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Relevance of Biochar Properties for the Emission of Greenhouse Gases in Agricultural Soils

Relevancia de las Propiedades del Biochar para la Emisión de Gases de Efecto Invernadero en Suelos Agrícolas

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5. Pascual, M.B., Macan, G.P.F., Sánchez-Monedero, M.A., Cayuela, M.L., Lidón, A., Belda, R.M., Fornes, F. Capacidad indirecta del biochar para secuestrar carbono al estimular la producción de glomalina. VII Workshop REMEDIA, Elche-Online (SPAIN). 22-23 September 2020. *Poster Presentation*
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Abbreviations

A	Abiotic
ABTS	2,2′-azino- bis(3-ethylbenzthiazoline-6-sulfonicacid) diammonium salt
AFOLU	Agriculture, forestry and other land use
ANOVA	Analysis of variance one-way
AOA	Ammonia-oxidizing bateria
AOB	Ammonia-oxidizing archaea
Ar	Argon
B	Biotic
BC	Biochar
BET	Brunauer–Emmett–Teller surface area
Bulkρ	Bulk density
C	Carbon
°C	Celsius degrees
CEBAS	Centro de Edafología y Biología Aplicada de Segura (Murcia, SPAIN)
CEC	Cation exchange capacity
CH_4	Methane
CO_2	Carbon dioxide
C_{org}	Organic carbon
CSIC	Consejo Superior de Investigaciones Científicas (SPAIN)
DIC	Dissolved inorganic carbon

DNRA	Dissimilatory nitrate reduction to ammonium
DOC	Dissolved organic carbon
e^-	Electron
EAC	Electron accepting capacity
EC	Electrical conductivity
EDC	Electron donating capacity
EEC	Electron exchange capacity
EU	European Union
GC	Gas chromatograph
GC-MS	Gas chromatograph–mass spectrometer
GHG	Greenhouse gases
GI	Germination index
GS	Grape stalks (biochar feedstock)
GTP	Global temperature change potential
GWP	Global Warming Potential
H	Hydrogen
Ha	Hectare
HHT	Highest treatment temperatures
HNO_2	Nitrous acid
HPLC	High-performance liquid chromatography
IBI	International Biochar Initiative
IPCC	Intergovernmental Panel on Climate Change
k	Oxidation/reduction rate constant
ks	Saturated hydraulic conductivity
Lig/Cell	Ratio lignin cellulose

LOQ	Limit of quantification
M	Molarity
MEO	Mediated electrochemical oxidation
MER	Mediated electrochemical reduction
N	Nitrogen
N_2	Nitrogen gas
NET	Negative emission technology
NH_3	Ammonia
NH_4^+	Ammonium
NH_2OH	Hydroxylamine
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
N_2O	Nitrous oxide
N_2OR	Nitrous oxide reductase
Nu^-	Nucleophile
O_2	Oxygen
Oak	Oak tree (biochar feedstock)
$OH^\cdot$	Hydroxyl radical
Olv	Olive oil tree (biochar feedstock)
OM	Organic matter
PAHs	Polycyclic aromatic hydrocarbons
ppm	Parts per million
PC	Principal component
PCA	Principal Component Analysis

PCR	Principal Component Regression
RI	Biochar reduction index
Ri	Rice straw (biochar feedstock)
SD	Standard deviation
SIM	Selected Ion Monitoring (GC-MS)
SOC	Soil organic carbon
TDC	Total dissolved carbon
TDN	Total dissolved nitrogen
Tg	Teragram (10^{12} g)
To	Tomato plants (biochar feedstock)
TOC	Total Organic Carbon
TporeA	Total pore area
V	Volt
WFPS	Water filled pore space
WHC	Water holding capacity
XRD	X-ray diffractometer

Resumen

El cambio climático es hoy en día una realidad cuyo avance se ha acelerado en los últimos años debido, principalmente, a cambios de carácter antropogénico. Una de las principales consecuencias de estas trasformaciones es el aumento en la temperatura de nuestro planeta, favorecido en gran medida por el gran incremento en la concentración atmosférica de los llamados Gases de Efecto Invernadero (GEIs). Además del dióxido de carbono (CO_2), a la cabeza de los GEIs más prejudiciales y con un índice de calentamiento global (GWP-acrónimo del inglés Global Warming Potential) más alto, se encuentran el metano (CH_4) y el óxido nitroso (N_2O). Sus GWP se encuentran entre 28-36 y 265-298 veces el del CO_2, respectivamente. Debido a las graves consecuencias de carácter socioeconómico y medioambiental que se desencadenarían de no frenar el cambio climático, desde numerosas instituciones internacionales se está animando a los países a poner en marcha políticas y medidas de choque. Entre los organismos más activos se encuentra el Grupo Intergubernamental de Expertos sobre el Cambio Climático (o más conocido en inglés como el Inter-Governmental Panel on Climate Change, IPCC) de las Naciones Unidas.

La agricultura y el uso que, en general, hace el ser humano del suelo, es un sector que contribuye en un 23% al total de las emisiones antropogénicas de GEIs. El suelo representa uno de los mayores reservorios de carbono (C) y nitrógeno (N) del planeta, pudiendo actuar como emisor o sumidero de GEIs. Por cientos de años, existió un equilibrio por el cual los suelos no eran un importante generador de estos gases. Sin embargo, debido al incremento constante de población mundial que se registra desde mediados del S. XX (fenómeno comúnmente conocido como 'La Revolución Verde'), los modelos de gestión que el ser humano ha aplicado al suelo han ido cambiando. Sin embargo, en su implantación, no se tuvo en cuenta el impacto que estas prácticas supondrían para el medio ambiente. Entre otros, los suelos comenzaron ser un importante generador de GEIs.

La presente tesis se centra en el estudio de los mecanismos de formación y consumo de dos GEIs en el suelo, el CH_4 y el N_2O. Las emisiones de CH_4 se encuentran reguladas por dos procesos biológicos, la metanogénesis y la metanotrofia. La metanogénesis comprende la producción de CH_4 por microorganismos metanogénicos a partir de un gran número de sustratos de C. Es un proceso que ocurre únicamente bajo condiciones anaerobias, por lo que abunda en ambientes con mucha humedad o inundados. Otros factores que lo regulan son la cantidad de materia orgánica en el suelo, la salinidad o la presencia de ciertos metales, entre otros. La metanotrofia es el proceso por el cual el CH_4 se oxida a CO_2 a través de una serie de reacciones que llevan a cabo bacterias aerobias. Además de una presencia suficiente de oxígeno (O_2), las condiciones ideales para la oxidación de CH_4 incluyen un pH cercano a la neutralidad, temperaturas entorno a 25˚C o una humedad superior al 20% de su capacidad de retención de agua.

