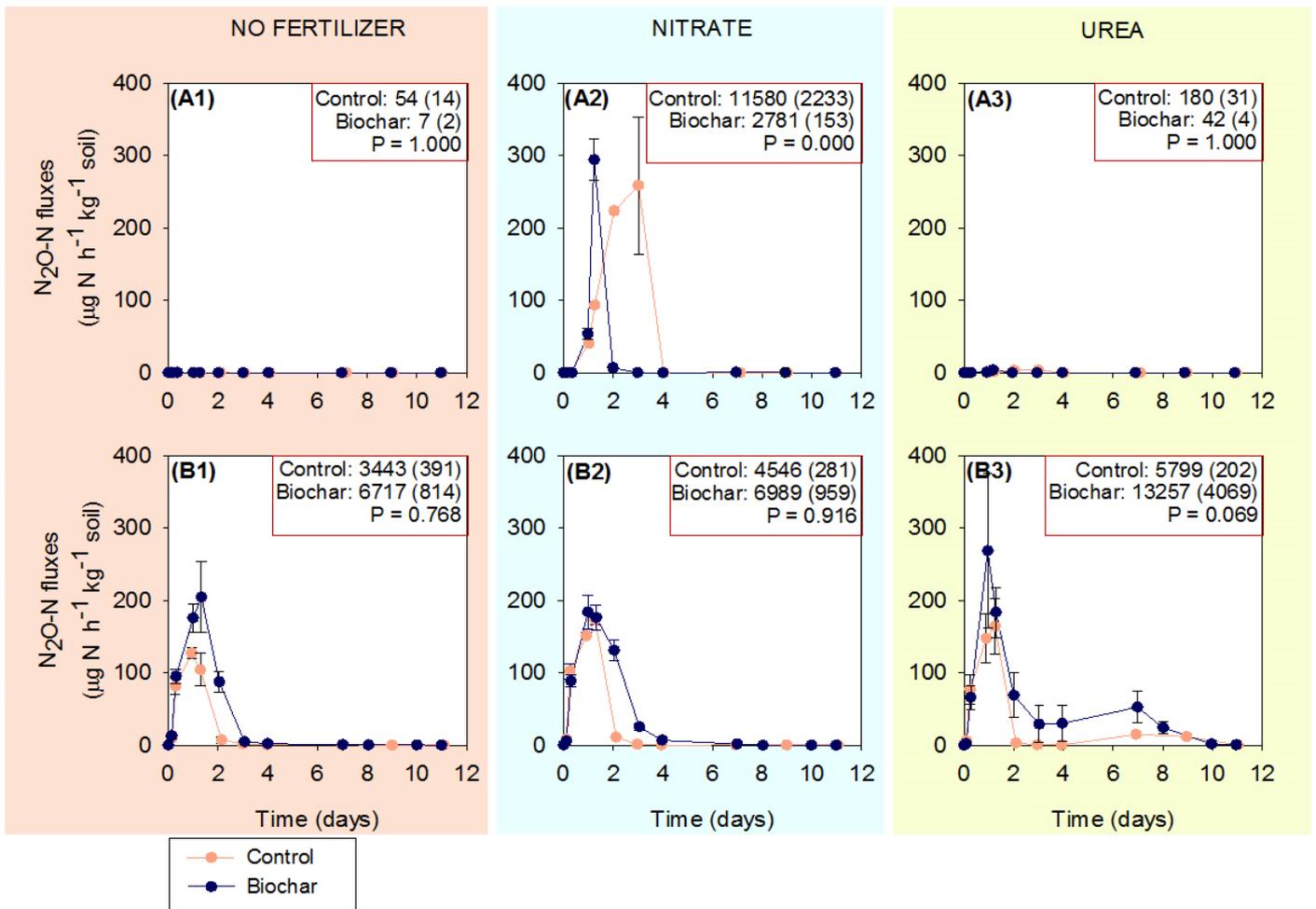


Figure 3. 1. Fluxes of N₂O in soils HP and HC in control and biochar treatments (mean ± standard error; n=4). (A1-A3) correspond to soil HP unfertilised, fertilised with KNO₃ and fertilised with CO(NH₂)₂ respectively. (B1-B3) correspond to soil HC unfertilised, fertilised with KNO₃ and fertilised with CO(NH₂)₂ respectively. Cumulative N₂O emissions (mg N₂O-N kg⁻¹ soil) and standard errors by the end of the incubation are reported in the right-above box for each treatment.

Figure 3.2 shows NH_4^+ -N concentration in soils HP and HC at the end of the experiment. The original concentration of NH_4^+ in soil HP was 19.3 mg N kg⁻¹ soil. After 14 days of incubation, soil HP underwent a significant increase in NH_4^+ content for all fertilisation treatments (74.5 - 110.4 mg N kg⁻¹ soil). The highest increase was observed when soil HP was fertilised with urea. Biochar addition did not have a significant impact on the final NH_4^+ concentration in this soil.

Soil HC similarly increased its NH_4^+ concentration throughout the incubation (initial concentration: 2.8 mg kg⁻¹ soil), excluding the KNO_3 treatment. In this soil biochar significantly decreased the amount of NH_4^+ by the end of the incubation for the non-fertilised soil. Biochar also decreased mean NH_4^+ concentration in the urea treatment, although not significantly due to the high variability in the biochar samples.

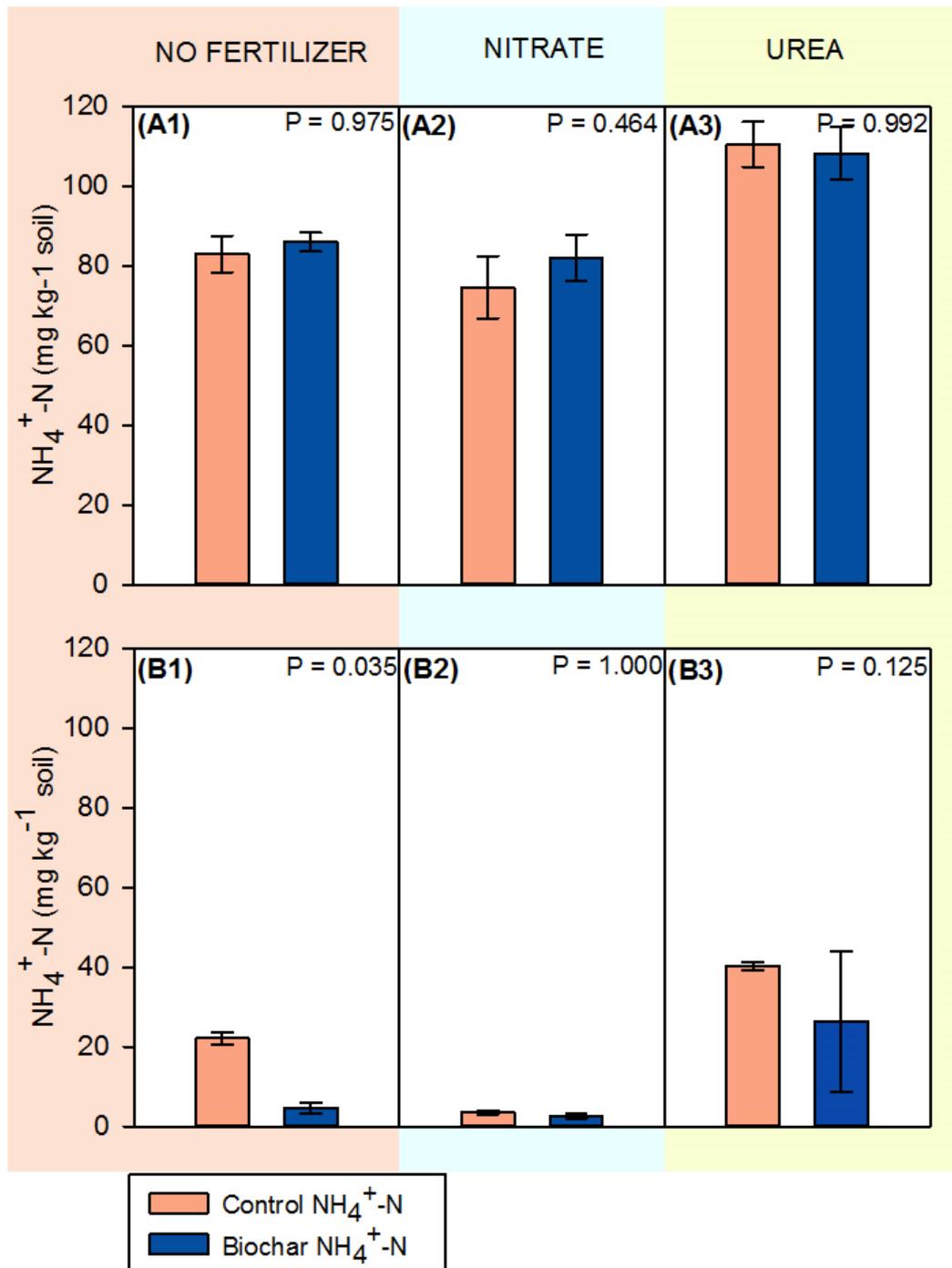


Figure 3. 2. NH₄⁺-N concentrations in soils HP and HC after 14 days of incubation (mean ± standard error; n=4). (A1-A3) correspond to soil HP unfertilised, fertilised with KNO₃ and fertilised with CO(NH₂)₂ respectively. (B1-B3) correspond to soil HC unfertilised, fertilised with KNO₃ and fertilised with CO(NH₂)₂ respectively.

Figure 3.3 shows $(\text{NO}_3^- + \text{NO}_2^-)$ -N concentrations in soils HP and HC. The concentrations of $(\text{NO}_3^- + \text{NO}_2^-)$ -N in soil HP were very low ($<2.0 \text{ mg kg}^{-1}$) for all fertilisation treatments and biochar did not have a significant impact. However, NO_2^- was detected in biochar amended soils and not in the control. Soil HC had low $(\text{NO}_3^- + \text{NO}_2^-)$ -N concentrations when no fertiliser was added or after the addition of urea. In contrast, $33.3 \text{ mg of NO}_3^- \text{-N kg}^{-1}$ were found in the KNO_3 treatment irrespective of the biochar addition.

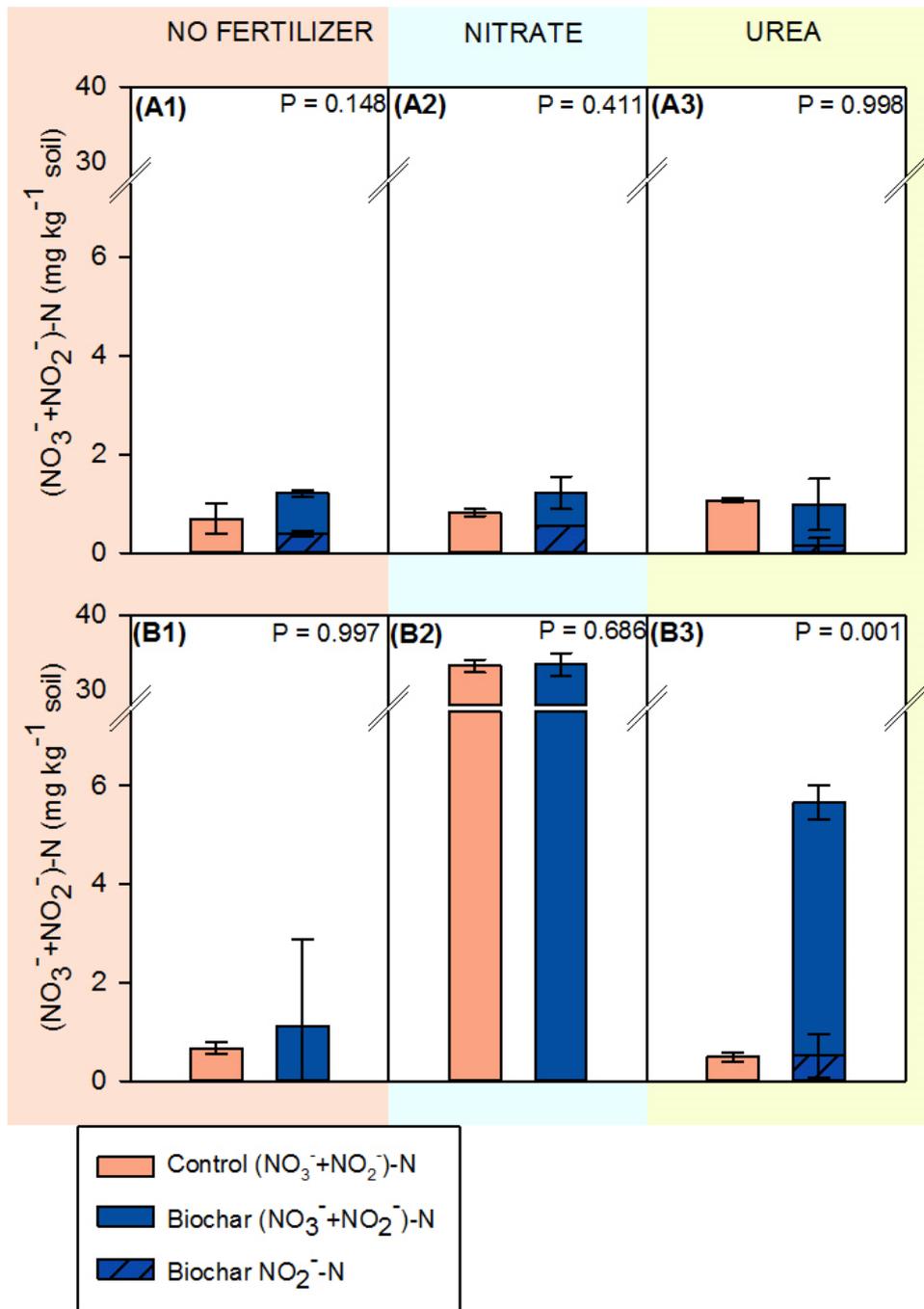


Figure 3. 3. $(NO_3^- + NO_2^-)\text{-N}$ concentrations in soils HP and HC after 14 days of incubation (mean \pm standard error; n=4). (A1-A3) correspond to soil HP unfertilised, fertilised with KNO_3 and fertilised with $CO(NH_2)_2$ respectively. (B1-B3) correspond to soil HC unfertilised, fertilised with KNO_3 and fertilised with $CO(NH_2)_2$ respectively.

Experiment 2. Isotopic composition of N₂O emitted from soils HP and HC

Figure 3.4 shows the ¹⁵N atomic fraction in N₂O emitted from soils HP and HC in experiment 2. When ¹⁵NO₃⁻ was added, the initial ¹⁵N atomic fraction in N₂O emitted from soil HP was 0.74, decreasing gradually to reach 0.04 at day 10 (Figure 3.4 (A)). In contrast, the ¹⁵N isotopic composition in soil HC followed totally different dynamics: the initial ¹⁵N atomic fraction in N₂O was only 0.18; it increased slightly to 0.33 by day three, and reached a final value of 0.10 by day 10 (Figure 3.4 (B1)). Biochar altered the isotopic composition of N₂O emitted in both soils.

When urea was added, soil HP did not emit N₂O (Figure 3.1 (A3)). In soil HC (even when emissions were high) the initial ¹⁵N atomic fraction in N₂O was zero (Figure 3.4 (B2)), it successively increased, but always remained beneath 0.15. The biochar and control treatments showed identical ¹⁵N-N₂O concentration dynamics.

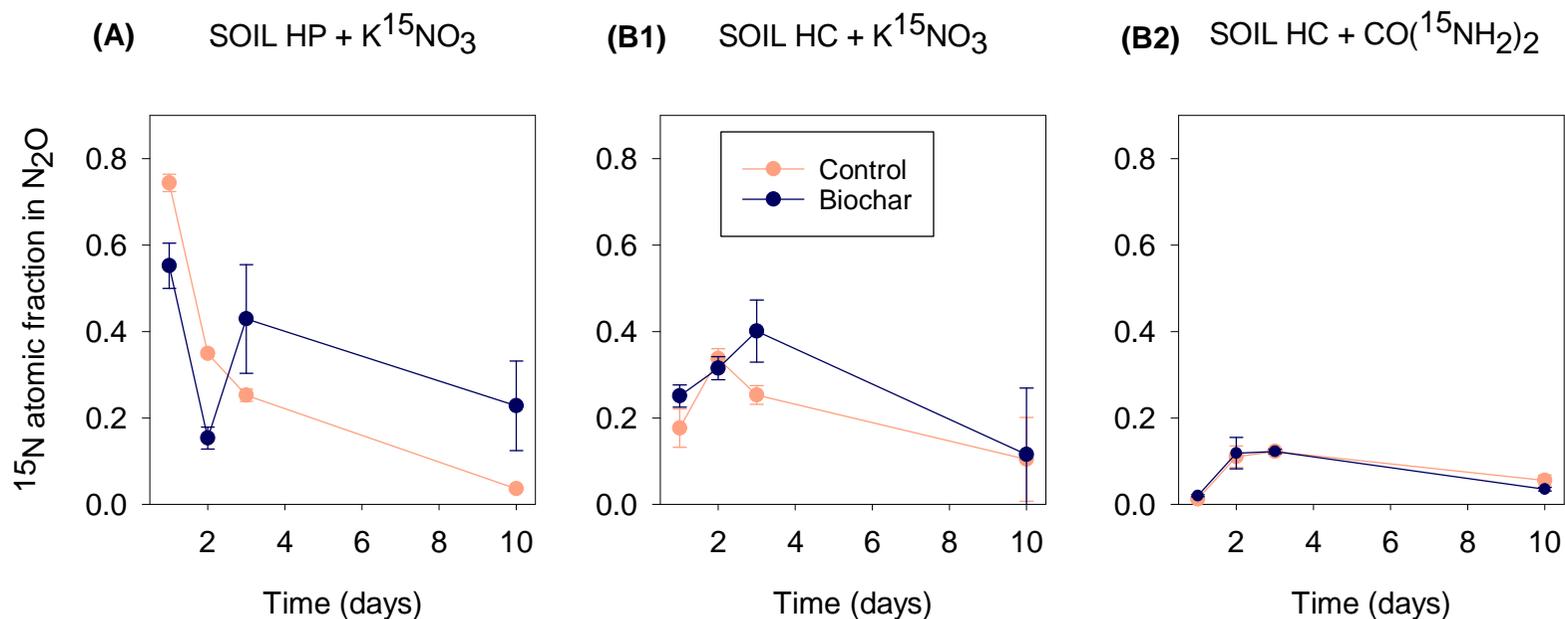


Figure 3. 4. ¹⁵N atomic fraction in N₂O emitted from soils HP and HC in control and biochar treatments after 1, 2, 3 and 10 days of incubation (mean ± standard error, n=4). **(A)** corresponds to soil HP fertilised with K¹⁵NO₃ (>99% enrichment). **(B1)** and **(B2)** correspond to soil HC amended with K¹⁵NO₃ and CO(¹⁵NH₂)₂ respectively (both at >99% enrichment).

Table 3.2 shows the molar fraction of $^{15}\text{N-NO}_3^-$ and the ratio $\text{N}_2\text{O}/(\text{N}_2+\text{N}_2\text{O})$ calculated by the ^{15}N gas flux method (Mulvaney and Boast, 1986) and the contribution of codenitrification to N_2O formation according to Spott and Florian Stange (2011) in soil HP. The ratio $\text{N}_2\text{O}/(\text{N}_2+\text{N}_2\text{O})$ was very high during the first three days, which demonstrates that most N was lost as N_2O . Biochar decreased the $\text{N}_2\text{O}/\text{N}_2$ ratio, particularly at day three (the peak of emissions in the control soil). The contribution of codenitrification was zero (see C in Table 3.2). This method of calculation could not be applied to soil HC, since other mechanisms than denitrification were operating in this soil and we could not calculate the enrichment of the source ($^{15}\text{NO}_3^-$ in soil ($^{15}\text{X}_\text{N}$)) (Mulvaney and Boast, 1986). Nonetheless, we found a high proportion of N_2O with a hybrid bond ($^{45}\text{N}_2\text{O}$) in soil HC.