Los mecanismos que regulan la emisión de N_2O por el suelo son numerosos y complejos . Varios son los procesos que incluyen su producción y posterior reducción a N_2, aunque los predominantes en cuanto a producción de N_2O son la nitrificación y la desnitrificación. La nitrificación comprende la oxidación biológica del amoniaco (NH_3) a nitrito (NO_2^-) a través de varios pasos y con la generación de productos secundarios, entre los que se encuentra el N_2O. Entre los numerosos factores que lo regulan, los más limitantes son que requiere la presencia de oxígeno y que requiere de un pH en torno a 6.6-8.5. En cambio, la desnitrificación conlleva la reducción del NO_2^- a N_2O y a N_2 por una gran variedad de microorganismos del suelo. A diferencia de la nitrificación, este proceso ocurre bajo condiciones pobres de O_2. La producción de N_2O está bastante generalizada en ciertos ambientes debido a que es relativamente común que los microorganismos desnitrificantes no cuenten con las enzimas necesarias para llevar a cabo el último paso de este proceso, esto es, la reducción de N_2O a N_2. La nitrificación-desnitrificante (*nitrifier-denitrification),* la reducción disimilatoria de nitrato a amoniaco (*dissimilatory nitrate reduction to ammonium*, DNRA), quimiodesnitrificacion, *n-damo*, o la codesnitrificación son otros procesos que contribuyen a la emisión de N_2O por el suelo. En

particular, la codesnitrificación, es un mecanismo muy similar a la desnitrificación y relativamente recientemente descubierto. Su particularidad versa en la formación de especies híbridas de N_2O y N_2 al generarlos mediante la reacción de especies distintas de N, tales como el NO_2^- y un nucleófilo (por ejemplo, aminas primarias o el amonio).

A partir de 1997 y hasta la actualidad, los informes del IPCC han establecido objetivos específicos para frenar y estabilizar las emisiones de CH_4 y N_2O, incluyendo medidas concretas para el sector agrícola. Entre ellas se encuentran: el impulso de la economía circular, el control del gasto de agua, la limitación de la generación de residuos, la reducción en el uso de fertilizantes, la implementación de técnicas como la reforestación, etc. El biochar es otra de las técnicas sostenibles propuestas y que, cada día, adquiere un mayor protagonismo debido a los efectos positivos que genera para el suelo y no sólo por su potencial efecto para mitigar el cambio climático. El biochar es el producto resultante de someter a residuos orgánicos a un proceso de pirólisis a altas temperaturas (350-700˚C) y en ausencia de O_2. Durante este proceso, la biomasa sufre una serie de trasformaciones que resulta en un producto con un alto contenido en C aromático. Su estructura es principalmente porosa, amorfa y presenta, así mismo, pequeños cristales desordenados semejantes a los del grafito. Esto le confiere una composición también única. Ambos factores determinan sus características, propiedades físicas, químicas y biológicas y, en definitiva, su funcionalidad.

Como enmienda para el suelo, el biochar puede resultar excepcionalmente valioso y útil. Aunque no existe pleno consenso en que todos los efectos que tiene sobre el suelo sean beneficiosos, a través de numerosas investigaciones se ha ido descubriendo cómo el biochar es capaz de mejorar la estructura física y las propiedades químicas del mismo. Por ejemplo, es capaz de adsorber y trasportar sustancias a través de su red de poros, mejorar los parámetros hidráulicos, incrementar el pH o contenido en nutrientes del suelo, facilitar reacciones redox involucradas en las actividades que realizan los microorganismos que en el suelo habitan, etc. Todo ello repercute positivamente sobre la micro y macro biota del suelo, favoreciendo su crecimiento, abundancia e impulsando su actividad. Asimismo, tiene la capacidad de influir en

los mecanismos de producción de GEIs. Concretamente, son prometedoras las cada vez más claras evidencias de que el biochar reduce las emisiones de CH_4 y, especialmente, las de N_2O generadas en suelos. Sin embargo, este efecto del biochar aún sigue siendo controvertido, pues no existe un consenso pleno entre los científicos debido a los resultados contradictorios que se obtienen. Esta disonancia se ve favorecida, principalmente, por la extensa gama de biochars que pueden generarse, dándose lugar a una un sinfín de materiales cada uno con unas características y propiedades diferentes. Se requiere, por tanto, de más investigación focalizada, especialmente, en ser capaz producir biochar "a la carta" en función de un propósito específico. En concreto, y con el apremiante objetivo de frenar el calentamiento global y la emisión de GEIs por el suelo, sería conveniente determinar qué características hacen de un biochar ser más eficaz en la disminución de la producción de los mismos y a través de qué mecanismos lo realiza.

Teniendo en cuenta el contexto planteado y los antecedentes existentes, el objetivo de la presente tesis es entender la interacción e influencia entre el biochar y los ciclos biogeoquímicos de suelo que llevan a la producción de CH_4 y N_2O. Para llevar a cabo este objetivo, se sintetizarán una gran variedad de biochars con propiedades muy diferentes con los que se llevarán a cabo experimentos de laboratorio con suelo. Se estudiará cómo varían las emisiones de CH_4 y N_2O con y sin los biochars, lo que nos permitirá discernir qué materiales son más efectivos en cuanto a mitigación y cuáles de sus propiedades son las responsables de ese efecto.

Para alcanzar este objetivo global, se plantearon los siguientes tres objetivos parciales, cada uno de los cuales incluyó diferentes hipótesis de partida que se abordaron en los capítulos 3, 4 y 5:

Objetivo 1. Relacionar las propiedades del biochar con su capacidad para modificar el potencial oxidativo de CH_4 por suelos agrícolas aeróbicos

Este objetivo fue abordado en el Capítulo 3. La hipótesis de partida fue que los biochar con una alta área superficial y capacidad donadora de electrones son capaces de incrementar la capacidad de los suelos aeróbicos para oxidar CH_4. Para alcanzar éste primero objetivo se analizaron 10 tipos de biochar generados a partir de cuatro materiales de partida y producidos a dos temperaturas de pirólisis diferentes, dos de los cuales además fueron químicamente modificados para aumentar sus propiedades redox. Mediante incubaciones, se estudió la oxidación de CH_4 que generaban estos biochar al adicionarse a un suelo agrícola.

Objetivo 2. Relacionar las propiedades del biochar con su capacidad para modificar los flujos de N_2O procedentes de la desnitrificación en suelo, diferenciándolo de otros mecanismos

En el Capítulo 4 se analizó este objetivo. La hipótesis que se planteó fue que la capacidad del biochar para mitigar las emisiones de N_2O procedentes de la desnitrificación depende de tres de sus propiedades: su ratio C/N, concentración en compuestos aromáticos policíclicos (PAHs) y composición en grupos funcionales redox. Se llevaron a cabo incubaciones de suelo con ocho tipos diferentes de biochar, los cuales fueron analizados al detalle incluyendo su contenido en compuestos aromáticos policíclicos. A las mezclas de suelo y biochar se añadió un fertilizante marcado con ^{15}N para estudiar en exclusiva el proceso de la desnitrificación y poder analizar las dinámicas de las diferentes especies de N del suelo.

Objetivo 3. Investigar la capacidad del biochar para donar electrones al proceso de reducción de N_2O a N_2 que lleva a cabo la bacteria *Paracoccus denitrificans*

Este último objetivo fue acometido en el Capítulo 5. Como hipótesis iniciales se plantearon que el biochar incrementaría la capacidad de la bacteria *Paracoccus denitrificans* para reducir N_2O y que esta capacidad estaría directamente relacionada con el potencial del biochar para donar electrones. Mediante incubaciones bajo condiciones anóxicas, estériles y en ausencia de

cualquier fuente extra de C o electrones, la bacteria *Paracoccus denitrificans* se puso en contacto con diferentes tipos de biochar y N_2O. Se estudiaron los cambios en la concentración de N_2O, así como las propiedades redox de los biochar. Entre los tipos de biochar estudiados se incluyeron dos modificados con mayores capacidades redox y uno envejecido tras su uso en un suelo agrícola durante 5 años.

En los últimos años, se ha propuesto el uso del biochar como práctica para modular las emisiones de CH_4, tanto en suelos aerobios como anaerobios. Las investigaciones que hasta el momento se han llevado a cabo han arrojado resultados contradictorios. Por un lado, se ha observado que, gracias al efecto del biochar sobre la porosidad, pH y disponibilidad de N y C del suelo, éste es capaz de disminuir su producción de CH_4 mediante la modificación de la actividad de los organismos metanogénicos. Además, se ha observado que le biochar también puede incrementar la abundancia de las bacterias metanótrofas y así incrementar el potencial oxidante de CH_4 de los suelos. Sin embargo, por otro lado, hay estudios que han observado los efectos contrarios. Diferentes meta-análisis relacionan estos resultados contrapuestos con la alta variedad de biochars que se pueden sintetizar, pues dan lugar a materiales con características y efectos muy diferentes. Teniendo en cuenta los escasos y contradictorios resultados previos y la necesidad de ahondar en el área, en este trabajo se planteó analizar el efecto de una gran variedad de biochars sobre el potencial de oxidación de CH_4 por un suelo agrícola en condiciones aerobias. La hipótesis de partida fue que, específicamente, las propiedades redox de los biochar junto con su área superficial determinarían su capacidad de alterar la tasa de oxidación de CH_4 del suelo objeto de estudio.

En primer lugar, se sintetizaron diez biochar diferentes. Se produjeron como resultado de combinar cuatro materiales de partida de origen agrícola, dos temperaturas de pirólisis y dos procesos de modificación post-pirólisis. Estos últimos se realizaron con el objetivo de aumentar el número de grupos funcionales superficiales con oxígeno del biochar y así su capacidad

aceptora de electrones. Dos experimentos de incubación se llevaron a cabo, en los cuales el suelo en condiciones aerobias se mezcló con cada biochar independientemente. En ambas incubaciones se estudió la evolución en la concentración de CH_4 gas inyectado en el espacio de cabeza al inicio y en cada muestra. La diferencia entre los dos experimentos de incubación fue que, en uno de ellas, el CH_4 introducido contenía ^{13}C ($^{13}CH_4$), en vez de ^{12}C. Esto permite comprobar si una disminución en la concentración de CH_4 se ha producido como resultado de su oxidación a CO_2 por bacterias metanótrofas mediante la medición del $^{13}CO_2$ (gas desprendido) y el ^{13}C que permanece en el suelo.

Los resultados obtenidos arrojan un alto grado de oxidación biológica de CH_4 en el suelo estudiado en el que la adición de biochar tuvo un efecto limitado. Únicamente uno de los biochar empleados aumentó de manera clara y significativa la capacidad oxidativa de CH_4 del suelo, el sintetizado a partir de restos de poda de olivo y caracterizado por tener una gran área superficial. Éstos hechos, junto con que también registró una mayor concentración en $^{13}CO_2$ emitido y contenido en ^{13}C en el suelo, sugieren que el mecanismo responsable de que este biochar mitigue la emisión de CH_4 fue el incremento de la capacidad del suelo para difundir gases (O_2, CH_4). Las propiedades redox de los biochar, contrariamente a lo que inicialmente se pensó, no resultaron influir la capacidad del suelo para oxidar CH_4, aunque sí otras de sus características. El análisis estadístico de los resultados obtenidos de las incubaciones permitió concluir que los biochar más eficaces para aumentar de manera más sustancial el grado de oxidación de CH_4 del suelo fueron: los generados a partir de biomasa de madera, producidos a alta temperatura y que tenían un bajo ratio O/C y alta área superficial. Por el contrario, aquellos biochar con altas concentraciones en cenizas y alta conductividad eléctrica tendieron a disminuir la capacidad del suelo para oxidar CH_4. El presente estudio arroja luz sobre los posibles mecanismos implicados en la reducción en la emisión de CH_4 en suelos agrícolas enmendados con biochar y, así mismo, abre nuevos caminos para la investigación. Debido a que el estudio se realizó sobre un único suelo, son necesarios futuros experimentos que confirmen y generalicen los resultados.

Gracias a estudios de investigación, hoy en día hay evidencias de que la aplicación de biochar al suelo es capaz de modificar los procesos de generación y reducción de N_2O. En el Capítulo 4 se aborda este papel del biochar. Numerosos son los mecanismos que se contemplan a través de los cuales el actuaría: incremento del pH del suelo, cambio en la abundancia y actividad de las comunidades de microorganismos que intervienen en la producción de N_2O, liberación de compuestos tóxicos, incremento de la aireación y porosidad del suelo, variación en la disponibilidad de diferentes especies de N o la limitación/promoción de procesos redox involucrados en la formación de N_2O. Todos estos mecanismos vienen respaldados por estudios que los sugieren, prueban y corroboran, pero, a su vez, existen investigaciones que los ponen en duda y otras que no consiguen explicar mediante ninguno de ellos sus resultados. La gran variabilidad de materiales y procesos a partir de los cuales se puede generar el biochar, así como lo diferentes que pueden llegar a ser los suelos sobre los que éstos se aplican, es lo que podría estar detrás de los resultados contradictorios obtenidos. Por tanto, existe aún un gran desconocimiento acerca de qué propiedades reunirían los biochar que resultarían más eficaces para reducir la emisión de N_2O por el suelo y sobre qué mecanismos del ciclo del N ejercerían su efecto. En concreto, hay una mayor falta de estudios e investigaciones que empleen suelos calcáreos con pH>8. Aunque estos suelos no son los más abundantes en el planeta y están delimitados en áreas concretas, son relativamente comunes en áreas de clima Mediterráneo y son también responsables de importantes emisiones de N_2O.