Table 3. 2. Means and standard deviations (n=4) of $^{15}\chi_N$, the ratio $N_2O/(N_2+N_2O)$ and the three fractions (A, B, C) of hybrid and non-hybrid N_2O (Spott and Florian Stange, 2011) in soil HP.

| Parameter | Treatment | Time (days) | | | |
|--|-----------|-----------------|-----------------|----------------|----------------|
| | | 1 | 2 | 3 | 10 |
| $^{15}\chi_N$ (molar fraction of ^{15}N - NO_3^- in soil, calculated by the ^{15}N gas flux method) | Control | 0.98 (0.00) | 0.99 (0.00) | 0.99 (0.00) | 0.84 (0.01) |
| | Biochar | 0.99 (0.00) | 0.99 (0.00) | 0.92 (0.06) | 0.91 (0.09) |
| $N_2O/(N_2+N_2O)$ (calculated by the ^{15}N gas flux method) | Control | 1.01 (0.12) | 0.99 (0.01) | 0.99 (0.00) | 0.14 (0.27) |
| | Biochar | 0.93 (0.05) | 0.89 (0.08) | 0.04 (0.05) | 0.05 (0.08) |
| A (fraction of nonhybrid N_2O from the unlabeled source) | Control | 0.19 (0.05) | 0.02 (0.02) | 0.00 (0.00) | 0.95 (0.00) |
| | Biochar | 0.03 (0.03) | 0.01 (0.01) | 0.48 (0.28) | 0.75 (0.22) |
| B (fraction of nonhybrid N_2O from the labeled source) | Control | 0.81 (0.05) | 0.98 (0.02) | 1.00 (0.00) | 0.05 (0.00) |
| | Biochar | 0.99 (0.03) | 1.00 (0.01) | 0.49 (0.31) | 0.24 (0.22) |
| C (fraction of hybrid N_2O formed by a 1:1 linkage of labeled and unlabeled sources) | Control | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| | Biochar | -0.02 (0.00) | -0.02 (0.00) | 0.04 (0.03) | 0.01 (0.00) |

Experiment 3. N₂O emissions, ¹⁵N isotopic composition and mineral N after fertilisation of soil HC with NO₂⁻

Addition of NO₂⁻ to soil HC produced the highest N₂O emissions peak monitored in this soil (Figure 3.5 (B1)); fourfold higher than that of the non-fertilised soil (Figure 3.1 (B1)). Under these conditions, the biochar amendment did not modify cumulative N₂O emissions.

The ¹⁵N atomic fraction in N₂O (Figure 3.5 (B2)) followed a different pattern than with ¹⁵NO₃⁻ (experiment 2; Figure 3.4 (B1)). The initial ¹⁵N atomic fraction in the N₂O emitted was 0.30, decreasing gradually to reach 0.06 at day 10 (Figure 3.4 (A)). Biochar did not significantly modify this pattern.

The biochar amended soil had a significantly lower concentration of NH₄⁺ at the end of the incubation (Figure 3.5 (B3)). The concentration of NO₃⁻ was low (below 5 mg kg⁻¹ soil) and not affected by biochar addition.

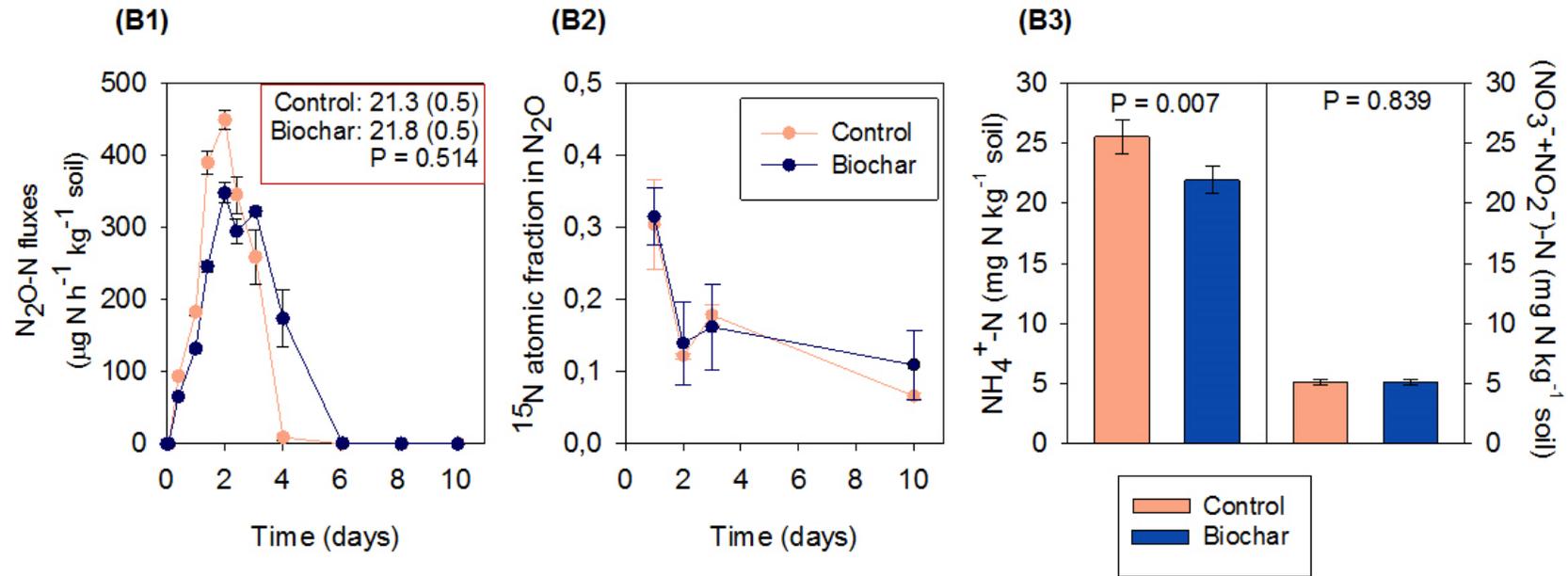


Figure 3. 5. Fluxes of N₂O (B1); ¹⁵N atomic fraction in N₂O at days 1, 2, 3 and 10 (B2) and NH₄⁺-N and NO₃⁻-N concentrations after 14 days of incubation (B3) in soil HC (mean ± standard error, n=4). Soil HC had been fertilised with Na¹⁵NO₂ (>98% enrichment). Cumulative N₂O emission (mg N-N₂O kg⁻¹ soil) and standard error by the end of the incubation is reported in the right-above box in B1.

Experiment 4. Impact of the nitrification inhibitor dicyciandiamide (DCD) on N₂O emissions and mineral N concentration in soil HC.

N₂O emissions almost ceased when DCD was added to soil HC (Figure 3.6). The highest emissions were observed when the soil was fertilised with NO₃⁻ (Figure 3.6 (B2)), but still represented less than 0.4% of the added N (compared to 12.7% without DCD (Figure 3.1 (B2))).

The highest NH₄⁺ concentrations were found in the soil amended with urea, followed by the non-fertilised soil and the soil amended with KNO₃. Biochar (compared to the control) systematically decreased the concentration of NH₄⁺ by the end of the incubation for all treatments (non-fertilised soil, KNO₃ and urea). NO₃⁻ concentration was lower than the original in soil (16.9 mg NO₃⁻-N kg⁻¹ soil).

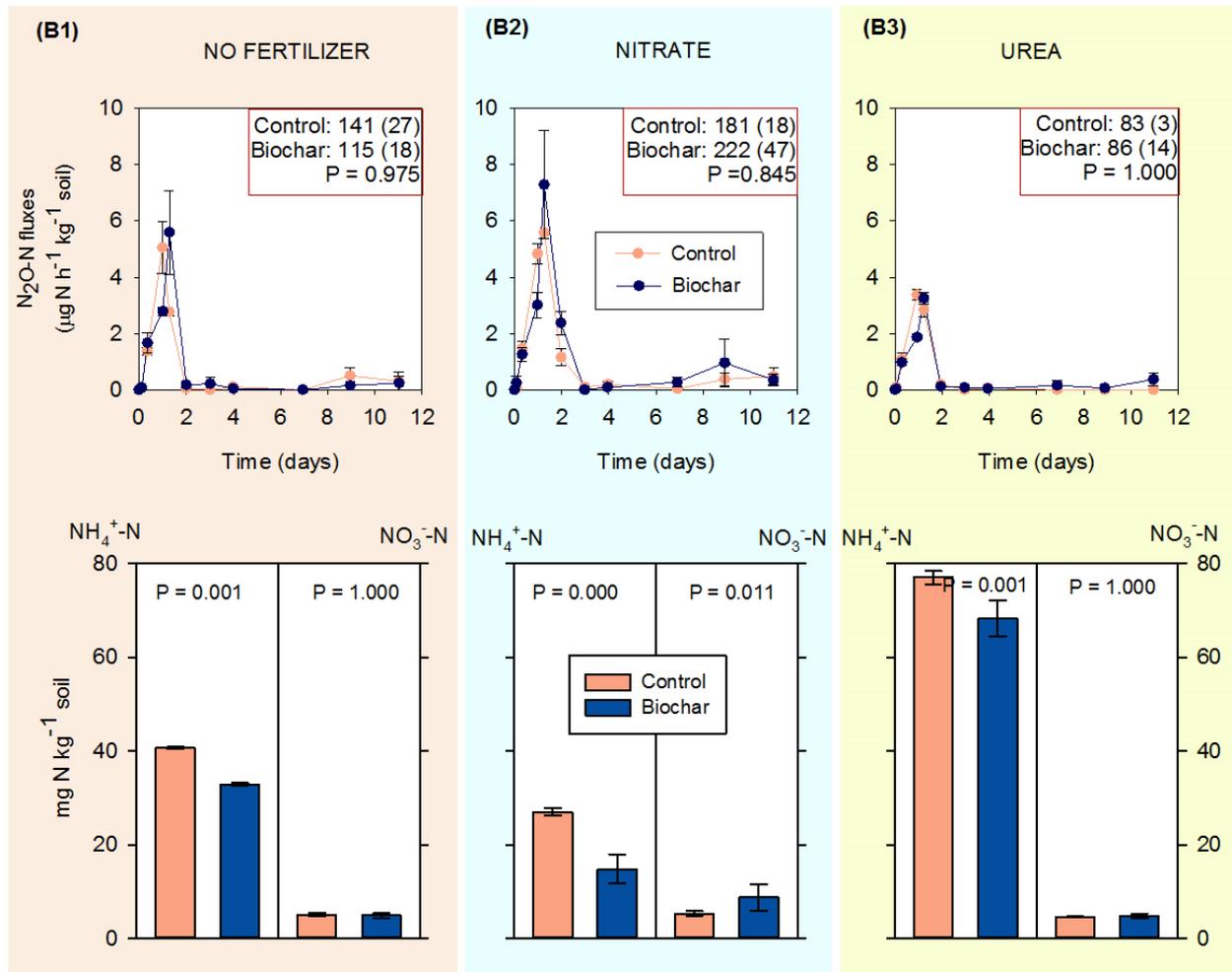


Figure 3. 6. Fluxes of N₂O (above) and NH₄⁺-N and NO₃⁻-N concentrations during 14 days of incubation (below) in soil HC containing DCD (30 mg kg⁻¹ soil) in control and biochar treatments (mean ± standard error, n=4). (B1-B3) correspond to unfertilised soil, fertilised with KNO₃ and fertilised with CO(NH₂)₂ respectively. Cumulative N₂O emissions (mg N-N₂O kg⁻¹ soil) and standard errors by the end of the incubation are reported in the right-above box for each treatment.

3.5. Discussion

3.5.1. Predominant N₂O formation pathways in soil HP and HC

Nitrous oxide emissions patterns and their response to the addition of different N fertilisers were different in soils HP and HC, which clearly reflected the different N₂O production pathways involved.

Figure 3.7 illustrates the main pathways for N₂O formation in soil. Ammonia oxidation takes place in two steps: first NH₃ is oxidised to NH₂OH, which is then oxidised to NO₂⁻. N₂O may be directly released as a by-product of ammonia oxidation (nitrifier-nitrification) (Hooper and Terry, 1979) or it can be produced through a denitrification pathway where NO₂⁻ is reduced to N₂O (nitrifier-denitrification) (Kool et al., 2011). The ability to denitrify is a widespread, if not ubiquitous, attribute in ammonia oxidisers (Shaw et al., 2006). Classically, denitrification (from NO₃⁻) has been considered the main N₂O formation pathway in soils. However, other pathways that have been systematically overlooked in soil studies could play a more important role than originally estimated (Baggs, 2011; Spott et al., 2011). This is the case for codenitrification, which is potentially a widespread pathway of microbial N transformation in terrestrial environments (Spott et al. 2011) and dissimilatory nitrate reduction to ammonia (DNRA) (Giles et al., 2012).

Although our knowledge of microbial N transformation in soil has evolved significantly over the last decades, recent findings show that, even today, our understanding of N₂O formation and consumption in soil is still very limited (Long et al., 2013; Sanford et al., 2012).

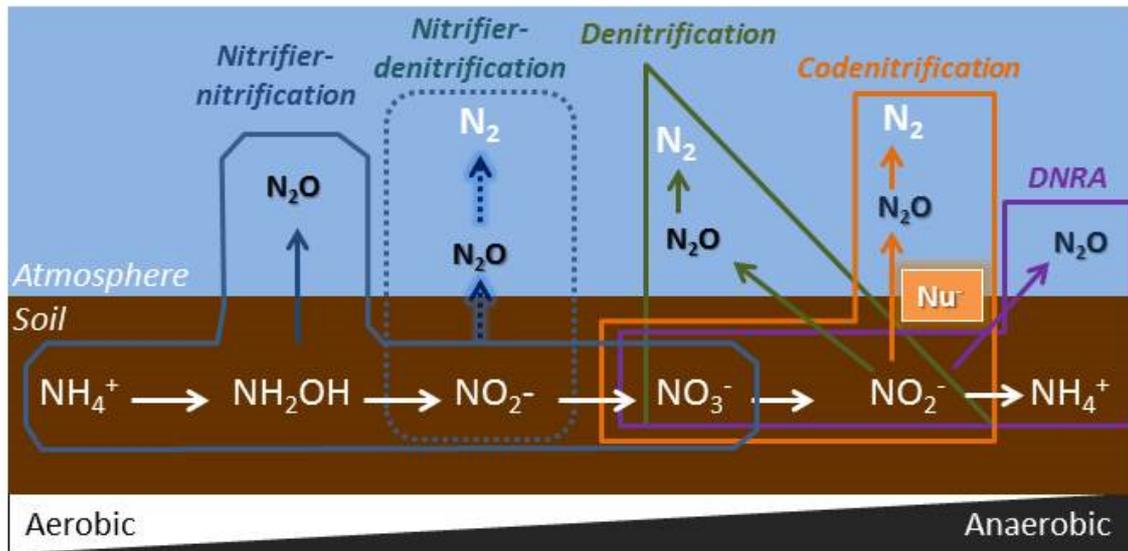


Figure 3. 7. Microbial sources of N₂O during transformations of mineral nitrogen in soil. Nu⁻: nucleophile (e.g. R-NH₂, NH₄⁺, amino acids or other organic N compounds). During codenitrification, nitrous acid reacts with a nucleophile in soil through nitrosation reactions forming a hybrid N-N bond (Spott *et al*, 2011)); DNRA: dissimilatory nitrate reduction to ammonium.

In the nearly water-saturated soil conditions used in our experiments (90% WFPS), N₂O production is expected to be dominated by denitrification of NO₃⁻. This was the case in soil HP, where emissions were clearly controlled by the conventional denitrification pathway. This can be deduced from the following facts: (i) This soil only emitted N₂O after the addition of NO₃⁻ (Figure 3.1 (A2)); (ii) the ¹⁵N atomic fraction of the N₂O emitted at day one was 0.74 (Figure 3.4 (A1)), which shows that N₂O was

primarily produced from the added $^{15}\text{NO}_3^-$. The ^{15}N atomic fraction decreased over time, showing the depletion of the labelled source; (iii) given the limited nitrification activity detected in this soil, addition of NO_3^- did not increase the final NH_4^+ concentration (with respect to the non-fertilised soil), which suggests that DNRA was not a relevant pathway and (iv) applying the equations developed by Spott and Florian Stange (2011), codenitrification was found to be null (Table 3.2).

As previously found in other soils under analogous optimal denitrifying conditions (Cayuela et al., 2013), biochar significantly decreased total N_2O emissions in this soil.

In soil HC, the weak response of N_2O emissions to NO_3^- addition pointed out to a low contribution of denitrification or DNRA in this soil. Given that the original NO_3^- concentration in the soil was $16.9 \text{ mg N kg}^{-1}$ at a natural abundance of 0.364% ^{15}N , and that we added 55 mg N kg^{-1} of $^{15}\text{NO}_3^-$ (>99% enrichment), the ^{15}N - NO_3^- enrichment in the soil at the beginning of the incubation was 75.8%. Yet, the ^{15}N atomic fraction in the N_2O emitted at day one (Figure 3.4 (B1)) was only 0.18, which demonstrates that some N_2O originated from denitrification, but also that NO_3^- was not the only source of N_2O . Moreover, the low C:N ratio of this soil and the NH_4^+

concentration at the end of the incubation in the KNO₃ treatment (Figure 3.2 (B2)) indicates that DNRA was not a major N₂O formation route in this soil (Giles et al., 2012). Instead, we hypothesise that N₂O formation in soil HC was mainly the result of nitrification-mediated processes. The results supporting this hypothesis can be summarised: (i) The addition of extra NO₃⁻ did not increase N₂O emissions in this soil, whereas the addition of extra urea did; (ii) the ¹⁵N atomic fraction of the N₂O emitted at day one was 17.7% (Figure 3.4 (B1)), which shows that N₂O was not predominantly formed from the added ¹⁵NO₃⁻. (iii) The concentration of dissolved organic N in this soil was very high (35.9 mg N kg⁻¹soil), which can explain the low contribution of the labelled urea to the emitted ¹⁵N₂O (Figure 3.4 (B2)). However, significant hybrid N₂O (⁴⁵N₂O) was produced (data not shown) and we cannot discard the contribution of codenitrification to N₂O formation in soil HC.

To better understand which processes (within nitrification-mediated pathways) biochar might be modifying we performed experiments 3 and 4.

3.5.2. Impact of biochar in N₂O by nitrification-mediated pathways

In experiment 3 the addition of NO₂⁻ to soil HC showed that, under high moisture conditions, this soil was able to rapidly reduce NO₂⁻ to N₂O, which was emitted in large quantities (38% of added NO₂⁻-N). It is very unlikely that the N₂O emitted was just the product of the chemical decomposition of NO₂⁻ (chemodenitrification), since this process, largely controlled by soil pH, only occurs in neutral and acidic soils (Bremner, 1997). Instead, NO₂⁻ was most probably used as electron acceptor for microbial respiration (nitrifier-denitrification). The high N₂O production in experiment 3 (21.3 mg N kg⁻¹ compared to 3.4 mg N kg⁻¹ in experiment 1) may be related to enhanced nitrifier-denitrification for detoxifying NO₂⁻ (Jung et al., 2014).

The subsequent tracer experiment with application of ¹⁵NO₂⁻, demonstrated that significant nitrite reduction to N₂O occurs (the N₂O originating from the added ¹⁵NO₂⁻ at day one was 31.5%, see Figure 3.5 (B2)), but also that it could not be the only process leading to N₂O emissions. This experiment demonstrated that biochar was not increasing N₂O emissions through the nitrifier-denitrification pathway, since N₂O emissions in the biochar and control treatments were not statistically different.

In our final experiment (experiment 4), the high NH₄⁺ and low NO₃⁻ concentrations by the end of the experiment demonstrate the effectiveness of the DCD treatment to inhibit ammonia oxidation, which correlated with a large decrease in N₂O emissions for all treatments. We assumed that DCD did not inhibit other possible N₂O formation pathways. Although the impacts of DCD on other aspects of microbial N transformation in soil are largely unknown, Bremner and Yeomans (1986) demonstrated that DCD does not inhibit N₂O and N₂ emissions by denitrification when applied at similar rates to those used in this study. More recently, Wakelin et al. (2013) also demonstrated in a field study that the application of DCD had a minor impact on denitrifying bacteria activity (*nirS*).

Addition of biochar significantly and consistently decreased the NH₄⁺ concentration in soil HC. These results reinforce our conclusion that the production of N₂O in soil HC must be the consequence of nitrification processes (nitrifier-nitrification and associated nitrifier-denitrification). It seems that biochar does not promote the denitrification from NO₂⁻ (as was deduced from experiment 3), but it does promote the oxidation of ammonia and concomitantly the formation of N₂O through nitrifier-nitrification. Clearly, if biochar raises the production of NO₂⁻ in soil, it will

intrinsically enhance its denitrification (nitrifier-denitrification) when the soil is under low oxygen conditions (as in our experiments).

Our results are in agreement with recent findings by Prommer et al. (2014), who showed that biochar promotes soil ammonia-oxidiser populations and accelerates gross nitrification rates in a calcareous arable soil. The importance of nitrifier-nitrification and nitrifier-denitrification for N₂O production in calcareous soils has been recently documented by Huang et al. (2014), who demonstrated that these processes accounted for 35-53% and 44-58% of total N₂O emissions, respectively.