El presente capítulo tiene como objetivo estudiar y relacionar las características que hacen que un biochar sea más o menos efectivo en la reducción de N_2O por un suelo agrícola de pH básico y a un 90% WFPS (acrónimo del inglés ‘water filled pore space’). Las condiciones que se establecieron fueron de elevada humedad y con fertilización inorgánica con NO_3^-, las cuales favorecen la desnitrificación. La hipótesis de partida fue que las propiedades fisicoquímicas del biochar determinarían su capacidad para modificar la producción de N_2O por el suelo

proveniente de la desnitrificación. Se prevé que sean especialmente relevantes su área superficial, capacidad redox y ratio C/N.

Ocho fueron los biochar que se sometieron al estudio, provenientes de cuatro materiales de partida diferentes y producidos a dos temperaturas. Todos ellos fueron analizados física y químicamente, incluyendo su índice de germinación y concentración en PAHs. En dos incubaciones independientes se mezcló el suelo con los biochar en condiciones de elevada humedad (90% WFPS) y se añadió KNO_3 o $K^{15}NO_3$ respectivamente. Durante 15 días, se midió la emisión total de N_2O o bien la emisión de N_2O proveniente de desnitrificación. Además, se analizó el contenido de N inorgánico en el suelo con el tiempo. La adición del fertilizante nitrogenado marcado con ^{15}N permite discernir entre el mecanismo de desnitrificación y otros mecanismos potenciales que también resultan en la formación de N_2O.

Los resultados obtenidos muestran que, el biochar es capaz tanto de disminuir como de incrementar o no ejercer ningún efecto sobre la desnitrificación del suelo. Los materiales empleados se separaron en dos grupos según su comportamiento. El grupo más numeroso de biochars tuvo un efecto limitado. Disminuyeron en un 10-26% la producción total de N_2O del suelo, pero la variación no fue significativa. El efecto más sustancial de reducción se observó durante las primeras horas (24-48). Contrariamente, otros biochar aumentaron de manera muy significativa las emisiones de N_2O, tanto a corto como a largo plazo (60-760%). Uno de los biochars empleados, generó un efecto no comparable con ningún otro. A pesar de no disminuir con diferencia las emisiones de N_2O con respecto al suelo control, mostró una evolución del flujo de N_2O con el tiempo anormalmente plana. Además, acumuló nitrito en el suelo y redujo la producción de CO_2 del suelo (17%).

Para determinar las propiedades que caracterizaron a cada uno los dos grupos en los cuales los biochar fueron separados y que resultaron determinantes sobre el efecto que generaron sobre la desnitrificación del suelo, se empleó una herramienta estadística, un PCR (acrónimo del inglés ‘Principal Components Regression’). Este análisis determinó que las propiedades de los biochar

que influyen en la capacidad que estos tienen para influir el proceso de desnitrificación del suelo o emisión de N_2O en general, son su ratio C/N, índice de germinación (GI), contenido en C orgánico soluble y grupos carboxilo/carbonatos en superficie. Concretamente, la correlación sería inversa con C/N y GI, es decir, biochars con altos valores C/N favorecerían menores producciones de N_2O. En cambio, el PCA concluyó que biochars con mayores contenidos en C orgánico soluble, grupos carboxilo/carbonatos y bajos índices de germinación, mayores emisiones de N_2O generarán por el suelo. Al contrario de lo que se esperaba, el PCA mostró que el contenido en PAHs así como la capacidad del biochar para donar, aceptar o intercambiar electrones no influyó en los resultados que se obtuvieron en las incubaciones. La exclusión de los PAHs esta probablemente favorecida por las bajas concentraciones que tuvieron los biochar empleados.

La utilización de fertilizante nitrogenado marcado reveló que, además de la desnitrificación, otros mecanismos ocurrieron de manera simultánea en el suelo y que contribuyeron a las emisiones de N_2O. Esta contribución fue variable. El porcentaje de N_2O no procedente de la desnitrificación para el suelo no enmendado fue del 58% y, dependiendo del tipo de biochar adicionado, este porcentaje aumentó o disminuyó (3-81%). Como posibles mecanismos responsables de estas emisiones se sugieren la codesnitrificación o la nitrificación-desnitrificante, aunque son necesarios futuros experimentos y análisis para poder determinar este aspecto con certeza.

El estudio anteriormente presentado analiza el efecto general de diferentes biochar sobre las emisiones totales de N_2O y las producidas específicamente por desnitrificación. Como siguiente paso, resulta necesario acotar las investigaciones para discernir qué papel tiene el biochar sobre cada una de las reacciones consecutivas que conforman la desnitrificación. Esto es lo que se analizó en el Capítulo 5. Recientemente, un importante número de investigadores han apuntado que el biochar podría tener un efecto relevante sobre el último paso de la desnitrificación, es

decir, la reducción de N_2O a N_2. Los estudios disponibles apuntan a que, el biochar, podría impulsar la actividad de los microorganismos que llevan a cabo la desnitrificación completa, hasta generar N_2, por diversos medios. Uno de ellos sitúa al biochar como mediador de esta reacción redox. Su papel consistiría, o bien en donar al microorganismo desnitrificante electrones o, actuando como 'electron shuttle', es decir el biochar adoptaría la función de especie redox intermediaria entre un dador de electrones presente en el medio y el N_2O. Ambos procesos dependen en gran medida de la presencia de grupos funcionales redox activos en la superficie del biochar y de su capacidad de actuar como medio conductor de electrones. Éstos son, principalmente, los grupos fenólicos, quinonas/hidroquinonas y estructuras aromáticas con electrones π deslocalizados, cuya proporción y abundancia en el biochar reside, esencialmente, en el material de partida y temperatura elegidos para su producción. Sin embargo, aún no existe un consenso ni certeza en cuanto a si el biochar realmente interviene en el paso de reducción biológica de N_2O a N_2 y si sus propiedades redox intervienen de manera activa y necesaria. Por estos motivos, se planteó el siguiente estudio. En él, se evaluó la respuesta de una bacteria del suelo, (*Paracoccus denitrificans*) que lleva a cabo la reducción de N_2O a N_2, a la presencia de biochars con diferentes propiedades redox. Entre ellos, se encontraban un biochar envejecido de forma natural en el suelo y otro modificado químicamente, dos procesos que aumentaron su número de grupos funcionales oxidados. Como hipótesis de partida se estableció que el potencial de reducción de N_2O por la bacteria en presencia de biochar estaría directamente relacionado con las capacidades redox de este último y, en especial, su capacidad para donar electrones.

Nueve biochars fueron producidos y caracterizados físico-químicamente, en especial, sus capacidades redox (capacidad donadora y aceptadora de electrones). Mediante incubaciones bajo condiciones anóxicas, estériles y en ausencia de cualquier fuente de C o electrones, las suspensiones de biochar se inocularon con *Paracoccus denitrificans*. A continuación, se inyectó una concentración conocida de N_2O en el espacio de cabeza de los tubos. La evolución de la concentración de N_2O, así como el cambio en la capacidad redox de los biochar, fueron

monitorizadas durante una semana. Las condiciones del experimento se establecieron para la adecuada supervivencia de la bacteria, pero, evitando su crecimiento y multiplicación, por lo que el biochar fue la única especie donadora/aceptadora de electrones disponible en la mezcla de reacción.

Los resultados obtenidos muestran como, en ausencia de biochar o la bacteria, no se registró una significativa reducción de N_2O, lo que prueba el carácter biológico del proceso y el papel esencial del biochar. Así mismo, un incremento en la concentración de biochar añadida supuso un aumento en la reducción de N_2O en la mayoría de los casos. En cuanto al potencial de los diferentes biochar, la reducción de N_2O por la bacteria *Paracoccus denitrificans* cambió dependiendo de la presencia de unos biochar u otros. Se encontró que el factor que principalmente determinó este resultado fue la RI (acrónimo del inglés Reduction Index) de los biochar (ratio entre su capacidad de donación y de intercambio de electrones), que depende de su capacidad donadora y de aceptora de electrones, pues se obtuvo una correlación entre ambas variables. Sin embargo, otros factores también intervinieron. Mediante un ensayo estadístico, se corroboró la importancia de las propiedades redox de los biochar y se concluyó que, además, los biochar producidos a bajas temperaturas y provenientes de materiales pobres en lignina promueven la reducción de N_2O por la bacteria *Paracoccus denitrificans*. Aunque se necesiten futuras investigaciones, gracias al presente estudio se prueba la relevancia del biochar como agente donador de electrones en reacciones biológicas. Así mismo, nos acerca a determinar y acotar qué tipo de biochars nos ayudarían a mitigar las emisiones de N_2O por el suelo de manera más eficaz.

Finalmente, las **conclusiones** que se derivan de los tres trabajos presentados son las siguientes:

1. En suelos agrícolas bajo óptimas condiciones para que se desarrolle la actividad de los organismos metanotrófos, el biochar tiene la capacidad de modificar la oxidación de CH_4. Su

efecto es variable y puede tanto incrementar esta capacidad de suelo (2-86%) como disminuirla (3-88%).

2. El biochar promueve el potencial del suelo para oxidar CH_4 mediante su influencia sobre los procesos biológicos que lo llevan a cabo. Este efecto está directamente relacionado con las propiedades fisicoquímicas de los biochar.

3. Los biochar que alcanzan mayores potenciales de oxidación de CH_4 son aquellos que reúnen las siguientes características: altas temperaturas de producción, materiales de partida leñosos, bajo contenido en cenizas, baja conductividad eléctrica y elevada área superficial.

4. El biochar es capaz de variar la producción de N_2O procedente de la desnitrificación de un suelo calcáreo en condiciones de anoxia. El efecto es variable, pudiendo ser de aumento, disminución o de ausencia de variación. Los biochar que redujeron la emisión de N_2O lo hicieron durante las primeras 24-48 horas, siendo no significativa esta disminución a largo plazo. En cambio, otros biochar aumentaron de manera muy sustancial la producción de N_2O (6-1840%) a corto y largo plazo.

5. Las propiedades de los biochar están directamente relacionadas con el efecto que éstos tienen sobre la desnitrificación del suelo. Son especialmente influyentes la ratio C/N, potencial toxicidad (GI), grupos funcionales superficiales o contenido en C orgánico soluble. No lo es, en cambio, su capacidad redox, ratio H/C o concentración en PAHs.

6. En un suelo calcáreo, al 90% WFPS y fertilizado con NO_3^-, el proceso de desnitrificación no es el único responsable de su producción de N_2O. Otros procesos contribuyen en un porcentaje que varía mediante la adición de biochar (3-81%). Se propone la nitrificación-desnitrificante y/o la codenitrificación como posibles mecanismos simultáneos a la desnitrificación.

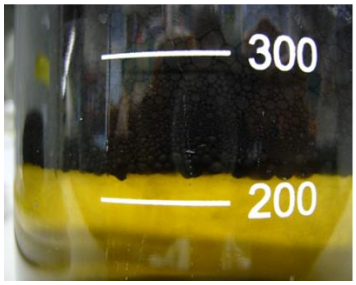
7. El biochar promueve la reducción biológica de N_2O a N_2 por la bacteria *Paracoccus denitrificans* bajo condiciones limitantes de nitrato y oxígeno. Es un efecto que aumenta con la concentración de biochar, pero no en la misma proporción para todos los materiales. La acción

del biochar está controlada, principalmente, por su capacidad para donar e intercambiar electrones.

8. Además de las capacidades redox del biochar, una combinación de otras de sus propiedades juegan un papel determinante en su efecto sobre la actividad reductora de N_2O de la bacteria *Paracoccus denitrificans*. Mayores grados de reducción de N_2O se obtienen con biochars producidos a bajas temperaturas, procedentes de materiales de partida no leñosos y que están caracterizados por altos H/C, contenido en cenizas y bajas áreas superficiales.

Chapter 1

General introduction




It is widely recognized that the reduction of greenhouse gases (GHG) emissions is crucial for mitigating climate change, as highlighted by the Kyoto protocol, the Paris agreement (UNFCCC, 2015) and numerous reports from the Inter-Governmental Panel on Climate Change (IPCC). Global warming has escalated by the rapid increase in the world population and the anthropogenic production of three main GHG: carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (KPG, 1998). Although CO_2 is the largest contributor, CH_4 and N_2O cannot be neglected since their global warming potential is 28 and 265 times greater than that from CO_2, respectively (IPCC, 2014). Without solid and urgent mitigation policies during the coming years, the global temperature is likely to increase by 1.5°C by 2030. This scenario will lead to the irreversible loss of the most fragile ecosystems and an endless serious socio-economic crisis for the most vulnerable people and communities (IPCC, 2018).

The recommended measures for avoiding these negative impacts in the forthcoming future include actions that involve all sectors and that affect economic growth, technology developments and lifestyles. These measures include increasing the use of energy derived from low-carbon-emitting sources (renewables, bioenergy), reducing coal-based power plants, shifting to more efficient vehicle technologies operating on lower-carbon fuels (hybrid or electric), reducing the rates of deforestation, transforming livestock management to make it more sustainable, etc. The IPCC Special Report on Global warming by 1.5°C (IPCC, 2018) has suggested that extra efforts need to be made in the agricultural sector for reducing GHG emissions, without compromising production. Between 2007 and 2016, Agriculture, Forestry and Other Land Use (AFOLU) activities have represented 23% of the total net anthropogenic emissions of GHGs. This percentage would increase up to 37% if pre- and post-production activities in the global food system were included. In particular, the contribution of each gas to the emissions of this specific sector was around 13, 44 and 82%, for CO_2, CH_4 and N_2O respectively (IPCC, 2019).