Here we present preliminary evidence that explains how biochar might affect N₂O emissions differently depending on the N₂O formation pathway operating in the soil. When denitrification was the main N₂O formation pathway (soil HP), biochar was found to decrease the N₂O/(N₂+N₂O) ratio (Table 3.2), which is in agreement with previous findings (Cayuela et al., 2013). Recent studies have reported that biochar promotes an increase in the abundance of nitrous oxide reductase (*nosZ*) in soil (Harter et al., 2014), an enzyme that enhances the reduction of N₂O to N₂ (the last step in denitrification). In contrast, when N₂O was produced by nitrification (soil HC), biochar addition might have increased emissions by

promoting gross nitrification. To our knowledge, there are not published studies explicitly relating to biochar and nitrification-N₂O production.

Another question that arises from this study is: why these two soils under identical experimental conditions follow different N₂O formation pathways, which we hypothesise might be linked to different soil microbial communities. In conclusion, predicting which N₂O formation pathway predominates in a certain kind of soil will be necessary for guaranteeing the success of biochar as a N₂O mitigation strategy.

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CHAPTER 4. COMPOST VS BIOCHAR AMENDMENT: A TWO-YEAR FIELD STUDY EVALUATING SOIL C BUILD-UP AND N DYNAMICS IN AN ORGANICALLY MANAGED OLIVE CROP



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4.1. Abstract

Background and Aims: Despite the high interest in biochar as soil amendment, the number of field studies is still limited, being experiments with perennial crops in arid lands particularly lacking. This study evaluated the application of compost and biochar in a drip-irrigated organic olive crop in a calcareous soil in South-East Spain.

Methods: During two consecutive years, changes in soil total organic C (TOC), dissolved organic C (DOC) and water soluble N (WSN), mineral N (NH_4^+ and NO_3^-), N_2O emissions, denitrifying enzyme activity (DEA) and number of *amoA* gen copies were monitored.

Results: Biochar increased TOC compared to the rest of treatments, whereas DOC and WSN significantly increased in plots amended with compost and the compost-biochar mixture. DEA, *amoA*-encoding genes and N_2O emissions were highest with the compost-biochar mixture, but results were not always significant.

Conclusions: Our results show that, in these N-limited and deficit irrigated semi-arid agro-ecosystems, compost amendment has an impact on soil microbiological activity with a link to N availability. Biochar applied alone does not alter the N dynamics, but markedly builds-up soil C. In both

cases only during the first year these effects were statistically significant. When applied in combination a synergistic effect was observed and the highest values of DEA, *amoA* gene copies and N₂O emissions were detected.

4.2. Introduction

Some of the most productive agricultural areas in the world stand in arid or semi-arid Mediterranean-climate regions (Andrews et al., 2002; Aznar-Sánchez et al., 2011). In spite of their high productivity, many of the agricultural soils in these areas have very low organic matter content and are exposed to severe degradation and persistent loss of fertility. Semi-arid soils are exposed to erosion by infrequent but intense precipitation, absence of plant cover and high organic matter mineralisation rates related to high temperatures (Álvaro-Fuentes et al., 2008). For this reason, the use of organic amendments to restore C stocks and soil biological functions is particularly important in these regions (Lal, 2004). In organic farming systems, the regular addition of exogenous organic matter has the additional key role of supplying the essential nutrients to maintain crop growth and yield.

Traditionally, organic amendments such as manures and composts have been applied in organically managed agricultural soils (Albiach et al., 2000). More recently other agro-industrial wastes have also attracted attention as organic fertilisers, like the residues from the meat industry (Mondini et al., 2008), or from bioenergy production (Cayuela et al., 2014a; Galvez et al., 2012). Among all bioenergy by-products, the use of biochar has recently captured the most attention within the soil science community. Biochar has been advocated as a multiple-win strategy with increases in crop productivity (Jeffery et al., 2015), significant N₂O reductions (Cayuela et al., 2014b) and long-term C sequestration potential (Whitman et al., 2013).

Despite the extensive increase of biochar studies, the number of field experiments is still low and not representative of a wide range of crops, climate zones, soil types and management practices. For instance, field studies on dry lands are particularly lacking (Farrell et al., 2014; Lentz et al., 2014). Arid and semi-arid soils developed on carbonated bedrock are characterised by a remarkable carbonate abundance, which prevents any biochar liming effect. This could reduce biochar benefits compared to its application in acidic soils. However, several experiments found positive effects after biochar amendment in calcareous soils: increasing soil water

holding capacity (Ippolito et al., 2014), decreasing N₂O emissions (Cayuela et al., 2014b) and increasing plant growth (Marks et al., 2014; Olmo et al., 2014).

Most field studies in calcareous soils have been performed under annual crops, typically cereals and grasslands (Felber et al., 2014; Lentz et al., 2014; Zhang et al., 2012). Even though there is particular interest and potential for biochar use in high value agroecosystems, only a very limited number of field studies focused on horticulture or perennial crops (Suddick and Six, 2013) and even less on organically managed systems (Pereira et al., 2015). In low-input cropping systems biochar might increase N use efficiency (Pereira et al., 2015) and positively impact the composition and function of soil communities (Domene et al., 2014; McCormack et al., 2013). To our knowledge there are no published studies comparing the implications of biochar and/or compost amendment on soil C and N cycling and N₂O emissions on the conditions of our study: a perennial irrigated crop grown under organic farming in a semi-arid area.

The objectives of our study were: (i) to evaluate medium-term N₂O emissions after one-time amendment with compost, biochar or a compost/biochar mixture in an olive crop grown in a semi-arid calcareous soil, (ii) to investigate the impact of these three treatments on soil C accrual,

C and N availability and microbiological activity in relation to N cycling at the field scale and (iii) to compare the nutritional status of the olive trees after two years of amendment.

We hypothesised that biochar amendment would reduce N₂O emissions and increase C build-up compared to compost, whereas compost would increase nutrients availability. We theorised an interaction between biochar and compost, with increased N turnover rates in the compost/biochar mixture treatment and lower N₂O emissions compared to the traditional management, consisting in compost amendment every two years.

4.3. Materials and Methods

4.3.1. Field site

The experiment was carried out from May 2013 to July 2015 in a commercial organic olive orchard within the farm “SAT Casa Pareja” (38°23’N; 1°22’W). The area belongs to a semi-arid Mediterranean climate. It has an annual rainfall of 250 mm, which mainly falls during the autumn and spring months, although the year 2014 was particularly dry, with only 192 mm (Supplementary Information, Figure S-4.1). The mean daily

maximum temperature is 20.7 °C and the mean daily minimum temperature 11.5 °C (SIAM Murcia, <http://siam.imida.es>).

The soil at the experimental site is a Haplic Calcisol (WRB classification, 2007), with 57% sand and 16% clay, 30% carbonate and a pH of 8.01. The total organic C (TOC) was 1.68%.

Trial plots were planted with 20 years old olive trees in a framework of 4 x 7 m².

During the last 15 years the olive orchard had been organically managed (EEC/834, 2007) and fertilisation consisted exclusively of compost application at 4 tons ha⁻¹ (10 kg tree⁻¹) every two years, being each application equivalent to approximately 47 kg N ha⁻¹ year⁻¹. No mineral fertilisers, herbicides or pesticides had been applied. The characteristics of the applied compost are detailed in the section below. Plots were cultured under low tillage intensity (three times per year) and deficit irrigation (drip irrigation in the summer; see irrigation volumes and water chemical analyses in Supplementary Information, Table S-4.1).

4.3.2. Amendments used

Two organic amendments were used: compost and biochar. The compost was prepared on-site following the optimised composting methodology developed in the farm since its conversion to organic farming (Cayuela et al., 2004). Composts were prepared by windrow turning, mixing two-phase olive mill waste with sheep manure and olive tree pruning (all of them generated in the farm) at volume proportion of 50%, 25% and 25%, respectively.

Biochar was made from holm oak wood by slow pyrolysis at 650°C, at atmospheric pressure, with a residence time of 15 hours. The biochar (particle size <10 mm) had a relatively high concentration of total organic C (67%) and a high degree of aromatic condensation (H/C_{org} molar ratio=0.32). It was produced by Proiniso Inc. (Málaga, Spain) within the EU project FERTIPLUS (www.fertiplus.eu). The main chemical characteristics of the amendments are summarised in Table 4.1.

Table 4. 1. Main chemical characteristics of the amendments used in the field experiment (results expressed in dry weight).

| | pH ^a | EC ^a (dS m ⁻¹) | TOC (%) | TN (%) | DOC ^b (g kg ⁻¹) | NH ₄ ⁺ -N ^c (mg kg ⁻¹) | NO ₃ ⁻ -N ^b (mg kg ⁻¹) |
|---------|-----------------|--|-----------------|----------------|---|--|--|
| Biochar | 9.26 (0.13) | 0.57 (0.04) | 67.30 (1.02) | 0.84 (0.03) | 0.38 (0.03) | 3.20 (-) | 0.8 (0.0) |
| Compost | 8.71 (0.05) | 2.70 (0.08) | 35.80 (0.84) | 2.35 (0.17) | 32.09 (6.33) | 189.24 (-) | 117.6 (5.3) |

^aWater extract 1:20 w:v 25°C; ^bwater extract 1:10 w:v; ^cKCl 2M solution extract 1:10 w:v. Standard deviation in brackets (n=3).

4.3.3. Experimental design

Figure 4.1 shows the field trial layout with four treatments: (i) control (no amendment) (ii) compost, (iii) a mixture of compost/biochar at 90:10 ratio (dw/dw) and (iv) biochar, and three replicates, each including six trees from two adjacent tree rows. Amendments were applied at 20 tons ha⁻¹ along the irrigation pipelines, i.e. 1 meter width at each side of the tree, following the common practice in the area. This corresponds to 16 kg tree⁻¹ or 6 tons ha⁻¹ considering the whole plot area. Therefore, no N nor C were added to control plots, whereas compost, mixture and biochar treated plots received 141, 132 and 50 kg N ha⁻¹ and 2.15, 2.34 and 4.04 tons C ha⁻¹ respectively, considering the whole plot area. Each experimental plot was separated by a buffer strip of olive trees. Amendments were manually

applied on May 2013 and 2015 and immediately incorporated into the soil by tractor ploughing at 15 cm. No other fertilisers (organic or inorganic) were incorporated, neither herbicides nor pesticides.

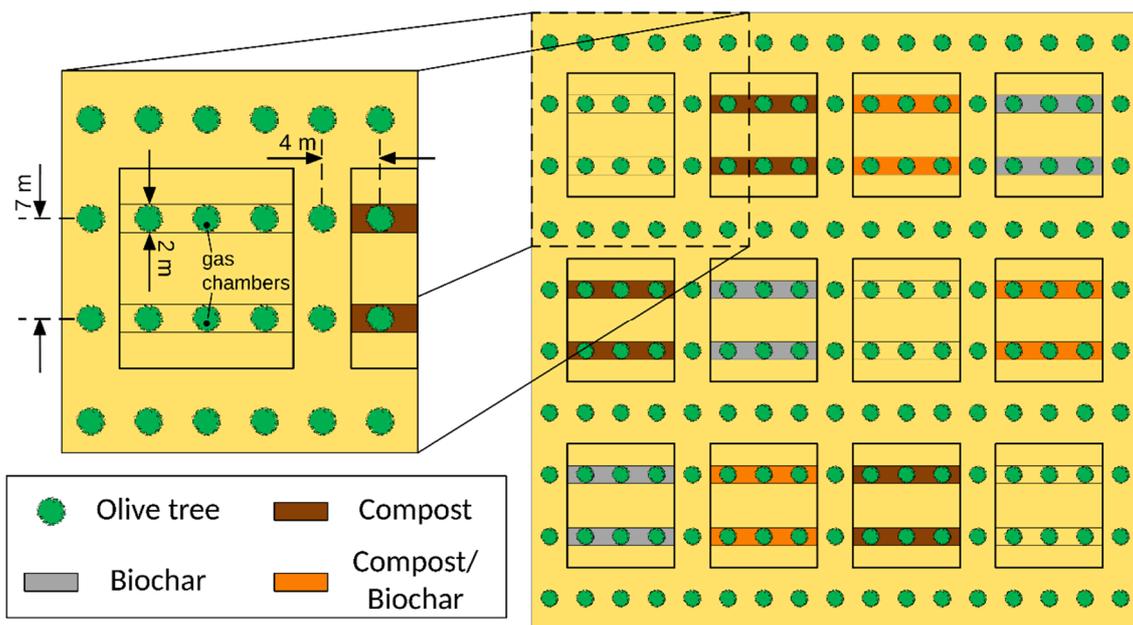


Figure 4. 1. Field trial layout showing the distribution of the different plots with controls, biochar, compost and a mixture compost/biochar (90/10 dry weight) treatments. The amendments were applied at 20 tons ha⁻¹ along the irrigation lines. Each experimental plot was separated by a buffer strip of olive trees.

4.3.4. *N₂O and CO₂ sampling and measurements*

A static chamber technique was used for gas collection. Chambers were constructed following the procedure specified by Parkin and Venterea (2010). The day after amendment, headspace chamber bases (300 mm diameter, 150 mm length, made from PVC) were installed in the field. Two chamber bases were installed per plot (6 chambers per treatment) next to the middle trees under the morning shadow.

Gas sampling was always performed between 9:00-11:00 am, considering that this has been calculated as the best sampling time for the estimation of daily mean N₂O flux from soils (Alves et al., 2012). Headspace gas samples were collected using a polypropylene syringe immediately and 1 h after closing the chambers and stored in pre-evacuated septum-capped, 12 ml vials (Exetainer Labco Limited, UK). Gas samplings were carried out weekly during the first three months, decreasing sampling's frequency to every two weeks the following three months until winter started. Additional gas samples were taken after rainfall or irrigation events, with varied frequency after these first six months depending on weather conditions, with longer intervals during winter. A total of 34 gas samplings were performed throughout the experiment (Figure 4.2).

CO₂ and N₂O were quantified using a gas chromatograph (VARIAN CP-4900 Micro-GC, Palo Alto, CA, USA). N₂O and CO₂ fluxes were calculated assuming a linear increase during the accumulation (closed) period, an approach that was corroborated prior to the experiments. Cumulative N₂O was calculated assuming linear changes in fluxes between adjacent measurement points.

Gas measurements were only performed in the crop rows (see Figure 4.1 for gas chamber locations). Field N₂O emissions were extrapolated to the orchard scale using measurements from the crop rows and considering N₂O emissions from the alleys negligible.

4.3.5. Soil and foliar sampling and analyses

Concurrent with each day of gas sampling, soil samples from each plot were taken using a 5 cm diameter core to 5 cm depth in the wet bulb (drip-irrigated area). Soil samples were oven-dried at 105 °C for determination of gravimetric water content, and water-filled pore space (WFPS) was calculated by dividing the gravimetric water content by the total soil porosity. Total porosity was determined as follows:

$$\text{Porosity (\%)} = 100 - \frac{\text{Bulk density}}{\text{Particle density}} \times 100,$$

where particle density was calculated from the densities of the fractions of clay, silt and sand (2.84, 2.80 and 2.66 g cm⁻³, respectively) in the soil.

Additionally, the topsoil (0 to 15 cm) was sampled next to all 24 chambers every three months. Four soil cores were taken from the amended area and combined to produce a composite sample associated with each plot. Denitrifying enzyme activity (DEA) was determined from unaltered subsamples and mineral N concentrations were determined from subsampled sieved soils (<2 mm) within one day after sampling. Dissolved organic carbon (DOC), water soluble nitrogen (WSN) total organic C (TOC) and total N (TN) were determined in air dried samples. The nutritional status of the olive crop was tested by foliar analysis, which was performed twice, in July 2013 and 2015.

DEA was determined according to Čuhel et al. (2010). Briefly, soil slurries were made by mixing 25-g field-moist soil samples in 120-ml serum bottles with 25 ml of a solution containing 1 mM glucose, 1 mM KNO₃ and 1 g l⁻¹ chloramphenicol. Bottles were capped with rubber stoppers, evacuated, and flushed four times with 99.99% He. The slurries were then incubated without and, subsequently, with acetylene to block the conversion of N₂O to N₂ (10%, v/v) on an end-to-end shaker at 25°C. After 30 and 60 min N₂O was quantified using gas chromatography (see above).

Finally, DEA was calculated from the N₂O increase during half an hour incubation (30-60 min) from the acetylene treated soils. The N₂ fraction was calculated as the difference between N₂O concentration in the slurries incubated with acetylene and the same slurries incubated without acetylene during the same period.

Soils were extracted by shaking moist soil (1:10 (dw/v) with 2.0 M KCl (for NH₄⁺) or water (for NO₃⁻ and NO₂⁻) for 2 h. Extracts were centrifuged (2509 G) and filtered (0.45 µm) before analysis. NH₄⁺ was determined by a colorimetric method based on Berthelot's reaction (Sommer et al., 1992). NO₃⁻ and NO₂⁻ were determined by ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland). DOC and WSN were determined in water extracts using a Photometer (Nanocolor 500 D MACHEREY-NAGEL, Germany). After removing all inorganic carbon, DOC was measured from the CO₂ formed by the digestion of organic carbon (reaction basis according to DIN EN 1484) by spectrophotometry. WSN was determined after the conversion to nitrate by oxidative digestion of all inorganic and organic nitrogen according to the Standard Method DIN EN ISO 11 905-1. Total N and TOC were determined by automatic elemental analysis (LECO CHNS-932, USA).

Foliar analyses were performed from leaves samples taken from the olive trees according to Fernández-Escobar et al. (2004). Three leaves from four branches in each plot (total: 216 leaves per treatment) were sampled during July from year shoots located in basal to middle position containing the petiole. Leaves were washed with deionised water, oven dried at 60 °C during 72 h and milled. Total concentrations of macro and micronutrients were measured after HNO₃/H₂O₂ digestion by using inductively coupled plasma (ICAP 6500 DUO THERMO, England). Total N was determined by automatic elemental analysis (see above).

4.3.6. DNA isolation from soils

DNA was extracted from soils sampled 15 months after amendment (July 2014). For each soil-sample replicate, the total DNA was separately extracted from four 0.5 g subsamples by the bead-beating method, following the manufacturer's instructions for the MoBio PowerSoil DNA Isolation kit (MoBio laboratories, Solana Beach, CA, USA). The extracts were pooled and further concentrated at 35 °C with a Savant Speedvac® concentrator to a final volume of 50 µl. The concentration and quality of the final DNA samples were checked using Nanodrop® ND-100 spectrometer (Nanodrop Technologies, Wilmington, DE, USA).

4.3.7. Quantitative real-time PCR assays

Real-time quantitative PCR assays of nucleic acids to quantify the bacterial biomass were performed as described by Moreno et al. (2009) with some modifications at the real-time PCR program. The bacterial biomass was determined with the universal primers for the V3 hypervariable region of 16S rRNA eubacteria P1 and P2 (Muyzer et al., 1993). Each 21 μ l of PCR reaction contained 3–6 ng of the DNA, 400 nM of each primer and 10.5 μ l 2 \times IQ SYBER Green Supermix (Bio-Rad, Munich, Germany). The real-time PCR program consisted of 2 min at 50 $^{\circ}$ C for carryover prevention, 5 min at 94 $^{\circ}$ C for enzyme activation, followed by 10 cycles consisting of 94 $^{\circ}$ C for 15 s, 60–55 $^{\circ}$ C for 15 s, decreasing the temperature by 0.5 $^{\circ}$ C every cycle, 72 $^{\circ}$ C for 30 s, and by 23 cycles consisting of 94 $^{\circ}$ C for 15 s, 55 $^{\circ}$ C for 15 s, 72 $^{\circ}$ C for 30 s; the fluorescence signal was measured at 72 $^{\circ}$ C step in both cases. The final step consisted of 7 min at 72 $^{\circ}$ C. PCR amplification procedure was checked with a heat dissociation protocol (from 70 $^{\circ}$ C to 100 $^{\circ}$ C) after the final cycle of the PCR.

The abundance of the ammonia-oxidising bacterial community was estimated by real-time PCR quantification of DNA samples using the

primers *amoA*-1F (5'-GGGGTTTCTACTGGTGGT-3') and *amoA*-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') designed by Rotthauwe et al. (1997). Agarose-gel electrophoresis was carried out to confirm the specific PCR product, resulting in a single band of the expected size, approximately 491 bp. Each 21- μ l PCR reaction contained from 1-5 ng of the DNA, 10.5 ml 2x iQ SYBR Green Supermix (Bio-Rad, Munich, Germany), and 400 nM each primer. The real-time PCR program consisted of 2 min at 50 °C for carryover prevention, 5 min at 94 °C for enzyme activation, followed by 12 cycles consisting of 94 °C for 30 s, 60–54 °C for 30 s, decreasing the temperature by 0.5 °C every cycle, 72 °C for 1 min, and by 23 cycles consisting of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min; the fluorescence signal was measured at 72 °C step in both cases. The final step consisted of 7 min at 72 °C. PCR amplification procedure was checked with a heat dissociation protocol (from 70 °C to 100 °C) after the final cycle of the PCR.