Recommended measures in the agriculture sector include limiting food waste, improving water management, shifting away from emissions-intensive livestock products, encouraging people to

follow healthier and more sustainable diets, improving agricultural practices towards Circular Economy methods (cascading, composting, feedstock recycling …), or applying techniques as afforestation, reforestation, biochar soil amendments, reduction of the use of mineral fertilizers, etc. The adaptation of agriculture to these strategies would also positively influence soils, leading to an improvement in their quality, fertility, biodiversity and carbon storage ability. Moreover, soil degradation and erosion would be lessened (IPCC, 2018).

1.1. Soils as source/sink of greenhouse gases

Soils represent one of the largest terrestrial carbon (C) and nitrogen (N) pools. They can act as a source or sink of GHG depending on how they are managed, since soil management practices have a direct impact on C and N fluxes between soil and the atmosphere (Oertel et al., 2016). Due to the increase in world's population since 1961 (by 111% between 1961 and 2005), the agricultural production model changed from mainly extensive to intensive, focusing on boosting crop yields. Indeed, a production increase of 162% was achieved during the 1961-2005 period worldwide (Burney et al., 2010). Agricultural practices such as the increased use of pesticides, fertilizers or the development of higher-yielding crop varieties were adopted, leading to the so-called 'Green Revolution' (Evenson and Gollin, 2003). However, these changes were carried out without taking into consideration the impact they may cause on the environment, e.g. soil degradation, chemical pollution, aquifer depletion or soil salinity. It was not until the 1970s when the important contribution of agriculture and soil to the global GHG budget started to be considered (e.g. Schmidt et al., 1988, Hutchinson and Mosier, 1979). Eventually, the imbalance caused by anthropogenic activities on soil CO_2, CH_4 and N_2O fluxes became obvious, and were considered by the United Nations at the United Nations Framework Convention on Climate Change (UNFCCC, Rio de Janeiro, 1992), which included the Agriculture, Forestry and Other Land Uses (AFOLU) sector in their commitment to stabilize GHG atmosphere concentrations. Afterwards, in 1997, specific goals for this sector were proposed in the first Kyoto Protocol (FAO, 2015).

This thesis focuses on non-CO_2 GHG, i.e. CH_4 and N_2O, whose specific production and consumption mechanisms in soil will be covered in the following sections (1.1.1 and 1.1.2.).

1.1.1. Mechanisms involved in soil CH_4 emissions

Methane is a potent GHG and the second (after CO_2) in total annual emissions (IPCC, 2014). Its concentration in the atmosphere has remained stable for thousands of years but, since 1750, it has shown a 150% increase due to human activities. At present, its concentration in the atmosphere is about 1800 ppb (IPCC, 2014). The atmospheric CH_4 that originates from soils is the result of the balance between two mechanisms: methanogenesis (CH_4 production) and methanotrophy (CH_4 oxidation) (Figure 1.1.).

About 69% of the **production of CH_4** is the result of microbial processes, i.e. methanogenesis (Figure 1.1.) (Conrad, 2009). It is carried out by methanogens, microorganisms in the archaea and bacteria domain that are divided into five groups depending on the substrate they use (in brackets) to generate CH_4: hydrogenotrophs (H_2+CO_2), formatotrophs (formate, HCOOH), acetotrophs (acetic acid, CH_3COOH), methylotrophs (methanol and methylamine, CH_3OH and CH_3NH_2) and alcoholotrophs (isopropyl alcohol, $CH_3CHOHCH_3$). They are strictly anaerobic; therefore, their activity occurs only at low redox potentials. Hence, their optimal growing conditions in soils are achieved in anoxic microsites or in waterlogged soils, such as paddy soils. Methanogens can only function as members of a microbial community as they depend on other microorganisms to produce the suitable conditions they need (Topp and Pattey, 1997). They are quite versatile since they can survive and grow in different environments in a wide range of temperatures (4-110°C) and pH (5.6-9.2) (García et al., 2000). Nevertheless, they are sensitive to other factors, such as organic matter (OM), salinity, trace metals and electron acceptors (Serrano-Silva et al., 2014). Significant emissions of CH_4 are generally observed at redox potentials lower than 100 mV, which are favoured in soils with high organic C substrates. Together with a C supply, methanogens require an electron acceptor such as acetate or formic acid and a low content in certain ions (Fe^{3+}, Mn^{4+}, SO_4^{2-} or NO_3^-) that may compete with CH_4.

They also need sufficient Ni and Co, whereas the presence of Cr, Se or high levels of salinity inhibit soil CH_4 production (Dalal et al., 2008). In addition to microbial mediated processes, around 6% of the emitted CH_4 can be generated abiotically from soils (Conrad, 2009). Chemical formation of CH_4 can be triggered during the degradation of soil organic matter under favourable conditions such as high temperatures, intense ultraviolet radiation, or the presence of reactive oxygen species (Liu et al., 2019; Gu et al., 2016; Jugold et al., 2012).

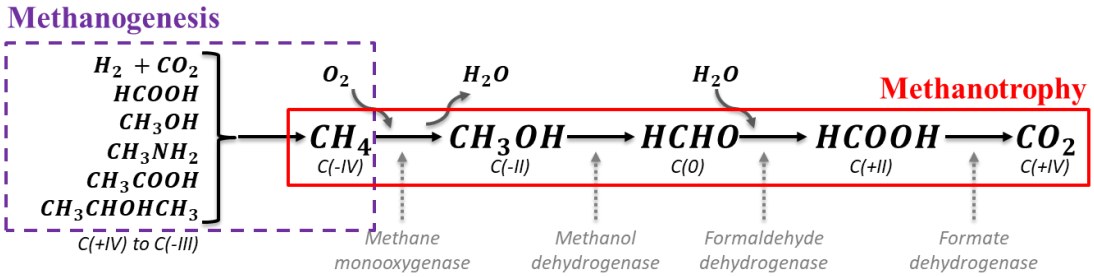


Figure 1.1. Soil processes of CH_4 formation and oxidation, i.e. methanogenesis and methanotrophy. The oxidation state of carbon (*C*) in each involved specie is included as well as the enzymes catalysing every step (Archa et al., 2002; Hütsch, 2001; Topp and Pattey, 1997).

Methane oxidation occurs mainly in the lowest layer of the atmosphere (troposphere) by hydroxyl radicals ($OH^{\cdot}$) and accounts for around 90% of the global CH_4 sink. However, around 4% is carried out biologically in soil, which is of great importance for achieving a balance with CH_4 production (Kirschke et al., 2013). Methane oxidation (Figure 1.1.) is accomplished by aerobic bacteria (methanotrophs), although, additionally, it can be carried out by a consortium of anaerobic archaea in association with anaerobic bacteria. This process involves the oxidation of CH_4 to CO_2 in a series of steps with CH_3OH, formaldehyde (HCHO) and HCOOH as intermediate species (Archa et al., 2002). The enzymes catalysing this reaction are methane monooxygenases and three dehydrogenases (Hütsch, 2001).

Two types of methanotrophs have been reported depending on the available CH_4 concentration. High-capacity/low-affinity methanotrophs grow in environments with high CH_4 concentrations (several-thousands parts per million in the air) often found in waterlogged soils and sediments. Meanwhile, low-capacity/high-affinity methanotrophs are able to make use of trace amounts of CH_4 (around 1.8 parts per million) (Reay, 2003). Although high-affinity microorganisms remain

poorly understood (Conrad, 2009), both of them are found in a wide range of environments. Their optimal activity is observed at neutral pH, temperatures around 25°C and in low salinity habitats. Soil aeration, low concentrations of CO_2 and NH_4^+, or a level of moisture higher than 20% water holding capacity are also essential requirements for methanotrophs activity (Serrano-Silva et al., 2014).

1.1.2. Mechanisms involved in soil N_2O emissions

Nitrous oxide is the most important GHG associated to the soil N cycle. This gas has an average lifetime of 121 years in the atmosphere and a global warming potential (GWP) of 264 (compared to CO_2). Currently, its approximate concentration in the atmosphere is around 320 ppb (IPCC, 2014). The high contribution of this gas to the total GHG emissions from agriculture (82%) has been primarily due to an indiscriminate N application or a poor synchronisation with crop demand (IPCC, 2018). The classical view states that N_2O was mainly formed by denitrification and nitrification pathways. However, its updated examination involves other pathways as well. The main pathways associated to agricultural soils (Figure 1.2.) will be adressed below.

Nitrification is the process in which ammonia (NH_3) is oxidized to nitrate (NO_3^-). It is a multistep process in which the oxidation of NH_3 generates hydroxylamine (NH_2OH), nitric oxide (NO) and nitrite (NO_2^-) as intermediate species. The enzymes that catalyze these steps are ammonia monooxygenase, hydroxylamine oxidoreductase and NO oxidoreductase, respectively. The incomplete oxidation of intermediate species or side reations can result in the formation of N_2O (Figure 1.2.). Lastly, NO_2^- is oxidized to NO_3^- by means of nitrite oxidoreductase (Lancaster et al., 2018). It is generally accepted that autotrophic microorganisms are responsible for nitrification, although there is some evidence of heterotrophic nitrification performed by fungi (Hayatsu et al., 2008). Autotrophic nitrification is performed by ammonia-oxidizing bacteria (AOB) and archaea (AOA) whereas nitrite transformation to nitrate is carried out by nitrite-oxidizing bacteria. There are numerous factors that have influence on the rate of soil

nitrification. Soil pH is one of the major limitations, as it needs to reach values of 6.6-8.5. Moreover, nitrifying organisms are obligate aerobes since they require oxygen (O_2) to form NO_2^- or NO_3^-. Levels of soil porosity between 50-60% are optimum for nitrification (Jalota et al., 2018). In addition, other soil properties such as temperature, organic C, NH_4^+ availability, or C/N ratio can greatly impact nitrification (Hu et al., 2015; Subba Rao et al., 2006; Bremner, 1997).

Nitrifier-denitrification is the pathway in which NH_3 is oxidized to NO_3^- followed by the reduction of NO_2^- to NO, N_2O and N_2 (Figure 1.2.). Only NH_3-oxidizers (AOB) carry out this process by means of the same set of enzymes that catalyze nitrification and denitrification, i.e. nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Wrage et al., 2001). Low concentrations of O_2 appear to favor nitrifier-denitrification. In aerobic environments or under changing cycles of oxic-anoxic conditions, nitrifier-denitrification is not supressed but N_2O and NO emissions increase at the expense of N_2. Other factors that may stimulate this mechanism of N_2O production are low C contents, large concentrations of NO_2^- and temperature. Nevertheless, the knowledge on the environmental aspects that influence nitrifier-denitrification is still at an early stage (Wrage-Mönnig et al., 2018).

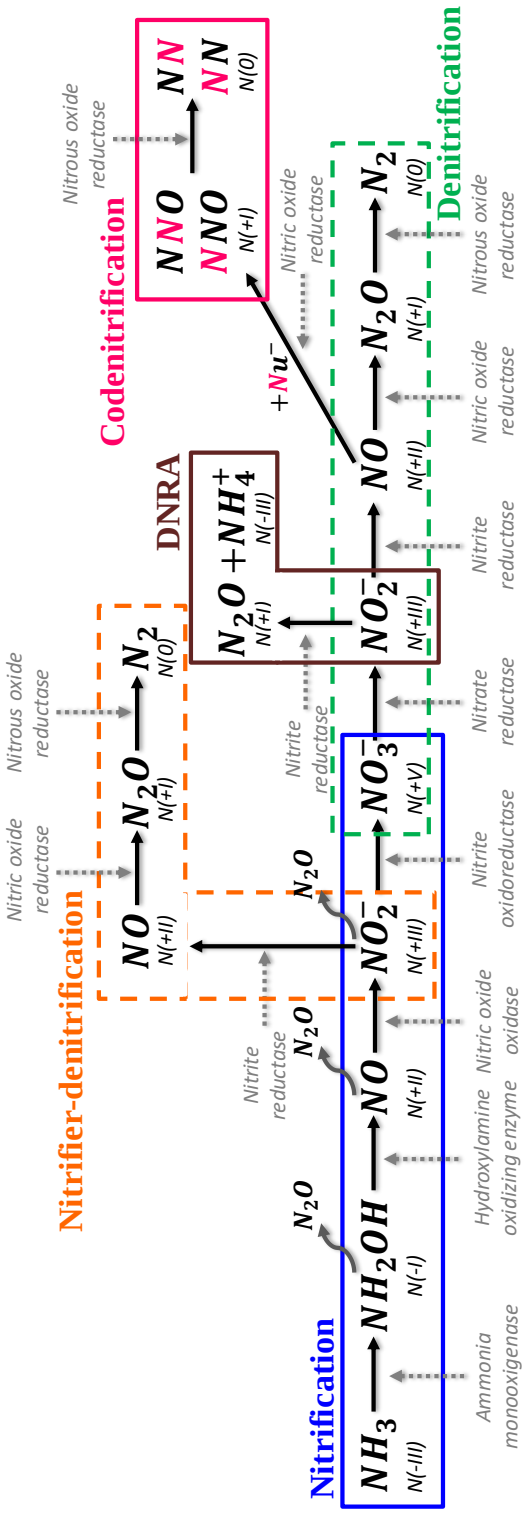


Figure 1.2. Pathways of N_2O production and reduction in soil. The oxidation state of nitrogen (N) in each molecule is included as well as the enzymes that catalyse every step (in light grey). DNRA=Dissimilatory nitrate reduction to ammonium; Nu^-=nucleophile (NH_4^+, R-NH_2, etc) (Based on: Quick et al., 2019; Jalota et al., 2018; Hu et al., 2015; Spott et al., 2011; Wrage et al., 2001).