Two different standard curves were generated by using a recombinant plasmid containing one copy of 16S rRNA fragment and a recombinant plasmid containing one copy of *amoA* gene from soil bacteria. The curves were drawn according to Moreno et al. (2009). Quantification of the DNA copy number was performed on an iQ5 thermocycler using iQ5-Cycler software (Bio-Rad, Munich, Germany). The relationship between

threshold cycle (Ct) and the target-gene copy number and the copy numbers of the real-time standard were calculated as described by Qian et al. (2007). For each extracted DNA, real-time PCR experiments were carried out three times with the threshold cycle (Ct) determined in triplicate. The equation describing the relationship between Ct and the log number of 16S rRNA copies was $Ct = -3.437 \times \log(16S \text{ rRNA}) + 26.541$, $R^2 = 0.991$. Target molecules were linear from 10^4 to 10^9 copies. The equation describing the relationship between Ct and the log number of LCMO gene copies was $Ct = -3.5773 \times \log(amoA) + 31.286$, $R^2 = 0.9873$. Target molecules were linear from 10^4 to 10^9 copies.

The potential presence of qPCR inhibitors was tested by mixing 1 μ l (4-8 ng) of soil DNA extracts with a known amount of recombinant plasmid DNA (pCR[®]2.1, Invitrogen, Carlsbad, CA, USA) with the appropriate primers. Controls, where DNA templates were replaced by filter-sterilized milliQ water, were carried out simultaneously. Ct values were not significantly different between the DNA extracts and the controls.

4.3.8. Statistical analyses

One-way ANOVA was used to assess global differences between treatments in N₂O emissions and mineral concentrations between treatments. Additionally, gas fluxes were analysed by repeated measures ANOVA with time as the within subject factor (Stein and Arp, 1998). The relationships between studied parameters were addressed through a correlation analysis (Pearson correlation coefficient) for each plot. This analysis was also performed for N₂O-N and CO₂-C to study their correlation with T, WFPS and the combination of both, which was calculated as the product TxWFPS (Table 4.2). For all analyses, significance was defined as P<0.05. Statistical analyses were performed with IBM SPSS Statistics 21, Sommers, USA.

4.4. Results

4.4.1. Gas emissions

Both CO₂ and N₂O emissions followed a seasonal pattern mainly affected by temperatures (T) and water filled pore space (WFPS) (Figure 4.2). Different amendments did not have a statistically significant effect on soil WFPS. The highest gas flux peaks were detected during the summer season and always associated to irrigation events. Table 4.2 shows Pearson correlations between N₂O-N, CO₂-C, WFPS, T and the product of WFPS x T. Both N₂O and CO₂ were best correlated with the product WFPS x T.

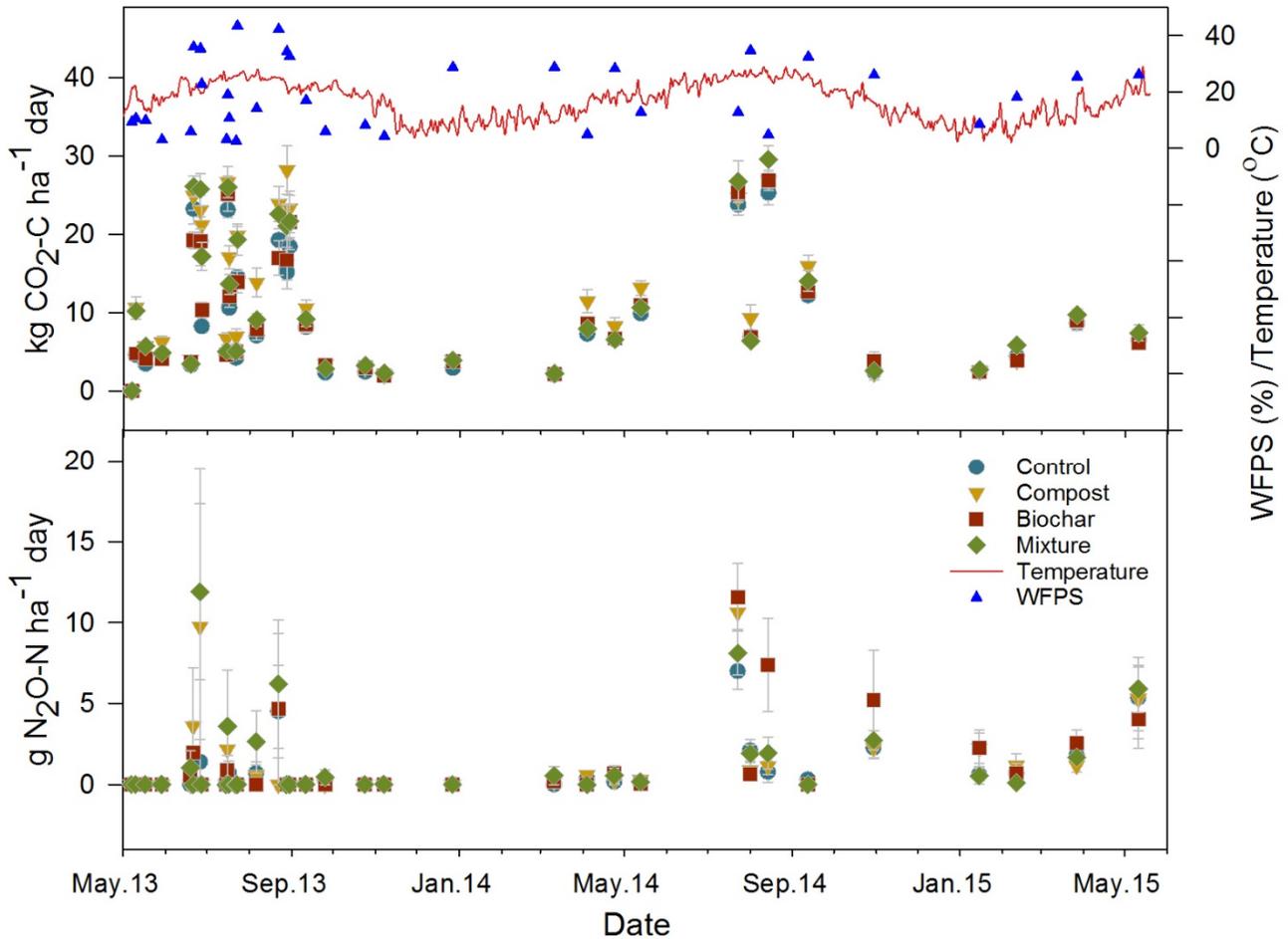


Figure 4. 2. CO₂-C and N₂O-N fluxes measured in the crop rows over two years for control, compost, biochar and mixture treatments. Average temperature and WFPS are showed on the upper figure. Error bars represent the standard error of the mean (n=3).

Table 4. 2. Pearson correlations between N₂O-N, CO₂-C, water filled pore space (WFPS), temperature (T) and the product of WFPS and T. Significance in brackets (N=34).

| Treatment | N ₂ O-N | | | | CO ₂ -C | | |
|----------------|--------------------|------------------|------------------|-------------------|--------------------|--------------------|--------------------|
| | CO ₂ -C | WFPS | T | T x WFPS | WFPS | T | T x WFPS |
| Control | 0.307 (0.077) | 0.197 (0.265) | 0.156 (0.377) | 0.232 (0.186) | 0.464** (0.006) | 0.416* (0.014) | 0.833** (0.000) |
| Compost | 0.316 (0.068) | 0.319 (0.066) | 0.084 (0.635) | 0.260 (0.060) | 0.731** (0.000) | 0.494** (0.003) | 0.847** (0.000) |
| Mixture | 0.454** (0.007) | 0.311 (0.073) | 0.268 (0.126) | 0.387* (0.024) | 0.768** (0.000) | 0.435* (0.010) | 0.870** (0.000) |
| Biochar | 0.467** (0.005) | 0.289 (0.097) | 0.116 (0.512) | 0.328 (0.058) | 0.704** (0.000) | 0.424* (0.012) | 0.803** (0.000) |

CO₂-C and N₂O-N represent average values for each treatment

**Significant at level 0.01; *Significant at level 0.05

N₂O fluxes were very low in all treatments during the whole experiment (below 12 g N₂O-N ha⁻¹ day⁻¹). During the first year of the experiment the highest N₂O flux peaks corresponded to the mixture (compost/biochar) treatment and, during the second year, to the biochar treatment. The estimated cumulative annual N₂O-N emissions in our experiment were 144.2, 193.2, 193.8 and 241.5 g ha⁻¹ year⁻¹ for control, compost, mixture and biochar amended plots respectively. The highest CO₂-C release corresponded to the mixture treated plots. However, there were not significant differences in average CO₂ or N₂O fluxes for the different treatments (P>0.05).

4.4.2. Carbon and Nitrogen cycling. TOC, TN, DOC, WSN, NH_4^+ and NO_3^- dynamics

Total organic carbon (TOC) was always higher in biochar amended plots compared to the rest of the treatments. Despite the high variability between plots, this difference was statistically significant ($P < 0.05$) during the first year (Figure 4.3a). Control plots always showed the lowest TOC concentrations and the difference was still evident two years after amendment. On average, total N (TN) concentrations were higher in all amended plots (compost, mixture and biochar), compared to the control, although there were not statistically significant differences between treatments (Figure 4.3b).

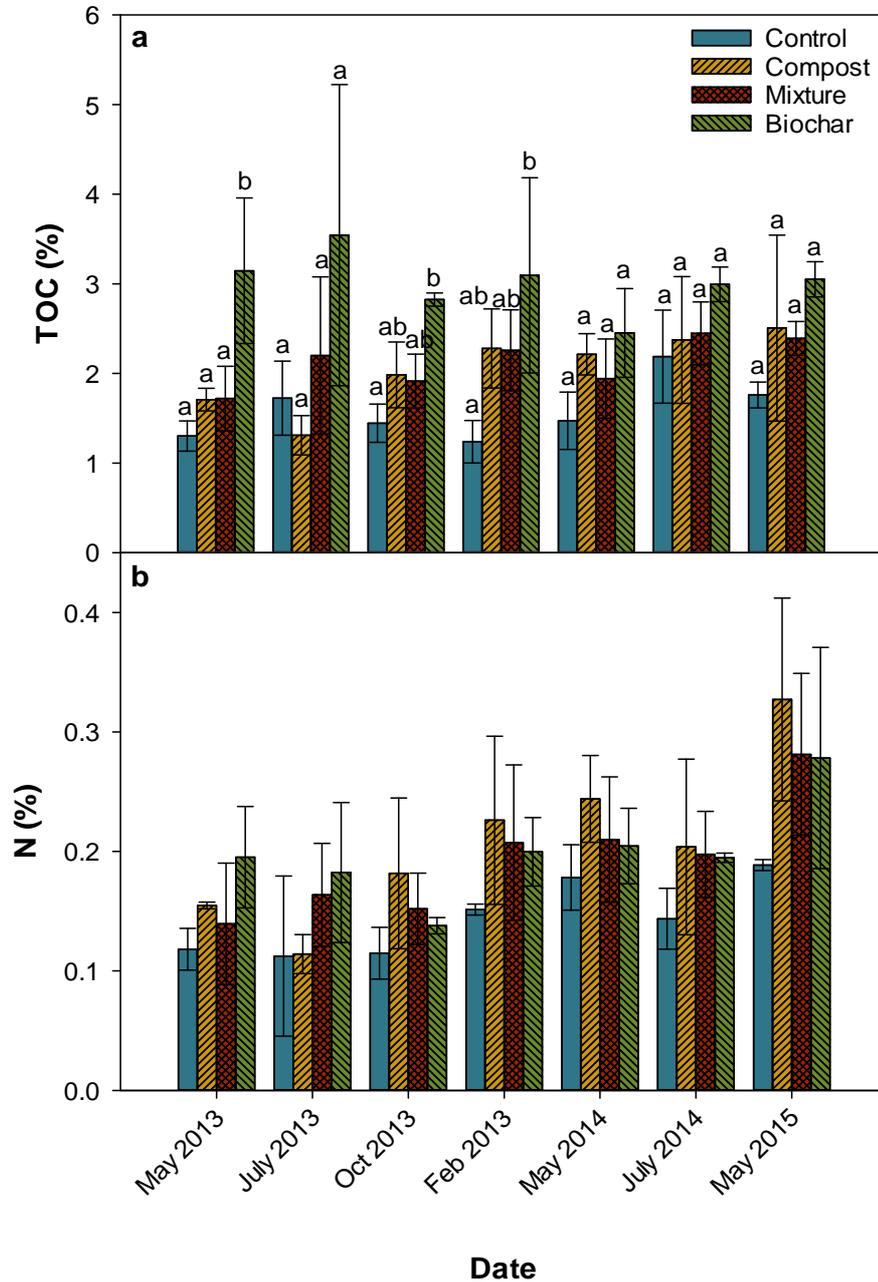


Figure 4. 3. Evolution of total organic carbon (TOC) (a) and total nitrogen (N) (b) over the experiment for control, compost, mixture and biochar treatments. Error bars represent the standard deviation of the mean (n=3). The different letters next to the bars indicate significant differences between treatments. The absence of letters in (b) indicates that there were not significant differences at $P < 0.05$.

Dissolved organic carbon (DOC) was repeatedly and consistently higher in the compost and mixture treatments compared to the control and biochar treated plots during the first year (Figure 4.4a). This difference in average DOC between compost treated vs not treated plots was statistically significant in three out of four sampling times.

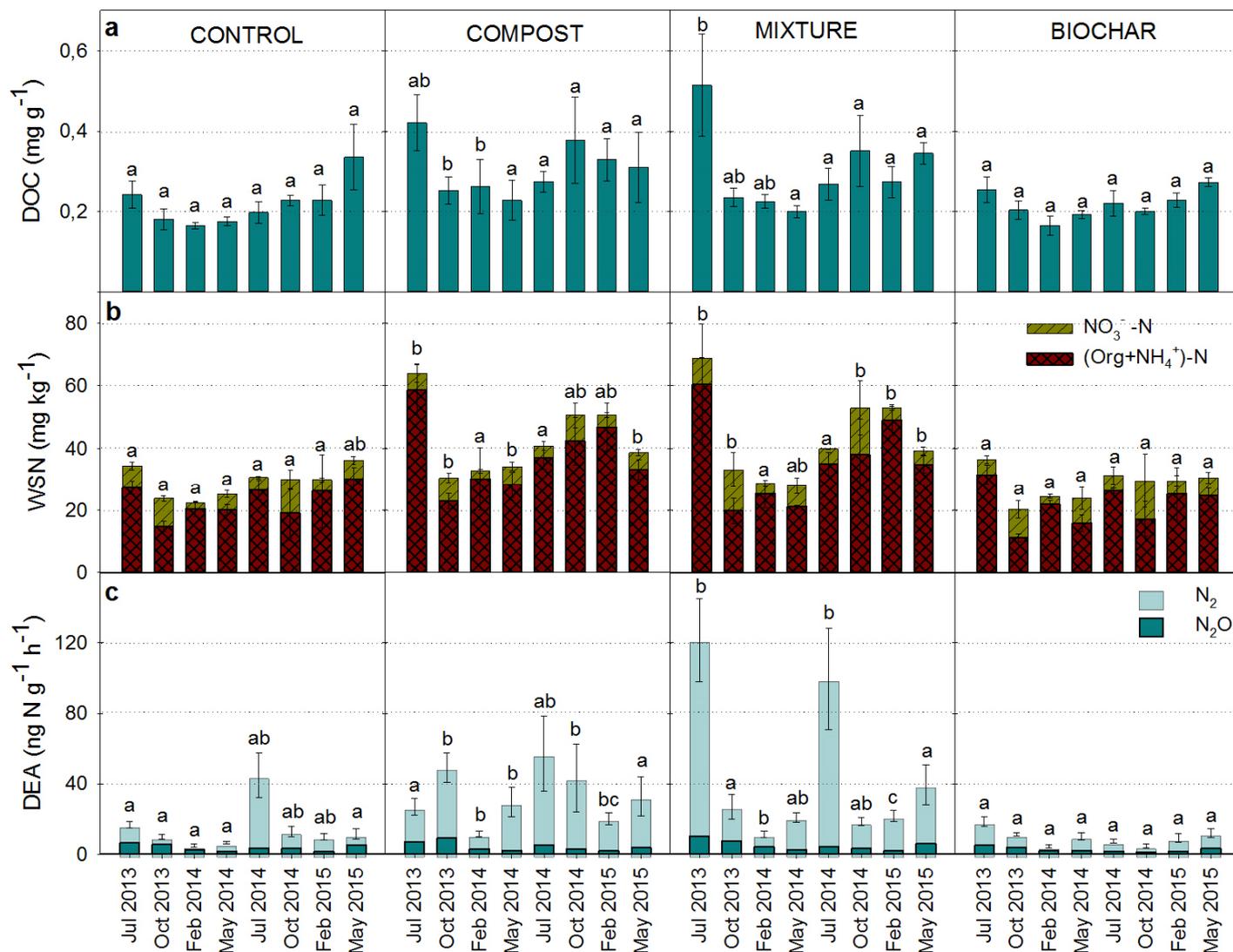


Figure 4. a. Evolution of dissolved organic carbon (DOC) over the experiment for control, compost, mixture and biochar treatments. Error bars represent the standard deviation of the mean (n=3). The different letters next to the bars indicate significant differences between treatments ($P < 0.05$). **b. Evolution of water soluble nitrogen (WSN), comprised by (organic + NH₄⁺)-N and NO₃⁻-N fractions over the experiment.** Error bars represent the standard deviation of the mean (n=3). The different letters next to the bars indicate significant differences in WSN between treatments ($P < 0.05$). **c. Evolution of denitrifying enzyme activity (DEA) over the experiment for control, compost, mixture and biochar treatments.** Denitrified N rates are represented separately as N₂ and N₂O. Error bars represent the standard error of the mean (n=3). The different letters next to the bars indicate significant differences in (N₂O+N₂)-N release between treatments ($P < 0.05$).

Water soluble nitrogen (WSN) followed a similar pattern as DOC, with a consistent higher value for the compost and mixture treatments, which was statistically significant ($P < 0.05$) in six out of eight sampling times (Figure 4.4b). Although water soluble NH_4^+ was not determined, we measured KCl extractable NH_4^+ (Supplementary Information, Figure S-4.2), which was always below 5.5 mg N kg^{-1} soil. Therefore, organic N was always the largest fraction of the WSN and responsible for the significant differences found in total water soluble nitrogen (WSN). No significant differences between treatments were measured in the NO_3^- -N fraction during the experiment.

4.4.3. Olive orchard nutritional status

Table 4.3 shows the macro and micronutrients concentrations in the olive trees leaves for the different treatments. According to Fernández-Escobar et al. (2004), N concentration in olive leaves was in the low limit of the recommended levels (1.5-2.0%) for all treatments in July 2013. Similarly, the concentration of Ca was close to 1%, which is considered the minimum for an adequate nutritional status. The concentration of other macro and micronutrients was within the normal range.

Nutrient concentrations were similar in all the plots three months after amendment (July 2013). Moreover, the different treatments did not have a significant impact on the nutritional status of the olive trees at the end of the experiment (July 2015).

Table 4. 3. Nutritional status of olive trees determined by foliar analysis for control, compost, mixture and biochar treatments (results expressed in dry weight).

| Treatment | N | P | K | Ca | Mg | Na | Mn | Cu | Zn | B |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|---------------------|--------------|---------------|---------------|
| | % | | | | | | mg kg ⁻¹ | | | |
| July 2013 | | | | | | | | | | |
| Control | 1.43 (0.09) | 0.13 (0.01) | 1.29 (0.03) | 1.01 (0.09) | 0.16 (0.02) | 0.01 (0.00) | 36.8 (3.9) | 4.2 (0.3) | 12.4 (0.7) | 33.3 (0.6) |
| Compost | 1.42 (0.06) | 0.14 (0.02) | 1.26 (0.07) | 0.82 (0.12) | 0.15 (0.02) | 0.01 (0.00) | 35.2 (3.8) | 4.1 (0.4) | 11.9 (1.3) | 31.8 (1.4) |
| Mixture | 1.58 (0.05) | 0.14 (0.01) | 1.27 (0.04) | 0.98 (0.08) | 0.18 (0.02) | 0.01 (0.00) | 36.3 (2.7) | 5.2 (0.5) | 13.6 (1.9) | 33.0 (0.4) |
| Biochar | 1.51 (0.06) | 0.12 (0.01) | 1.24 (0.07) | 0.91 (0.18) | 0.16 (0.03) | 0.01 (0.00) | 35.47 (3.9) | 4.4 (0.4) | 12.1 (0.7) | 31.7 (1.9) |
| July 2015 | | | | | | | | | | |
| Control | 1.73 (0.01) | 0.18 (0.02) | 1.15 (0.06) | 1.49 (0.16) | 0.20 (0.02) | 0.02 (0.00) | 23.1 (1.7) | 6.7 (0.5) | 11.2 (0.3) | 36.7 (3.9) |
| Compost | 1.80 (0.08) | 0.18 (0.02) | 1.20 (0.12) | 1.37 (0.23) | 0.20 (0.03) | 0.03 (0.00) | 24.5 (3.3) | 7.3 (0.5) | 13.3 (1.3) | 36.1 (1.7) |
| Mixture | 1.82 (0.16) | 0.17 (0.02) | 1.25 (0.20) | 1.34 (0.08) | 0.21 (0.01) | 0.03 (0.01) | 20.3 (2.6) | 7.8 (1.7) | 13.1 (3.2) | 33.2 (1.7) |
| Biochar | 1.71 (0.20) | 0.17 (0.01) | 1.19 (0.07) | 1.47 (0.18) | 0.21 (0.04) | 0.03 (0.01) | 25.8 (2.3) | 6.7 (1.2) | 12.1 (1.8) | 36.2 (3.6) |

Average nutrients concentration in olive tree leaves. Standard deviation in brackets (n=3). No significant differences were detected between treatments (P<0.05).

4.4.4. Denitrifying Enzyme Activity (DEA)

DEA was significantly higher in plots with the mixture amendment (Figure 4c) and showed, as N₂O fluxes, a seasonal pattern. The highest activity was registered in summer, precisely in July 2013 and July 2014 with values of 121.6 and 99.7 ng (N₂+N₂O)-N g⁻¹ h⁻¹ respectively in mixture amended plots. Biochar amended plots reached the lowest values of DEA in the same period, 18.5 and 7.4 ng (N₂+N₂O)-N g⁻¹ h⁻¹ in July 2013 and July 2014, respectively. Biochar treated plots consistently showed the lowest DEA values during the whole experiment. There was not a significant correlation between DEA and N₂O-N emissions, WSN or its fractions, but DEA was positively correlated with DOC in almost all measurement points (except the two last measurements, in February and May 2015).

The N₂O/(N₂+N₂O) ratio was always very low, below 0.15 in all measurements (data not shown). The N₂O fraction was very similar in all treatments during the experiment, being the increments in N₂ fraction responsible of the largest DEA registered values.

4.4.5. Real-time PCR assays

Figure 4.5 shows the 16S rDNA copy numbers in the four treatments. No significant differences could be detected for bacterial biomass between treatments. The size of the nitrifier community was estimated by quantification of *amoA* encoding genes from ammonia-oxidising bacteria. The use of compost or biochar did not cause effect on the natural soil bacterial *amoA* gene copies (Figure 4.5). However, when both amendments were combined, the size of the nitrifying bacteria population showed an increasing trend compared to the levels detected when they were used separately, despite no statistically significant differences with the control were noticed.

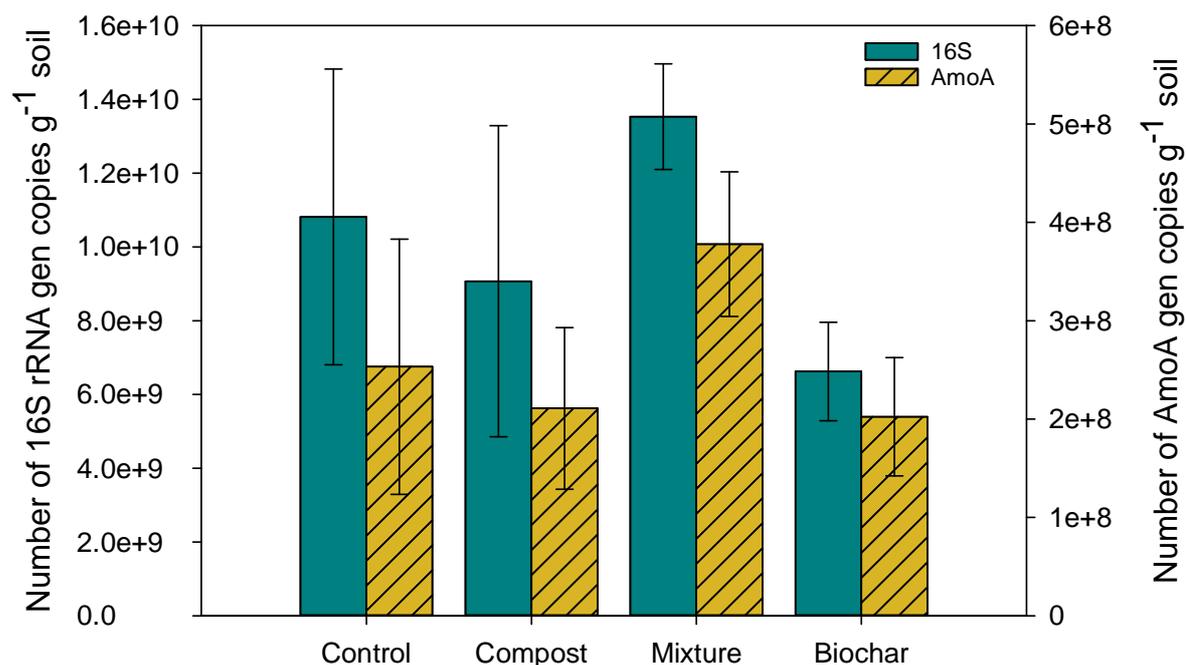


Figure 4. 5. Number of copies of 16S rRNA and *AmoA* gen number in soils 15 months after amendment (July 2014). Error bars represent the standard error of the mean. The absence of letters indicates that there were not significant differences at $P < 0.05$.

4.5. Discussion

4.5.1. Biochar vs compost impacts on N_2O emissions

N_2O emissions from olive crops in semi-arid regions have only been recently reported (Ussiri and Lal, 2013). Other few studies have been performed in comparable perennial cropping systems with similar climate, such as almond orchards (Alsina et al., 2013; Schellenberg et al., 2012) and vineyards (Garland et al., 2014). The average background N_2O emissions in these perennial cropping systems have been found to be low, although

largely influenced by management practices (Alsina et al., 2013; Garland et al., 2014).

In our study N₂O emissions were only observed when favorable conditions of both high temperature and water availability were reached (independently of the treatment). The highest N₂O-N flux peak in tree rows for most treatments corresponded to July 2014, with values of 7.0, 10.6, 8.1, 11.6 g ha⁻¹ day⁻¹ in the control, compost, mixture and biochar treated plots, respectively. Our results are in agreement with those reported by Maris et al (2015), who observed a maximum flux peak of 9.16 g N₂O-N ha⁻¹ day⁻¹ from the drip irrigated area of an olive orchard cultivated under similar environmental conditions but different management (i.e. conventional farming with synthetic fertilisation). These authors related N₂O-N flux peaks to irrigation as well as to fertilisation events. Previous studies in almond orchards in similar climate conditions also reported the highest N₂O-N flux peaks during the summer, e.g. Schellenberg et al. (2012) reported values of 25.5 g N₂O-N ha⁻¹ day⁻¹ and Alsina et al. (2013) measured instantaneous emissions of 60.5-121 g N₂O-N ha⁻¹ day⁻¹ after fertigation events. The higher fluxes reported in these studies are probably related to higher fertilisation rates (~240 kg N ha⁻¹ in both studies) and wetter soil

conditions (e.g. average WFPS of 40% in Alsina et al. (2013)) than in our study.

Our results show that biochar did not significantly decrease N₂O emissions compared to compost treatment and therefore we reject our first hypothesis. Although biochar has been found to decrease N₂O emissions in several field studies (Felber et al., 2014; Taghizadeh-Toosi et al., 2011), the few field experiments performed under Mediterranean climatic conditions showed small to not significant reductions (Castaldi et al., 2011; Suddick and Six, 2013) and even an increase was observed in a wine grape crop in California (Verhoeven and Six, 2014). The highest reductions in N₂O emissions with biochar have been generally found under denitrifying conditions i.e. high moisture contents and high organic C and NO₃⁻ availability (Cayuela et al., 2014b). However, under our specific field conditions these circumstances were never reached.

On the other hand, we predicted a rise in N₂O emissions with compost due to an increase in N and C availability, but the overall impact on N₂O release was minor.

In general, the annual N₂O emissions found in our study (between 0.14 and 0.24 kg N₂O-N ha⁻¹ year⁻¹) were lower than those previously reported in other perennial crops under Mediterranean climate. For

instance, Schellenberg et al. (2012) reported annual N₂O emissions of 0.53-0.8 kg N₂O-N ha⁻¹ year⁻¹ and Garland et al. (2014) of 3.92 kg N₂O-N ha⁻¹ year⁻¹ after cover crops were incorporated into the alleys of a vineyard system. Our lower emissions rates might be the consequence of the combination of several factors: (i) a well-aerated soil with coarse texture less prone to denitrification (Bollmann and Conrad, 1998), (ii) very low N availability (Subbarao et al., 2006), (iii) organic fertilisation (Burger et al., 2005) and (iv) the use of deficit drip irrigation (Schmidt, 1982).

4.5.2. Biochar vs compost impacts on carbon build-up, nitrogen cycling and olive orchard nutritional status

Both biochar and compost amendments have been proposed as promising strategies to increase carbon storage (Sánchez-Monedero et al., 2008; Whitman et al., 2013) and to promote soil biological activity and nutrient cycling also in calcareous soils (Lejon et al., 2007; Ventura et al., 2014). In our study, both amendments could be considered as highly stable in their respective categories (low H/C_{org} molar ratio of the biochar and high degree of maturity in the olive mill waste compost (Cayuela et al., 2004)). However, the higher recalcitrance of biochar compared to compost

was unequivocal. Biochar treatment led to the highest and most persistent increase in TOC in soil, which validates our second hypothesis. However, during the second year the differences in TOC between treatments were lower and not statistically significant. We hypothesise that biochar loss might be a consequence of physical processes leading to biochar transport off-site. This circumstance has been previously reported in field studies, as a consequence of physical dispersion derived by management practices, wind or rain (De Boer and Kowalchuk, 2001; Jeffery et al., 2015). On the contrary, DOC was always higher in compost amended plots and positively correlated with DEA. WSN was also higher in compost treated plots mainly due to their higher content in the organic N fraction. Mineral fractions of soil N (NO_3^- and NH_4^+) were not different between treatments and we did not find any correlation between these parameters and N_2O release nor DEA. In previous studies, the absence of correlation between NO_3^- and N_2O emissions have been explained by the insufficient NO_3^- concentration in soil, which is common in low-N input systems (Schmidt, 1982). In our study this could also indicate that denitrification was not the main source of N_2O formation and release.

DEA showed a seasonal pattern reaching the highest values (44.7, 57.1 and 7.4 $\text{ng (N}_2\text{O+N}_2\text{)-N g}^{-1} \text{ h}^{-1}$ in control, compost and biochar amended

plots, respectively) one year after amendment. Our results are lower than those previously reported by Steenwerth and Belina (2008) in similar conditions. These authors also reported a seasonal behaviour, with DEA values below $180 \text{ ng (N}_2\text{O+N}_2\text{)-N g}^{-1} \text{ h}^{-1}$ in every season in a rainfed vineyard cultivated in Mediterranean climate but registered the highest activity in winter. This difference in behaviour could be due to the absence of irrigation during the summer dry period. Compost amendment enhanced DEA activity, which can be related to higher nutrient availability and therefore higher microbial activity in compost treated plots (Burger et al., 2005). Biochar treated plots showed similar or even lower values of DEA than control plots. However, N_2O gas flux peaks were higher in biochar treated plots, suggesting that N_2O was primarily derived from nitrification pathways as previously found in a laboratory study in this soil (Sánchez-García et al., 2014). Contrary to our observations, Castaldi et al. (2011) reported an increase in DEA with biochar addition from 70 to $110 \text{ ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ 3 months after 30 tons ha^{-1} of biochar amendment. They explained the positive effect of biochar in microbial activity with the shift in soil pH, which differs from our conditions, since the soil of our experiment is alkaline. To our knowledge, there is no previous data of biochar effect in DEA in similar conditions to our experiment. Increases of DEA values were

reached mainly due to a rise of the N₂ fraction, confirming the ability of the soil microbial community to perform the last step of denitrification. This is consistent with Čuhel et al. (2010), who reported a DEA of 177.1 ng (N₂+N₂O)-N g⁻¹ h⁻¹ in alkaline soils where the major fraction of the DEA was also in the form of N₂.

The effects of biochar application on soil bacterial biomass and key functional taxa such as N cycling bacteria have been evidenced and then associated with its impacts on C and N cycling (Kool et al., 2011; Ritchie and Nicholas, 1972). Despite the spatial variability of the whole experiment, it is important to notice that some interesting correlations could be retrieved when plots were considered as independent units. In this framework, the size of total bacteria population showed a linear correlation with both nitrifying bacteria population and DEA activity in each plot regardless of their treatment (P=0.005 and P=0.000, respectively), evidencing the effect of organic amendments on the bacteria mediated processes transforming nitrogen.

Regarding the olive trees nutritional status, the differences between the different treatments were negligible and more linked to the variability in the field than to an amendment effect. However, we cannot discard a long term effect on the orchard nutritional status since olive trees have a

slow response to changes in fertilisation practices (Fernández-Hernández et al., 2014).

4.5.3. Biochar-compost interactions

Little is known about the interactions of biochar with organic amendments under field conditions. As hypothesised, we observed some interesting synergistic effects in microbial activity related to the N cycle when both amendments were combined. The greatest effect was observed in DEA, which was up to five times higher in the mixture amended plots compared to the rest of the treatments during the most active season, in July 2013 ($121.6 \text{ ng (N}_2\text{O+N}_2\text{)-N g}^{-1} \text{ h}^{-1}$) and 2014 ($99.7 \text{ ng (N}_2\text{O+N}_2\text{)-N g}^{-1} \text{ h}^{-1}$). No differences in DOC, organic N, NH_4^+ nor NO_3^- concentration were registered in compost and mixture treated plots, which may indicate that the increased DEA was a result of an overall higher microbial activity in mixture amended soils.

Regarding nitrifying bacterial populations, a high spatial variability between plots was detected and the effect of the organic amendments on many of the parameters measured was not clear. Nevertheless, we could report that nitrifying bacterial population was considerably larger in the

mixture treatment, suggesting some type of synergistic effect when compost and biochar were applied together. To date, there was limited support of a superadditive effect of biochar and other organic amendments or chemical fertilisers when used simultaneously (Biederman and Harpole, 2013). When used alone, previous research suggests that biochar may promote soil bacterial nitrifier populations accelerating nitrification rates (Prommer et al., 2014), while others report the opposite (Liu et al., 2014). In our study, no determinant effects of compost or biochar were noticed with respect to the non-amended soils. However, the combination of both amendments increased the size of ammonia monooxygenase gene *amoA*, which could be related to the highest N₂O emissions observed with the mixture treatment during the first year. In any case, the combined application of compost and biochar would not represent a risk in this type of soil and under these environmental conditions, since N₂O emissions were anyhow very low.

4.6. Conclusions

Our results suggest that under this type of semiarid Mediterranean agro-ecosystem (with low water availability, high soil pH and very low N inputs) N₂O emissions are negligible and biochar does not play a significant role in N₂O mitigation. On the other hand, compost application increased dissolved organic C and soluble N, without a parallel increase in N₂O emissions. The application of a compost/biochar mixture showed a synergistic impact on DEA and *amoA* genes in soil, suggesting a boost on the microbial processes transforming N.

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SUPPLEMENTARY INFORMATION

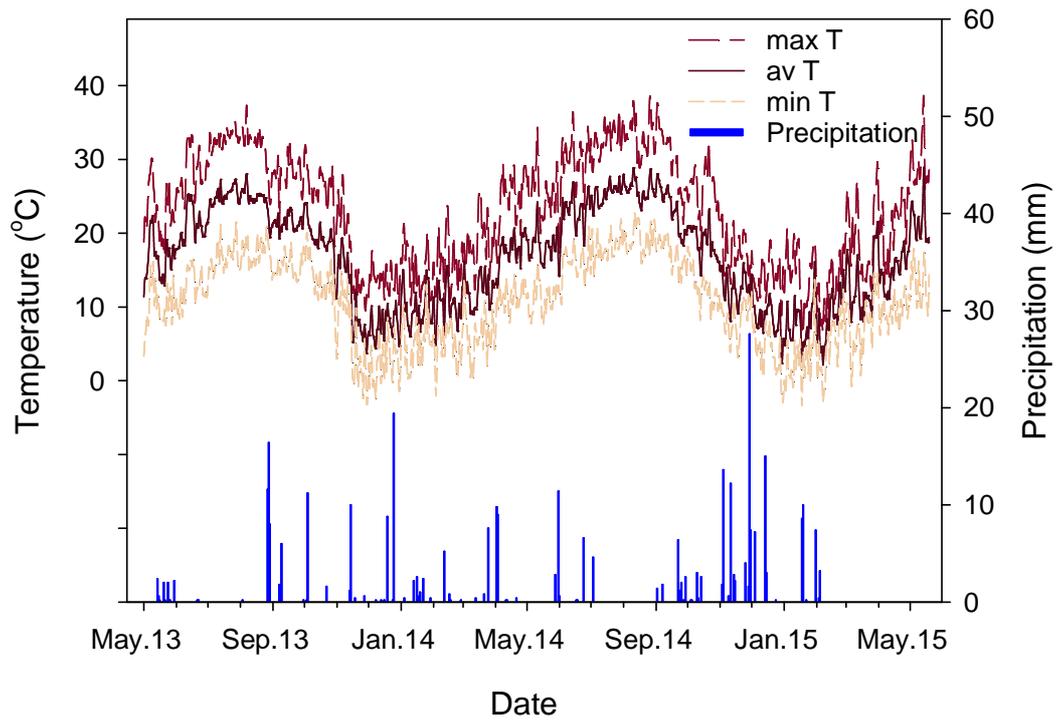


Figure S-4. 1. Precipitation and maximum, average and minimum daily temperatures from May 2013 to May 2015.

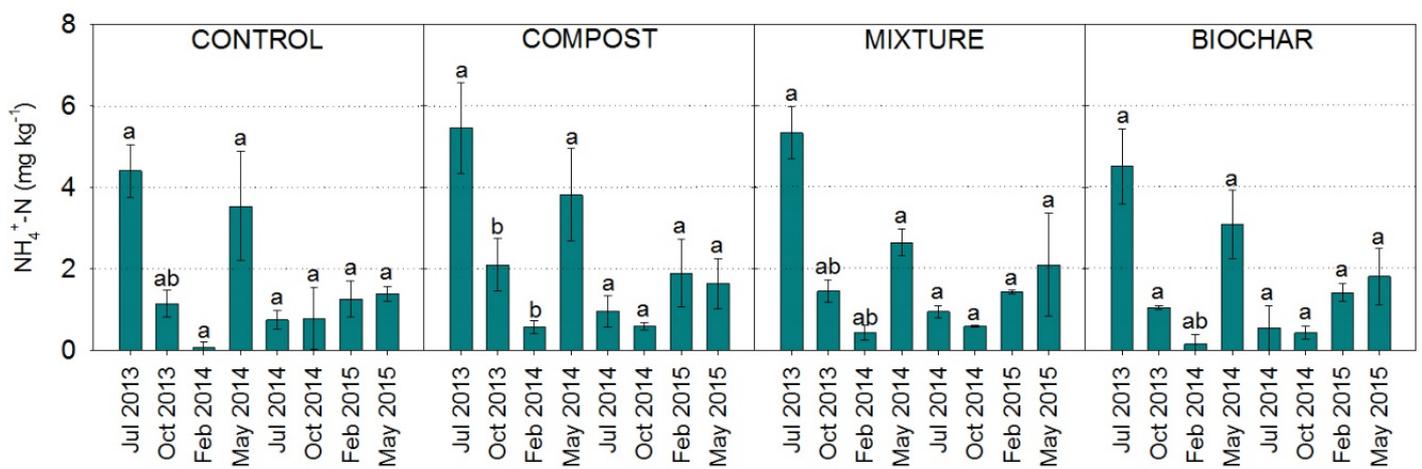


Figure S-4. 2. Evolution of NH₄⁺-N over the experiment. Error bars represent the standard deviation of the mean (n=3). The different letters next to the bars indicate significant differences in NH₄⁺-N between treatments (P < 0.05).

**CHAPTER 5. BIOCHAR ACCELERATES ORGANIC MATTER DEGRADATION
AND ENHANCES N MINERALISATION DURING COMPOSTING OF POULTRY
MANURE WITHOUT A RELEVANT IMPACT ON GAS EMISSIONS**



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5.1. Abstract

A composting study was performed to assess the impact of biochar addition to a mixture of poultry manure and barley straw. Two treatments: control (78% poultry manure + 22% barley straw, dry weight) and the same mixture amended with biochar (3% dry weight), were composted in duplicated windrows during 19 weeks. Typical monitoring parameters and gaseous emissions (CO₂, CO, CH₄, N₂O and H₂S) were evaluated during the process as well as the agronomical quality of the end-products.

Biochar accelerated organic matter degradation and ammonium formation during the thermophilic phase and enhanced nitrification during the maturation phase.

Our results suggest that biochar, as composting additive, improved the physical properties of the mixture by preventing the formation of clumps larger than 70 mm. It favoured microbiological activity without a relevant impact on N losses and gaseous emissions. It was estimated that biochar addition at 3% could reduce the composting time by 20%.

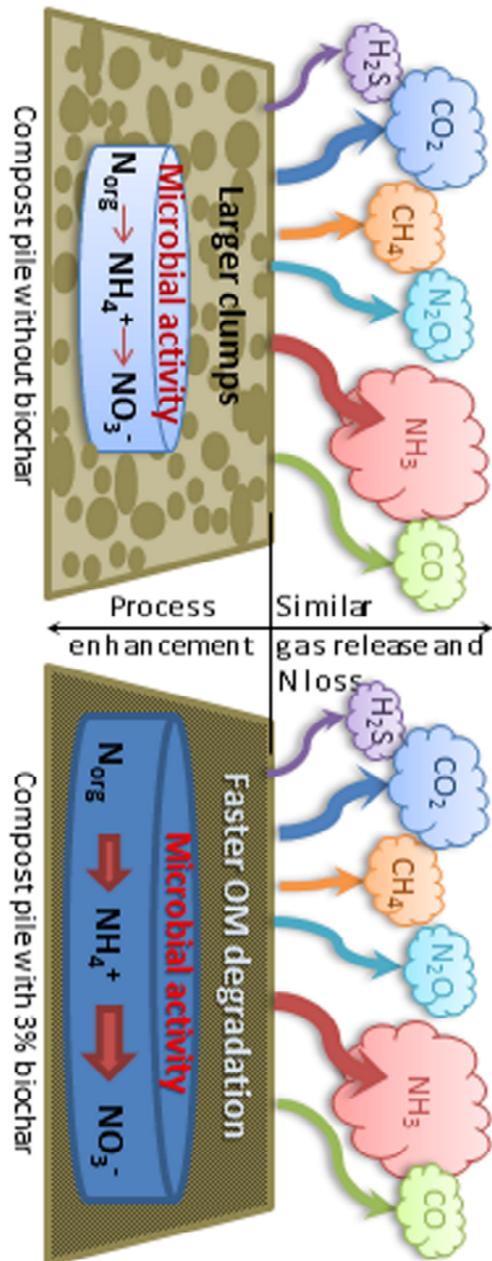


Figure 5. 1. GRAPHICAL ABSTRACT

5.2. Introduction

Intensive livestock production systems generate large amounts of manures and slurries with insufficient surrounding farmland for their safe application as fertilisers (Burton and Turner, 2003). Composting is a technology that can be applied to stabilise and sanitise these biodegradable wastes, representing a cost effective method of recycling nutrients and carbon in agriculture (Kelleher et al., 2002; Larney and Hao, 2007). However, manure composting has been regarded as a source of anthropogenic greenhouse gases and other gaseous emissions such as ammonia, hydrogen sulphide, volatile organic compounds and carbon monoxide (Amon et al., 2006; Sánchez et al., 2015). Therefore, special attention must be paid to reduce gas emissions during composting in order to minimise health and environmental problems and avoid the N-losses that can reduce the agronomical value of the end product. The use of co-substrates (bulking agents, additives) seems to be the most beneficial option to optimise the composting process, in terms of improving the nutritional value of the compost and minimising the potential environmental impacts. A proper mixture for co-composting must comply mainly with the following objectives: optimising aeration condition,

preventing the compaction of the composting piles, achieving an efficient balance of the C/N ratio, assuring biodegradable carbon fractions and energy source for microorganisms and reducing N loss during the process (Cayuela et al., 2009; Haug, 1993)

Biochar is a carbon rich material generated from the pyrolysis of biomass. The use of biochar as amendment in agricultural soils has been suggested as a strategy to maintain soil fertility and productivity, increasing soil carbon sequestration due to its recalcitrant nature (Jeffery et al., 2015; Lorenz and Lal, 2014) and reducing greenhouse gas emissions (Van Zwieten et al., 2015). However, the use of biochar as an additive during the composting process has been less investigated. Recent research suggests that biochar interacts positively with the composting mixture, decreasing bulk density and increasing aeration conditions and hence enhancing composting performance, as also retaining nutrients and improving the quality of the end-product (Steiner et al., 2015; Wei et al., 2014; Zhang and Sun, 2014). In addition, reductions of 52% N losses, 58% NH₃ and 71% H₂S emissions were registered in poultry litter composting mixtures containing 20% of biochar (Steiner et al., 2010). But still, the interactions between organic matter and biochar during composting and the impact on gaseous losses remain largely unknown (Wang et al., 2013).

We hypothesised that the addition of biochar to a composting mixture, even at a low rate, will induce positive effects on process performance, providing better physical properties and decreasing gaseous emissions. Therefore, the main objective of this study was to evaluate the impact of biochar on poultry manure composting by monitoring the composting process and gaseous emissions in order to validate the proposed hypothesis. A complete characterisation of the composts with and without biochar was performed in order to assess the agronomical quality of the end products.

5.3. Materials and Methods

5.3.1. Description of raw materials

A mixture of poultry manure and barley straw was used for the composting experiments. The poultry manure, bedding-free material, was collected directly from the conveyor belts located under the poultry cages in a commercial poultry farm located in Murcia (Spain). The barley straw was acquired from a conventional barley crop in Elche (Alicante, Spain). The biochar (particle size < 10 mm) had a high concentration of total organic C (67%) and a high degree of aromatic condensation (H/C_{org} molar ratio=0.32). It was produced from holm oak by slow pyrolysis at 650 °C and atmospheric pressure, being the residence time in the reactor chamber of 12-18 hours (Proinso Inc. Málaga, Spain). The main physico-chemical characteristics of the raw materials are provided in Table 5.1.

Table 5. 1. Physico-chemical characteristics of the raw materials used in the composting experiment (dry weight)

| | M (%) | pH | EC dS m ⁻¹ | TOC (%) | C/N | N | P | K (%) | Ca | Mg | Fe | Cu | Mn | Zn | Pb | Ni | Cr | Cd |
|----------------|-----------------|-----------------------------|---------------------------------|-------------------|---------------|----------------|----------------|-----------------|----------------|----------------|-------------|---------------|-------------|------------------------|--------------|---------------|--------------|----------------|
| | | | | | | | | | | | | | | (mg kg ⁻¹) | | | | |
| Poultry Manure | 69.6 (0.9) | 6.35 ^a (0.02) | 9.76 ^a (0.09) | 25.2 (0.1) | 4.8 (0.0) | 5.23 (0.02) | 1.17 (0.20) | 1.94 (0.35) | 5.22 (0.85) | 0.41 (0.07) | 486 (73) | 59.6 (9.5) | 224 (38) | 203 (34) | 3.0 (0.3) | 5.2 (0.7) | 8.6 (1.1) | 0.17 (0.03) |
| Barley Straw | 11.7 (0.6) | 5.84 ^a (0.15) | 4.33 ^a (1.03) | 34.7 (0.4) | 44.9 (0.8) | 0.77 (0.00) | 0.06 (0.00) | 2.01 (0.15) | 0.59 (0.04) | 0.09 (0.01) | 244 (10) | 4.9 (0.2) | 46 (3) | 16.5 (0.7) | 0.3 (0.0) | 2.4 (0.2) | 4.8 (0.4) | <0.10 (0.0) |
| Biochar | 35.5 (0.3) | 9.26 ^b (0.13) | 0.57 ^b (0.04) | 67.3 (1.0) | 80.2 (1.0) | 0.84 (0.03) | 0.19 (0.00) | 0.72 (0.01) | 5.25 (0.08) | 0.33 (0.00) | 928 (9) | 16.4 (0.2) | 604 (16) | 19.7 (0.1) | 1.3 (0.1) | 11.2 (0.2) | 3.4 (0.1) | <0.10 (0.0) |

M: Moisture; EC: Electrical Conductivity; TOC: Total Organic Carbon; ^aWater extract 1:10 w:v 25°C; ^bWater extract 1:20 w:v 25°C. Standard deviation in brackets (n=2)

5.3.2. Composting process

Two different composting mixtures were prepared with the following composition (dry weight basis):

- PM: poultry manure (78%) and barley straw (22%) as bulking agent.
- PM+B: the same PM mixture with 3% of added biochar.

To ensure the homogeneous composition of the piles, an initial mixture of poultry manure with barley straw (12000 kg on a fresh weight basis) was prepared. After that, one half was amended with the biochar. Finally, each treatment (PM and PM +B) was divided into two piles (3000 kg each per pile; fresh weight).

The mixtures were composted, in trapezoidal piles of 1.5 m high with a 2x3 m base in a pilot scale plant, by using the turned pile (windrow) system. The piles were turned weekly using a front-end loader tractor during the active phase of the process (approximately 90 days, Figure 5.1) in order to homogenise and aerate the mass, reactivate the process and avoid compaction. Moisture level was maintained during the process between 40 and 60% by adding water periodically. The temperature was measured by inserting a thermocouple into four different points of the pile every three days during the bio-oxidative phase and fortnightly afterwards.

The total duration of the composting process, including the maturation phase, was 135 days.

5.3.3.1. Sampling strategy

On days 0, 23 (3 weeks), 51 (7 weeks), 76 (11 weeks) and 135 (19 weeks), one representative composite sample per pile was taken from randomised sites around the pile. A portion of the composite sample was frozen (-18°C) and stored until extraction for the measurement of ammonium, while the remainder was air dried and ground to 0.5 mm for other chemical analyses. In the final product (week 19), an additional fresh subsample was used for microbiological determinations.

5.3.3.2. Gas sampling

Gas samples were taken every two weeks during the whole composting process. A static closed chamber technique was used to measure gas fluxes from the surface of the composting piles (Sommer et al., 2004). Gas samples were taken from three aluminium closed chambers (volume: 0.009 m³, area: 0.038 m²) placed randomly on the top of each pile,

summing up a total of six gas sampling points per treatment (Sánchez-Monedero et al., 2010). The chambers were pushed 5 cm into the composting pile and air samples were taken within the headspace after 0, 10, 20 and 30 minutes using disposable syringes. Gas samples were immediately transferred to 12 ml gas vials (Exetainer, Labco, UK), which had been previously purged with He and evacuated.

5.3.3. Analytical methods

5.3.3.1. **Physico-chemical analyses**

Moisture content was determined by drying at 105°C to constant weight. Organic matter (OM) was determined by loss on ignition in a muffle furnace at 550 °C. Total nitrogen (TN), carbon (TC) and organic carbon (TOC) were analysed by automatic elemental analysis (LECO CHNS-932, USA). Electrical conductivity (EC) and pH were determined in a 1:10 (w/v) water-soluble extract. NH_4^+ was extracted with 2.0 M KCl at 1:10 (w/v) and determined by a colorimetric method based on Berthelot's reaction (Sommer et al., 1992). NO_3^- and NO_2^- were extracted with water at 1:20 (w/v) and determined by ion chromatography (DIONEX ICS-2100, Sunnyvale, CA, USA). Total concentrations of macro-, micro-nutrients and heavy metals were measured after $\text{HNO}_3/\text{H}_2\text{O}_2$ digestion by using

inductively coupled plasma (ICAP 6500 DUO THERMO, England). Losses of OM and TN were calculated according to Cayuela et al. (2009). Stability and maturity were assessed by respirometric and phytotoxicity methods respectively. Stability was determined by CO₂ production according to Iannotti et al. (1993) and phytotoxicity by a Germination Index (GI) according to the method proposed by Zucconi et al. (1981). In order to evaluate the formation of clumps in the piles, the compost was screened according to gross particle size by sieving below 70 and 25 mm and weighting to quantify the different fractions (>70, 70-25 and <25 mm).

5.3.3.2. Gas emission measurements

Gas samples were analysed by gas chromatography (VARIAN CP-4900 Micro-GC, Palo Alto, CA, USA) to measure CH₄, N₂O and CO₂. In addition, CO and H₂S fluxes were measured using a portable NDIR analyser (Dräger X-am 7000, Dräger Safety, Lübeck, Germany) directly from the static chambers at the same sampling intervals. Emission fluxes were calculated by fitting the experimental data to a second-order polynomial equation (gas concentration vs time). The flux at time 0 was calculated by taking derivatives of the second-order polynomial (Hao et al., 2001).

5.3.3.3. Microbiological determinations

Fresh samples of compost were homogenised according to UNE-EN ISO 6887-4:2004. The most probable number technique was followed according to ISO 7251:2005 for the determination of *Escherichia coli* and ISO 6579:2002 horizontal method for the detection of *Salmonella*.

5.3.4. Calculations and statistical analyses

OM and N losses were fitted to a first-order kinetic model: $f(x) = A_0(1 - e^{-kt})$; where “ A_0 ” represent the maximum degradation/loss (% of initial content), “ k ” the rate constant (days^{-1}) and “ t ” the composting time (days). Plots were fitted using SigmaPlot 12.0 for Windows (Systat Software). Univariate analysis of variance was used to investigate the significant differences between PM and PM+B treatments. Additionally, gas fluxes were analysed by repeated measures ANOVA with treatment as independent variable and time as the within subject factor. Analyses of variance were performed with IBM SPSS Statistics 21, Sommers, USA. The relationships between studied parameters were addressed through a correlation analysis (Pearson correlation coefficient). For all analyses, significance was defined as $P < 0.05$.

5.4. Results and Discussion

5.4.1. Biochar effect on composting performance

Both treatments (PM and PM+B) showed a similar pattern of temperature evolution with a rapid activation of the composting process (Figure 5.1). Biochar addition slightly increased the temperature profile during the thermophilic phase. This fact is consistent with previous studies using biochar as co-substrate for manure composting (Jindo et al., 2012; Wang et al., 2013; Wei et al., 2014). They registered higher temperatures and shorter thermophilic phases with the addition of biochar, since biochar constitutes a suitable habitat for microorganism proliferation and exerts a positive effect on substrate properties such as porosity, surface area and moisture content. These changes promoted microbial activity and explained the temperature increase.

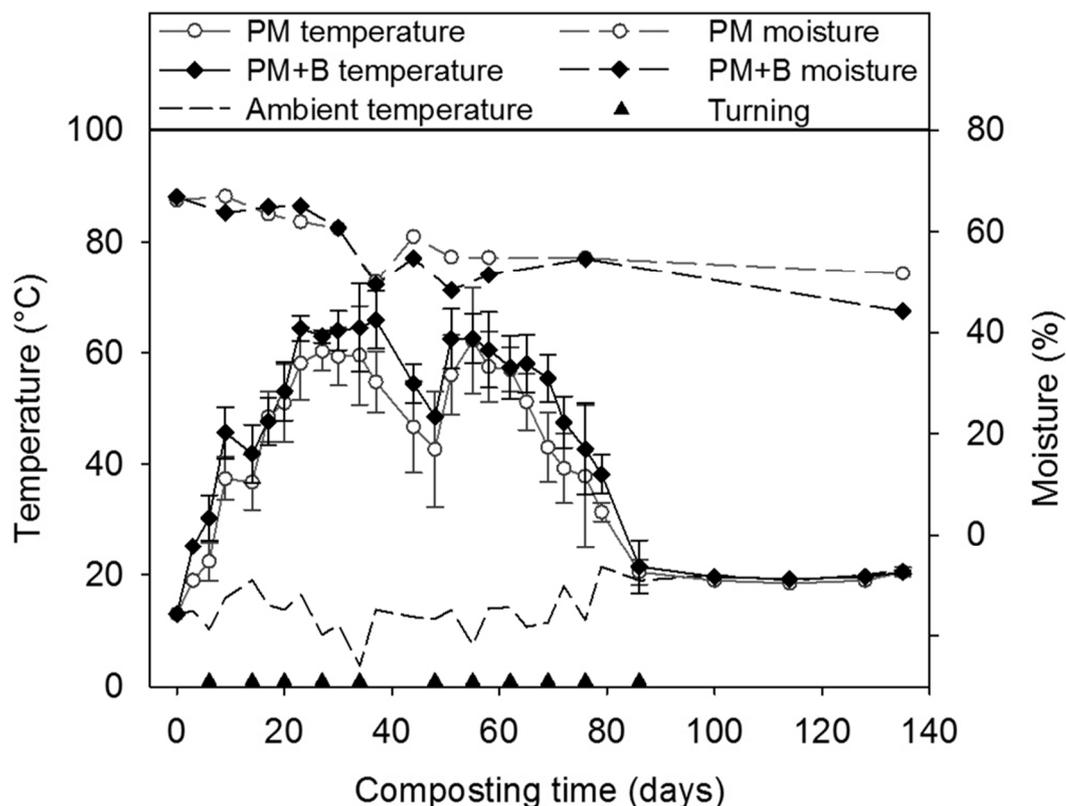


Figure 5. 2. Evolution of the average temperature and moisture during composting of poultry manure mixture (PM) and poultry manure mixture with biochar (PM+B). The arrows in the x-axis indicate turning events.

Biochar also affected the physical properties of the composting mixture. The formation of large clumps during composting can originate anaerobic conditions and reduce the effectiveness of the composting process (He et al. 2000; Yamamoto et al. 2011). In the present study, a progressive formation of clumps of variable size was observed in both treatments from the initial stages regardless of turnings. However, the

formation of large clumps was less pronounced in biochar-amended piles, which suggests that biochar avoided the creation of anaerobic sites, facilitating O₂ diffusion through the composting mixture. At the end of the process, the particle size distribution was measured in both composts. The average weight percentages corresponding to the fractions >70 mm, 25-70 mm and < 25 mm were 26, 38 and 36% for PM compost and 14, 31 and 55% for PM +B. Therefore, biochar addition decreased the formation of large clumps, increasing the proportion of small particles (< 25 mm). This had a positive effect on the composting progress as discussed in the following sections. Our results are consistent with a recent study where the addition of biochar increased the proportion of small particle fractions (Zhang et al., 2014).

The pH values increased throughout the composting process from initial values of 5.9 and 6.5 for PM and PM+B, to a final value of 9.2 for both treatments. Similarly to pH, as composting progressed, there was an increase of the electrical conductivity (EC) related to the substrate mineralisation process and the concentration of the mineral fraction, reaching a final value of 10.0 and 9.4 dS m⁻¹ for PM and PM+B respectively (Supplementary Information, Figure S-5.1).

Biochar addition exerted a clear effect on C mineralisation dynamics (Figure 5.2). The OM losses followed a first-order kinetic model, as generally found in composting experiments. Biochar-amended piles showed a lower maximum OM loss (A ; 71.6 vs 77.6%) but higher rate constant (k ; 0.06 vs 0.03 days⁻¹) and mineralisation rate ($A \times k$; 4.0 vs 2.24% OM days⁻¹). The reason behind the stimulation of the OM mineralisation in PM+B piles must be related to the improvement of the substrate structure above mentioned. Biochar is a highly porous material with a high capacity to retain the excess of water, improve aeration conditions and provide a suitable habitat for microorganisms, promoting metabolic activity and increasing organic matter decomposition (Steiner et al., 2015).

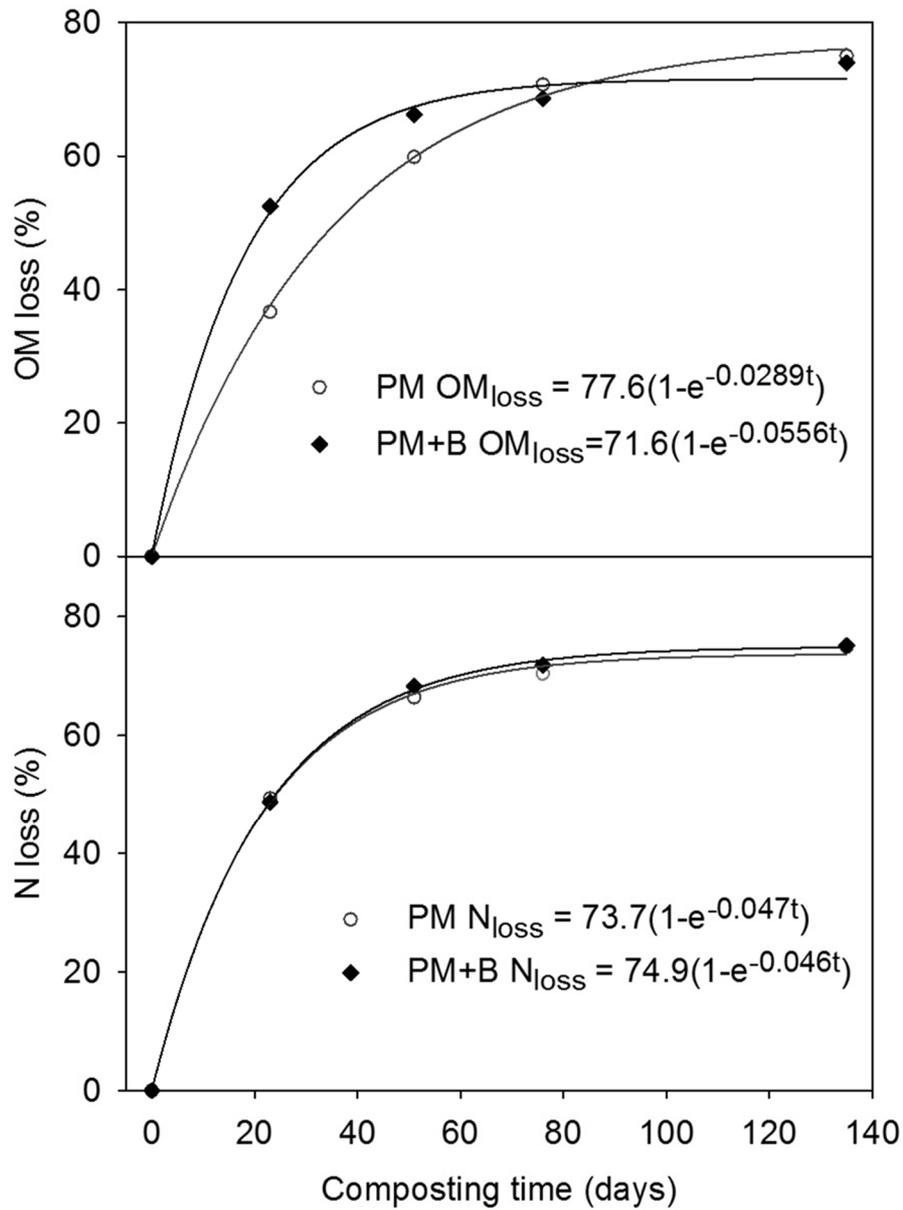


Figure 5. 3. OM and N loss patterns during composting of poultry manure mixture (PM) and poultry manure mixture with biochar (PM+B).

Associated to OM mineralisation, there was an increase in the concentration of NH_4^+ during the initial stage of the composting process (Figure 5.3). The increase was slightly higher in biochar-amended piles, which suggests an enhancement of the ammonification process associated to a higher mineralisation rate. In addition, the nitrification process was also promoted by biochar addition. As the nitrifying bacteria are aerobic microorganisms, it is probable that the better aeration conditions given by biochar enhanced the nitrification process. Zhang and Sun (2014) reported lower NH_4^+ and higher NO_3^- contents in the final product of the co-composting of green waste with biochar, and related it to a better microenvironment for the nitrifying bacteria in the biochar treated mixtures.

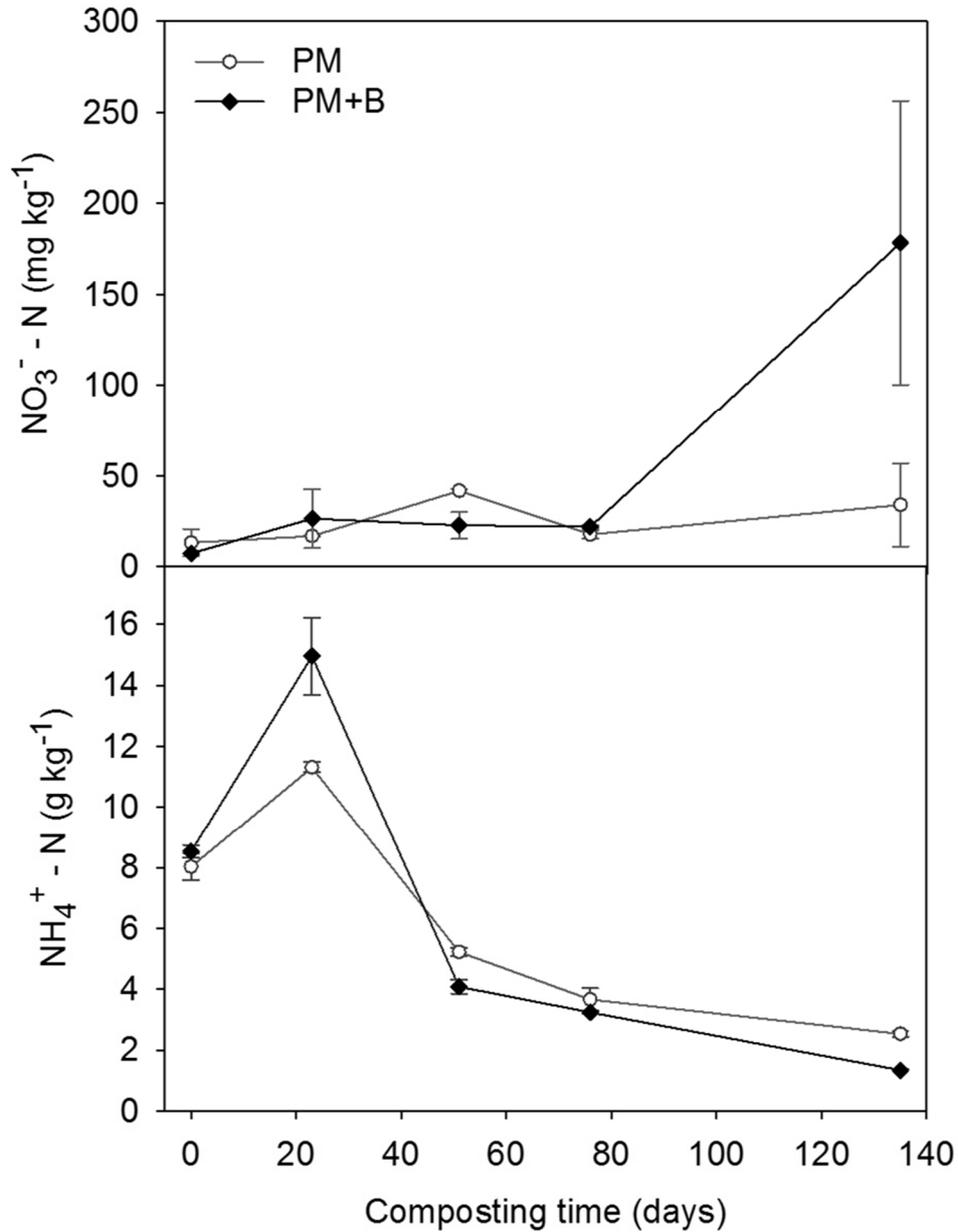


Figure 5. 4. Evolution of the mineral N ($\text{NO}_3^- - \text{N}$ and $\text{NH}_4^+ - \text{N}$) during composting of poultry manure mixture (PM) and poultry manure mixture with biochar (PM+B). Error bars mark standard error (n=2).

Contrary to OM loss, trends in N loss did not differ between treatments (Figure 5.3). As biochar accelerated OM loss, higher N losses would be expected in biochar treated piles. Nevertheless, N loss was similar in both treatments, suggesting a compensation effect by biochar addition. Other studies have found that biochar decreased N loss during composting. For instance, Hua et al. (2009) registered a decreasing total N loss with increasing amounts of added biochar during composting of sewage sludge. They found that biochar at 9% (dry weight) reduced N losses by 64%. In another study with poultry litter, treatments with biochar at 5% and without biochar registered similar N losses, but they were significantly reduced (by up to 52%) in the compost produced with biochar at 20% (Steiner et al., 2010). Apparently the amount of biochar added in the present study (3%) was not enough to induce a perceptible impact on N loss reduction.

The evolution of the stability and maturity indices during the composting process is presented in Figure 5.4. According to these parameters, both treatments achieved suitable degrees of stabilisation and maturity at the end of the process. However, biochar-amended piles were stabilised and detoxified faster than control piles. After fifty days of

composting, biochar treated piles were already stabilised whereas control piles needed extra four weeks to reach a similar degree of stability and maturity. Poultry manure is a substrate rich in water-soluble salts, causing relatively high mineral salt concentrations in the end-product, which are responsible of low GI values (Huang et al., 2004). Nevertheless, all piles reached germination indices above 50%, which is defined as a quality parameter for composts produced from manures and slurries (Bernal et al., 1998).

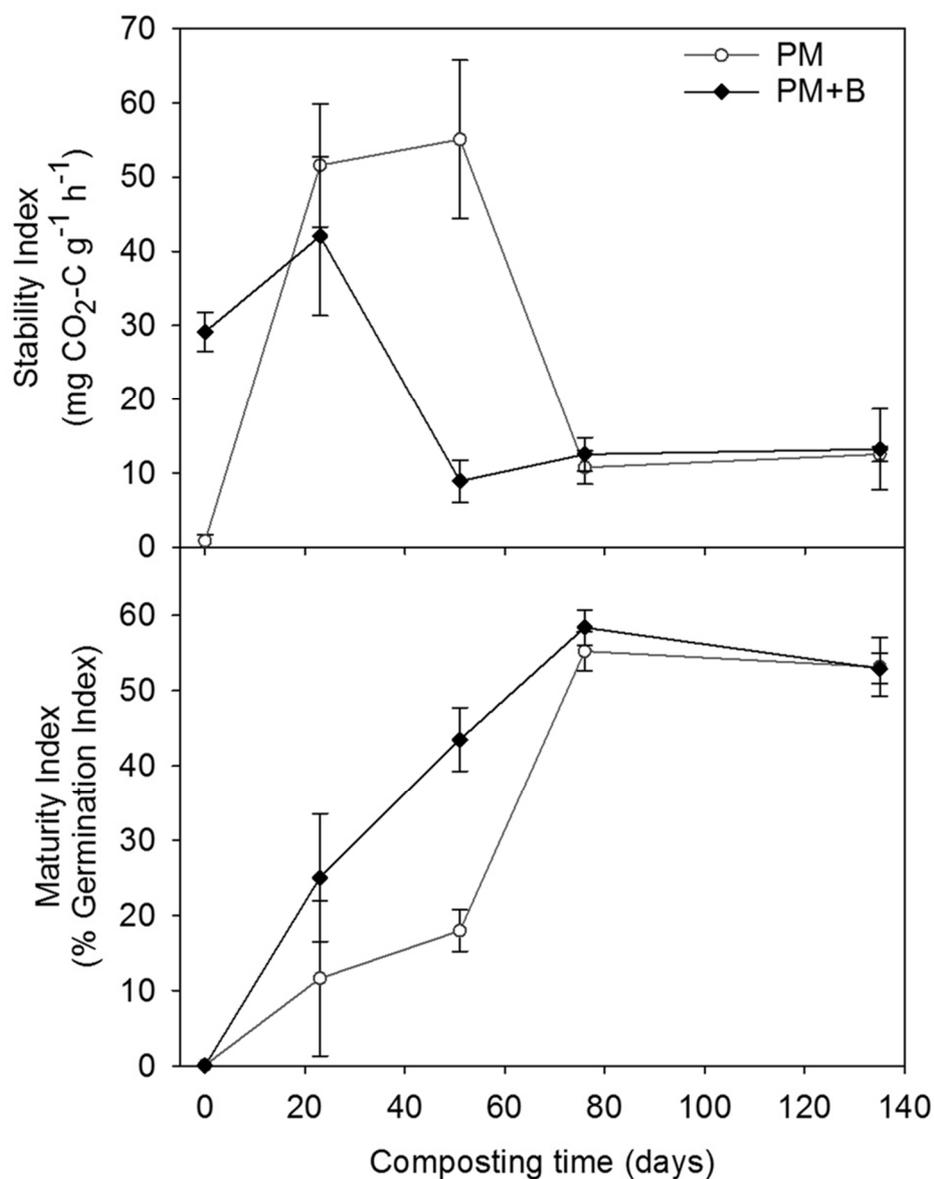


Figure 5. 5. Evolution of stability and maturity indices, according to Iannotti et al. (1993) and Zucconi et al. (1981) respectively, during composting of poultry manure mixture (PM) and poultry manure mixture with biochar (PM+B). Error bars mark standard error (n=2).

5.4.2. Biochar effect on gaseous emissions during composting

5.4.2.1. Carbon gaseous emissions

The fluxes of CO₂ and CH₄ emissions (Figure 5.5) fitted well with the temperature profiles (Figure 5.1), as products resulting from the microbial activity. CO₂ was the main gas generated during the bio-oxidative phase and its production correlated to temperature values at a significance level of 0.01 in both treatments. No significant differences in CO₂ fluxes were observed between treatments, showing an average emission flux over the composting process of 215 g CO₂-C m⁻² day⁻¹ for PM and 197 g CO₂-C m⁻² day⁻¹ for PM+B. In all piles, the highest CO₂ production occurred mainly during the initial 60 days of composting, with a marked decrease around day 35, which coincides with a decrease in temperature. Subsequently, CO₂ fluxes gradually decreased during the end of the thermophilic phase (70th day) to levels lower than 200 g m⁻² day⁻¹, and finally CO₂ emissions during maturation dropped to levels below 60 g m⁻² day⁻¹. This is indicative of a progressive stabilisation of the material since CO₂ production is frequently used as a respirometric index, which reflects microbial activity (Barrena et al., 2006).

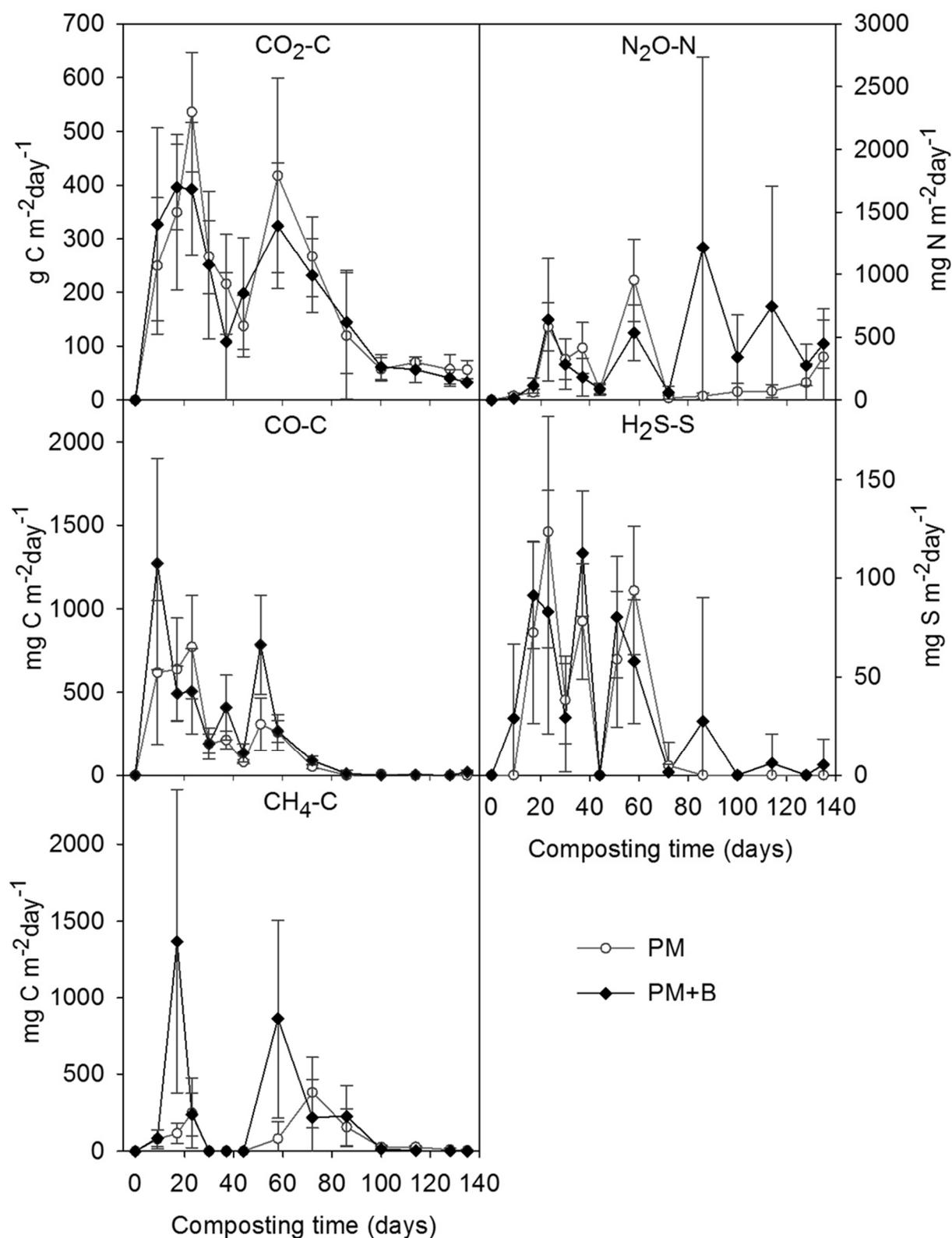


Figure 5. 6. Gaseous fluxes during composting of poultry manure mixture (PM) and poultry manure mixture with biochar (PM+B). Error bars represent the standard error of the media (n=2)

A similar pattern was followed by CO fluxes, which were correlated with CO₂ in all treatments, at a significance level of 0.01 in PM piles and of 0.05 in PM+B piles. The average CO fluxes did not statistically differ between biochar and control piles (298 and 224 mg CO-C m⁻² day⁻¹, respectively). The highest fluxes of CO were registered at the beginning of the composting process, as found in other composting matrices like greenwaste, manure and municipal solid waste (Hellebrand and Schade, 2008; Phillip et al., 2011). To date, the number of studies analysing CO emissions during composting is very limited. Hellebrand and Schade (2008) demonstrated that the CO produced during composting is generated by abiotic reactions and not by microorganisms even if they normally occur during the initial active stage when microbial activity is high.

CH₄ release correlated positively with CO₂ fluxes (P<0.05 in both treatments) and the biochar-amended treatment registered the highest flux-peaks (Figure 5.5). CH₄ production during composting is mainly associated to anaerobic processes. However, favourable conditions for CH₄ release from composting piles can be temporally found at the early stages of the composting process, where large amounts of nutrients and available sources of organic compounds stimulate microbial growth, depleting the oxygen levels in the pile (Sánchez et al., 2015). The two peaks of CH₄

registered in the biochar-amended piles presented high standard errors, reflecting the spatial variability of CH₄ emissions (Sánchez-Monedero et al, 2010). Although PM+B showed a higher average emission flux than PM (232 and 86 mg CH₄-C m⁻² day⁻¹, respectively) during composting, statistically significant differences between treatments could not be found due to the large variance of the data. Recent studies have shown that biochar was able to decrease CH₄ emissions during cow manure (Sonoki et al., 2013) and municipal solid waste composting (Vandecasteele et al., 2013). However, higher biochar application rates (10%) were used in these experiments. Most likely, the comparatively small biochar application rate used in our study was not enough to improve the aeration conditions at the required level.

5.4.2.2. Nitrous oxide emissions

During the thermophilic phase, N₂O emission peaks corresponded with the highest temperatures reached during this stage of the composting process. In this period, N₂O-N emissions in both treatments were similar. Nevertheless, in the subsequent mesophilic and maturation phases, higher peaks of N₂O-N emissions were registered in PM+B compared to PM piles, which led to a lower mean emission flux in the latter (380 mg N₂O-N m⁻²

day⁻¹ for PM+B and 240 mg N₂O-N m⁻² day⁻¹ for PM). The observed peaks (954 mg m⁻² day⁻¹ and 1217 mg m⁻² day⁻¹, PM and PM+B respectively) were lower than previous flux rates reported from other manures (swine manure: 1365 mg m⁻² day⁻¹; cattle dairy manure: 1800 mg m⁻² day⁻¹) and higher than those reported from other wastes (municipal solid waste: 261 mg m⁻² day⁻¹), which is consistent with manure characteristics favouring GHG emissions during composting (Sánchez et al., 2015). Although the mechanisms of N₂O formation during composting have not been studied in detail, N₂O emissions are mainly associated to nitrification and denitrification processes (Sánchez-Monedero et al., 2010). These microbial processes are regulated by the amount of mineral N, the presence of available carbon sources and the O₂ concentration in the composting substrate. Both nitrification and denitrification processes coexist in composting piles. As addressed previously, increasing microbial activity can temporarily lead to a decrease in O₂ availability creating anaerobic conditions favourable for denitrifying microorganisms (Rogers and Whitman, 1991). However, nitrification has been also found to substantially contribute to N₂O emissions during the maturation phase (He et al., 2001).

Recent studies have found that biochar decreases total N₂O emissions during composting of manures. For example Wang et al. (2013)

showed 31% lower emissions in biochar treated piles and linked this reduction to a change in the abundance and composition of denitrifying bacteria. In our experiment, the significance of differences between treatments could not be determined as a consequence of the high variability of the registered N₂O fluxes. Nevertheless, there was a direct relationship between N₂O emissions and NO₃⁻ formation in PM + B piles, as N₂O emissions occurred during a period of intense nitrification. This seems to indicate that biochar increased N₂O formation by the nitrification pathway (Beck Friis et al., 2000; Sánchez-Monedero et al., 2010).

5.4.2.3. Hydrogen sulphide emissions

Hydrogen sulphide (H₂S) release was positively correlated with temperature (P<0.01 in both treatments) and with CO₂ fluxes (P<0.01 in PM piles and P<0.05 in PM+B piles). Similarly to CH₄ and CO, its formation and release mainly occurred during the thermophilic phase of the composting process coinciding with the highest microbial activity. H₂S formation is promoted by reducing conditions and availability of sulphates and sulphur-containing organic compounds (Christian et al., 2010). Despite the enhanced microbial activity in PM+B piles, no significant differences were observed in H₂S fluxes between treatments with an average emission flux

over the composting period of 37 and 34 mg H₂S-S m⁻² day⁻¹ for PM+B and PM, respectively. Steiner et al. (2010) registered higher H₂S flux-rates in composting piles without biochar amendment, which was explained by the better aeration of the biochar treated mixtures.

5.4.3. Biochar effect on compost quality

Table 5.2 exhibits the main physico-chemical characteristics of the composts obtained. The composts had alkaline pH and relatively high electrical conductivity. Except for N, there was a general increase in the concentration of mineral elements when compared to the raw material, as a consequence of the concentration effect caused by the degradation of the organic matter. However, the presence of biochar did not have a distinctive effect on the total and water-soluble amount of macro and micronutrients. With respect to the maturity indices commonly used to define compost quality (Bernal et al., 1998), all composts verified GI > 50% and C/N < 12. The organic matter content in PM and PM+B piles were 44 and 45% respectively.

All composts reached the sanitary requirements according to the European guidelines with values of *E. coli* < 1000 CFU G⁻¹ and absence of

Salmonella. In addition, all the composts satisfied the thresholds established for maximum heavy metal content to be classified in class 1 except for zinc (Zn) concentrations (higher than 200 mg kg⁻¹). Thus, the composts obtained would be labelled as class 2 and shall be used in a quantity not exceeding 30 tonnes dry matter per hectare on a three-year average (European Commission, 2001).

Table 5. 2. Main physico-chemical characteristics of the composts (dry weight). PM: poultry manure+barley straw mixture and PM+B: poultry manure+barley straw mixture blended with biochar.

| | pH ^a | EC ^a (dS m ⁻¹) | C/N | | <u>N</u> | <u>P</u> | <u>K</u> | <u>Ca</u> | <u>Mg</u> | <u>Fe</u> | <u>Cu</u> | <u>Mn</u> | <u>Zn</u> | <u>Pb</u> | <u>Ni</u> | <u>Cr</u> | <u>Cd</u> |
|-------------|-----------------|--|--------|------------|----------|----------|----------|-----------|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| | | | | | (%) | | | | (mg kg ⁻¹) | | | | | | | | |
| PM | 9.12 | 10.02 | 6.87 | TC | 2.77 | 2.07 | 4.58 | 9.89 | 0.83 | 1507 | 93 | 380 | 365 | 21.0 | 11.4 | 19.0 | 0.32 |
| | (0.19) | (0.59) | (0.20) | | (0.05) | (0.49) | (0.75) | (2.89) | (0.26) | (258) | (17) | (72) | (58) | (3.9) | (1.8) | (3.6) | (0.04) |
| | | | | WSC | nd | 0.25 | 4.46 | 0.07 | 0.04 | 175 | 59 | 16 | 74 | 1.1 | 2.5 | 2.6 | <0.20 |
| | | | | | | (0.00) | 0.12 | (0.00) | (0.00) | (2) | (1) | (1) | (2) | (0.1) | (0.1) | (0.1) | (0.00) |
| PM+B | 9.09 | 9.37 | 8.26 | TC | 2.58 | 2.34 | 4.48 | 9.74 | 0.80 | 1245 | 96 | 441 | 358 | 10.6 | 9.9 | 16.1 | 0.34 |
| | (0.11) | (0.01) | (0.41) | | (0.01) | (0.32) | (0.57) | (1.37) | (0.10) | (135) | (12) | (52) | (46) | (1.6) | (1.1) | (1.9) | (0.04) |
| | | | | WSC | nd | 0.25 | 4.33 | 0.07 | 0.04 | 152 | 56 | 17 | 69 | 0.5 | 2.2 | 2.4 | <0.20 |
| | | | | | | (0.00) | (0.26) | (0.00) | (0.00) | (4) | (1) | (1) | (1) | (0.1) | (0.1) | (0.1) | (0.00) |

^aWater extract 1:10 w:w 25°C; EC: Electrical Conductivity. TC: Total Concentration; WSC: Water Soluble Concentration; nd: not determined; Standard deviation in brackets (n=2)

5.5. Conclusions

Our results demonstrate that even with a relatively small amount of biochar addition, the composting of poultry manure with barley straw was significantly enhanced. Biochar addition at 3% promoted rapid OM degradation, minimised the formation of large clumps and accelerated stabilisation and detoxification. Biochar also favoured N mineralisation (ammonification and nitrification). Considering these findings, it was estimated that biochar could reduce the length of the active phase and hence the composting time by four weeks (reducing production costs). Biochar addition neither had an impact on total N loss nor on gaseous emissions. Overall, the addition of a small amount of biochar for composting demonstrated to be a beneficial practice in the management of poultry manure.

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SUPPLEMENTARY INFORMATION

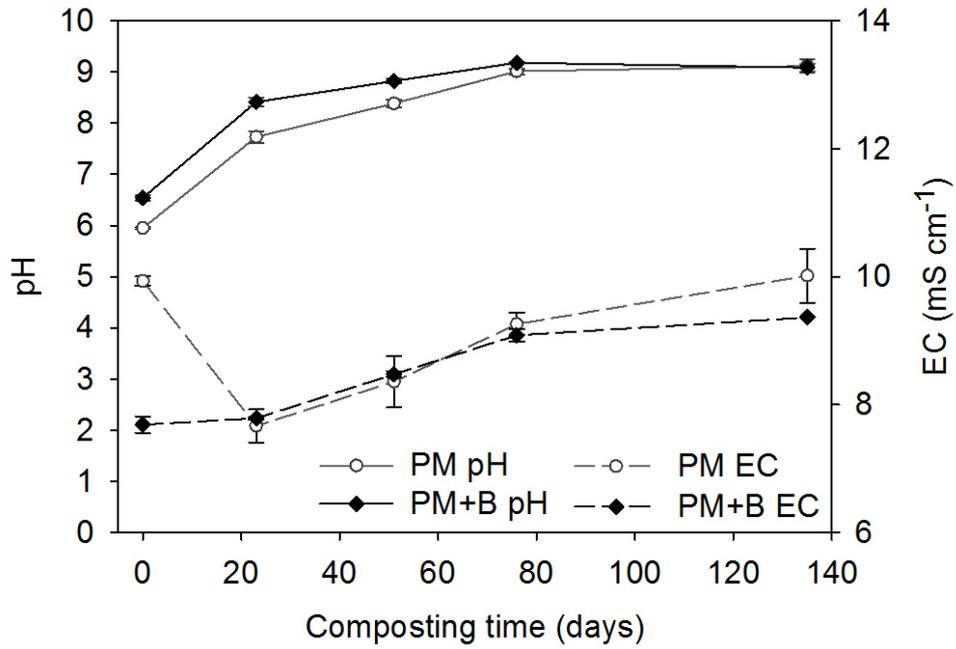


Figure S-5. 1. Variation in pH and EC values during the process of composting. Error bars mark standard error (n=2).

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS



The concentration of atmospheric N₂O has been increasing from a preindustrial concentration of 270 parts per billion by volume (ppbv) to 324 ppbv in 2013 (Ussiri and Lal, 2013). Agriculture is responsible of 60% of global anthropogenic N₂O emissions (IPCC., 2014), which are primarily derived from the use of mineral fertilisers, biological N-fixation and animal manures management (Davidson, 2009). The potential use of biochar as a N₂O mitigation strategy in agriculture is subject of ongoing scientific debate. Although several experiments have shown a substantial reduction in N₂O emissions (Cayuela et al., 2014), the rationale behind this phenomenon is still unknown. The general objective of this thesis was to reach a better understanding of the way biochar influences N dynamics involved in the release of N₂O from two relevant agricultural sources: soils and manure management through composting. With this purpose, the influence of biochar on N₂O emissions was studied in these two different environments. In chapters 3 and 4, biochar impact on N₂O emissions from soils was monitored under laboratory and field conditions, whereas chapter 5 focused on the effect of biochar addition on manure composting.

6.1. Biochar impact on soil N₂O emissions

Despite an increasing body of research has evaluated N₂O emissions from biochar amended soils, little is known about how the specific mechanistic pathways involved in N₂O emissions are affected by biochar. It has been shown that the effect of biochar on soil N₂O emissions depends on both the type of biochar and soil (Yoo and Kang, 2012), with results as diverse as the biochar-soil combinations tested. Many studies have reported decreases on N₂O release from soils after biochar addition (Cayuela et al., 2014) while others have shown no differences or even an increase (Clough et al., 2010; Saarnio et al., 2013; Suddick and Six, 2013). Moreover, Yanai et al. (2007) reported higher or lower N₂O emissions after biochar application depending on the water filled pore space (WFPS). Considering the previous findings, the first hypothesis of this dissertation was that different soils displaying different N₂O formation pathways would be differently affected by biochar amendment, even at identical environmental conditions.

Denitrification has been traditionally considered as the main source of N₂O emissions from soils (Bremner, 1997). This was the case of the acidic soil studied in chapter 3. N₂O fluxes were higher when NO₃⁻ was added to this soil, and ¹⁵N signature confirmed that N₂O was primarily derived from

NO_3^- addition, while other sources of N_2O were not relevant. Biochar addition to this soil led to lower N_2O emissions, which was consistent with previous findings reporting that biochar promotes the last step of denitrification (Cayuela et al., 2013) and an increase of nitrous oxide reductase (*nosZ*) in soils (Harter et al., 2014).

However, the second soil studied in chapter 3 (a Mediterranean calcareous soil) responded differently to the addition of biochar, increasing N_2O emissions. The results demonstrated that the principal N_2O formation pathway in this soil was either nitrifier-nitrification or nitrifier-denitrification. Biochar addition to this soil promoted ammonia oxidation and, possibly, nitrifier-denitrification as a consequence of the low O_2 availability in the nearly water-saturated conditions of the experiment. Recent research has demonstrated that other microbiological mechanisms than denitrification can play a relevant role in soil N_2O emissions. These are the dissimilatory nitrate reduction to ammonium (DNRA), ammonia oxidation (the first step in nitrification), nitrifier-denitrification and co-denitrification (Baggs, 2011; Spott et al., 2011). Nonetheless, the effect of biochar amendment on N_2O emissions derived from these pathways remains largely unknown, being chapter 3 one of the first studies looking

into the impact of biochar on N₂O produced by nitrification (Wells and Baggs, 2014).

Thus, as it was hypothesised, biochar addition affected N₂O emissions differently depending on the N₂O formation pathway operating in the soil. When denitrification was the main pathway, N₂O release decreased after biochar addition whereas it was promoted when nitrification was the prevailing operating mechanism.

The Mediterranean calcareous soil studied in chapter 3 was evaluated under field conditions in chapter 4. The experiment was carried out in an organic olive orchard under semiarid Mediterranean conditions, with compost and biochar application every two years, low-tillage intensity and deficit drip irrigation.

The application of exogenous organic matter is especially important in this kind of agro-ecosystems. Soil organic matter is a key factor preventing soil degradation, which can be especially intense in semi-arid to arid Mediterranean soils. This climate is characterised by reduced precipitation and high temperatures that reduces plant cover, biomass turn over, nutrient cycling and soil organic carbon storage. As a consequence, land degradation is accelerated and accompanied by higher GHG emissions

(Diacono and Montemurro, 2010). Little is known about the possible interactions between biochar and organic matter (Biederman and Harpole, 2013). However, during the field experiment some synergistic effects could be observed in the N related microbial activity. Amended soils treated with a mixture of compost and biochar showed the highest denitrifying enzyme activity (DEA) and increased the size of ammonia monooxygenase gene *amoA*. These effects were not observed in the plots amended with compost nor biochar alone. Since no differences in DOC, organic N, NH_4^+ nor NO_3^- concentration were registered between treatments, the observed differences in the mixture amended soils were probably a consequence of a higher microbial activity.

Although previous field studies have demonstrated a N_2O mitigation with biochar amendment (Felber et al., 2014; Taghizadeh Toosi et al., 2011), the impact of biochar on N_2O emissions from olive orchards do not appear to have been previously reported. The second hypothesis of this dissertation was that the use of biochar as soil amendment alone or in combination with compost would lead to lower N_2O emissions compared to the traditional compost fertilisation. However, the different treatments (biochar, compost or a biochar/compost mixture) did not have a significant impact on field N_2O emissions. In general, N_2O emissions were low in all

treatments, which was consistent with previous studies performed in similar environmental conditions (Castaldi et al., 2011; Suddick and Six, 2013). Therefore, contrary to our hypothesis, biochar amendment did not significantly decrease N₂O emissions compared to compost amendment.

The major reductions of N₂O emissions with biochar utilisation have been generally found under favourable conditions for denitrification, *i.e.* high moisture contents and high organic C and NO₃⁻ availability (Cayuela et al., 2014). However, these conditions were never reached in this field experiment. The low values of DEA in this soil also corroborate that denitrification would not be a predominant pathway. This was already demonstrated in the laboratory experiments carried out in chapter 3 even at favourable denitrifying conditions. During the field experiment, it was necessary the co-occurrence of high moisture and temperature to register N₂O flux peaks and biochar amendment did not reflect a significant impact on N₂O fluxes. Other field studies performed under Mediterranean climate conditions studies have reported similar findings. Angst et al. (2014) reported higher cumulative N₂O emissions in biochar treated plots although not significant in a one year field study under annual ryegrass. Moreover, Verhoeven and Six (2014) reported a significant increase of N₂O emissions in plots amended with pine chip biochar during two years of field

experiment in a vineyard system. These authors also found a seasonal behaviour suggesting a dependence of biochar interaction with N₂O related mechanisms on climatic conditions. However, little is known about the impact of biochar on N₂O emissions from agricultural soils under semiarid conditions where nitrification seems to be the dominant pathway.

These results indicate that biochar might not play a significant role for developing strategies aimed at N₂O mitigation in this type of agroecosystems since emissions are already very low. Moreover, N₂O formation and release might be increased by biochar amendment if N₂O is produced by ammonia oxidation pathways, as it was revealed by laboratory soil incubations in chapter 3.

6.2. Biochar impact on manure composting

Composting of manures is regarded as a source of anthropogenic GHG and other gaseous emissions such as ammonia, hydrogen sulphide, volatile organic compounds and carbon monoxide (Amon et al., 2006; Sánchez et al., 2015). Composting matrices are characterised by a high nutrient and organic matter content and an active microbial biomass,

properties that are markedly different from agricultural soil features. Inside the composting mixture there is a stronger gradient than in soils in biotic and abiotic factors promoting specific N₂O formation pathways. Therefore, different processes can be operating within the composting matrix at the same time. The occurrence of favourable conditions for N₂O production are common inside the composting matrix, *i.e.* a microaerobic environment, high mineral N availability and a high concentration of organic C substrate (Czepiel et al., 1996). N₂O formation and release during composting has been associated to biological nitrification and denitrification processes (Sánchez-Monedero et al., 2010). Although these processes can be operating simultaneously during composting, the changing environmental conditions during the process can promote one mechanism over the other. Moreover, it has been found that N₂O release mainly occurs during the maturation phase (Cayuela et al., 2012), when nitrification has been identified as the main operating pathway (He et al., 2001). Despite there are no studies evaluating nitrifier-denitrification, co-denitrification or DNRA during composting, He et al. (2000) suggested nitrifiers as the main microorganisms that reduce NO₂⁻ to N₂O after the depletion of organic carbon. Some practices such as modifying the aeration rate, turning frequency and bulk density have proved success in lowering N₂O emissions

from composting. However, the optimisation of these practices is elusive since they are dependent on the technology employed as well as on the production scale, being biochar addition during the process proposed as a potential low-technology solution (Wang et al., 2013). The interactions between biochar and organic matter during composting and the consequent impact on gaseous losses have been less investigated than in soils and remain largely unknown. However, there is recent evidence of a positive impact of biochar addition to composting processes. It has been suggested that biochar addition causes a diminution of the composting matrix bulk density leading to a better aeration and hence improving process performance while retaining nutrients and improving the quality of the end product (Steiner et al., 2015; Wei et al., 2014; Zhang and Sun, 2014). Moreover, Steiner et al. (2010) reported decreased N losses as well as lower NH_3 and H_2S emissions during composting of poultry manure with biochar whereas Wang et al. (2013) reported lower N_2O emissions as a result of a higher N_2O -consuming bacteria abundance in pig manure piles composted with biochar. Therefore, the starting hypothesis in the composting experiment was that biochar physical properties would enhance aeration of the composting poultry manure mixture, resulting in lower N_2O emissions and total N losses during the process.

Gaseous emissions from the composting piles surface registered a high spatial variability and, consequently, the significance of the differences between treatments could not be determined. However, some trends could be distinguished. Contrary to the initial hypothesis, N_2O flux peaks during the mesophilic and maturation phases were higher in piles containing biochar, which could be related to an increase in nitrification since NO_3^- concentration also increased. Nitrification has been previously reported as a relevant contributor to N_2O emissions during the maturation phase (He et al., 2001). CH_4 flux peaks also reached higher values in composting mixtures with biochar during the thermophilic phase, which is paradoxical since CH_4 production is associated to anaerobic sites. However, at the early stages of the composting process, when large amounts of nutrients and available sources of organic compounds stimulate microbial growth, oxygen levels are depleted and favourable conditions for CH_4 formation can be temporarily found (Sánchez et al., 2015). Biochar physical properties benefit microbial proliferation and exert positive effects on substrate properties such as enhanced O_2 diffusion through the composting mixture, preventing the formation of anaerobic sites (He et al., 2000). Apparently, the small rate of biochar incorporated to the mixture was not enough to improve the aeration conditions at the required level.

In the end, the presence of biochar did not have a distinct effect on the total and water-soluble amount of macro and micronutrients of the composts, being quality parameters similar in both types of composts (with and without biochar). Nonetheless, the low rate addition of biochar (3%) enhanced the composting process leading to a reduction in the composting time of four weeks, with the consequent alleviation of the production costs. Our experiment revealed that including biochar in the composting mixture prevented the formation of large clumps and favoured organic matter degradation and N mineralisation (ammonification and nitrification) while enhancing detoxification and stabilisation. However, contrary to the starting hypothesis and to the observed enhancement of OM loss, trends in N losses were not affected by the presence of biochar. Other studies have reported reductions of N losses when co-composting of higher rates of biochar with N-rich manures (Hua et al., 2009; Steiner et al., 2010), thus 3% addition probably was not enough to perceive an impact on N loss.

GENERAL CONCLUSIONS

The general conclusions of this thesis are:

1. Biochar affects N₂O emissions from soils differently depending on the N₂O formation pathway operating in the soil. When denitrification is the main process, biochar reduces N₂O release. However, it increases nitrification and the N₂O derived from this pathway.
2. The highest mitigation potential of biochar corresponds to denitrification processes. Therefore, the success of mitigation strategies based on biochar implementation will depend on the main mechanisms involved in the formation and release of N₂O operating in the targeted soil.
3. N₂O emissions from calcareous agricultural soils in semi-arid Mediterranean conditions under organic management are low and biochar has little or no effect on N₂O mitigation compare to traditional management practices.
4. Under Mediterranean agro-climatic conditions the combined use of biochar and compost boosts microbial processes related to the N cycle, with no significant impact on N₂O emissions.

5. The addition of biochar at a rate of 3% (weight basis) does not significantly affect N₂O emissions from a N-rich composting pile.
6. The addition of biochar, even at low rates, to manure composting piles is an effective tool to reduce the time required for manure stabilisation and sanitisation with the consequent benefits on cost savings.

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