Denitrification is a multistep reaction that reduces oxidized mineral forms of N (NO_2^- and NO_3^-) to NO, N_2O and N_2 under oxygen-limited conditions (Figure 1.2.). This is a heterotrophic process of anaerobic respiration conducted mainly by facultatively bacteria that can also respire aerobically. Apart form bacteria, fungi and archaea can also carry out denitrification. Each step is regulated by different enzymes (reductases) encoded by a wide variety of genes (Hu et al., 2015). Although denitrification is a widespread process occurring in either terrestial, marine or freshwater systems, it is regulated by a few factors: a sufficient availability of NO_3^-, electron donors and reduced O_2 concentrations. Nevertheless, denitrification does not always reach its final step to the production of N_2, with the consequence that, in numerous environments, the release of intermediates (NO, N_2O) is relatively common. This can occur under two circumstances. The first one is the existence of bacteria that do not have the complete set of enzymes to fully reduce NO_3^- to N_2. The key enzyme that microorganisms usually lack is nitrous oxide reductase, which is necessary for reducing N_2O to N_2 and that is encoded by the *nosZ* gene. The second occurs in soils with low pH, high NO_3^-/C_{org} ratios and under non-strictly anaerobic conditions (Kamman et al., 2017).

Dissimilatory nitrate reduction to ammonium (DNRA) is a pathway through which NO_3^- is reduced to NO_2^- and subsequently, to NH_4^+(Quick et al., 2019) (Figure 1.2.). Two different set of enzymes are needed for DNRA, one respiratory and the other fermentative. The microrganisms capable of reducing NO_3^- via a dissimilatory pathway are widely spread in soils, and are either bacteria (facultative anaerobes, obligate anaerobes, aerobes …) or fungi (Zhou et al., 2002). The importance of this process is that NO_3^- is transferred into another mineral N form which is less mobile, retaining N at the ecosystem level. Generally less than 1% of NO_3^- reduced to NH_4^+ is emitted as N_2O (Cole, 1988). Generally, denitrification is favored over DNRA. However, under strongly reducing (anoxic) conditions, high pH, high temperatures and low NO_3^- concentrations (or high C_{org}/ NO_3^-), DNRA predominates (Giles et al., 2012; Rütting et al., 2011). These conditions are often found in marine sediments or freshwater systems such as paddy soils, aquifer sediments or tropical and boreal forests (Quick et al., 2019).

Codenitrification is one of the less studied N_2O formation pathways. This microbially-mediated N-nitrosation reaction (a particular class of an electrophilic substitution reaction) produces N_2O and/or N_2 when NO_2^- or NO (nitrosating agent) is combined with one N atom from another N species (Figure 1.2.), i.e. a nucleophile (Nu^-, e.g. primary amines, NH_2OH, NH_4^+). It occurs sequentially, at a sufficient concentration of nucleophiles and by means of the same set of enzymes and similar conditions as denitrification (limited soil O_2 concentration, enough C_{org} availability). However, the essential difference between these two processes is that during codenitrification, the products N_2O and N_2 are hybrid N-N species, where the N-N bond originates from a combination of an N atom from the nitrosating agent and an N atom from a co-metabolized compound (i.e. Nu^-). During denitrification, all the intermediates and the N_2 end product are non-hybrid species (Spott et al., 2011). Due to the scarce research on codenitrification (Selbie et al., 2015; Long et al., 2013; Spott and Stange, 2011; Laughlin and Stevens, 2002), its relevance in the N_2O global budget is still unknown (Spott et al., 2011).

Other N_2O formation mechanisms. Additionally, there are other even less-studied pathways for N_2O formation. Among them is chemodenitrification. It is an abiotic mechanism in which NO_2^- is reduced to N_2O, via nitrous acid (HNO_2), by a chemical interaction with soil organic matter (SOM) or transition metals (e.g. Fe(II)). It has been reported that a substantial production of N_2O through this process takes place in acidic NO_2^--rich soils (Wei et al., 2019). Another recently discovered process that involves N_2O is the *n-damo*. It occurs in freshwater wetlands and couples the anaerobic oxidation of CH_4 to nitrite/nitrate reduction (Shen et al., 2015). Because of the scarce available research, it is still not clear if N_2O is produced. To date, most studies have focused on CH_4 oxidation and look at $^{13}CO_2$ formation from $^{13}CH_4$, but the gaseous products of nitrate reduction (theoretically N_2) have not been evaluated in depth (Shi et al., 2017).

1.2. Biochar

Biochar (BC) was defined by Lehmann and Joseph in 2009 as a carbon-rich product, intended for use as a soil amendment, obtained when biomass (wood, manure, leaves …) is subjected to thermal decomposition under the absence of O_2 and at temperatures in the range between 350 and 700°C. Its unique, complex structure and composition makes it a highly versatile product that is useful in many areas. Specifically, it has risen as a tool for mitigating climate change, managing waste and for improving soil quality (Brassard et al., 2016).

One of the most promising applications of BC is its potential role in soil C sequestration, as a consequence of its recalcitrant C structure that can remain in the soil for hundreds of years. Additionally, the use of BC as a soil amendment can provide other positive side effects on ecosystem services, agriculture and food security. For these reasons, BC has been included as a promising negative emission technology in a recent IPCC special report (Rogelj et al, 2018). Despite the interest of BC on soil C sequestration, its use as a soil amendment may have other side effects on the reduction of greenhouse gas emissions from agricultural soils, in particular N_2O and CH_4, which needs to be evaluated to understand the potential of BC as a greenhouse gas mitigation tool.

1.2.1. Biochar production

There are three main technologies applicable for BC production: pyrolysis, carbonization and gasification, although the first one is the most widely used (Narzari et al., 2015). The three technologies rely on thermal decomposition in the absence of O_2. Three products are always obtained: a solid, C-rich material (BC), a liquid fraction or bio-oil and non-condensable gases. The proportions among them can vary substantially depending on the process parameters (Bridgwater, 2012).

A wide range of biomass types can be used for BC production, from woody biomass and crop wastes to sewage sludge, manures or algae. During pyrolysis, the three main components of the

biomass, cellulose, hemicelluloses and lignin, are transformed. Each of them decomposes at different temperatures and rates (cellulose: 240-350°C; hemicellulose: 200-260°C; lignin at 280-500°C), undergoing a series of physical and chemical transformations (Liu et al., 2015). During the heating process, a range of condensation, polymerization, dehydration, decarboxylation and aromatization reactions take place, resulting in BC's amorphous structure with a certain degree of turbostratic crystallites, pyrogenic graphene like-sheets and pores (Keiluweit et al., 2010. Figure 1.3.). This complex structure defines BC. Therefore, its properties, morphology, functionality and characteristics are mainly driven by a combination of factors, where the original feedstock and its production conditions (i.e. temperature, residence time and heating rate) are the most determinant.

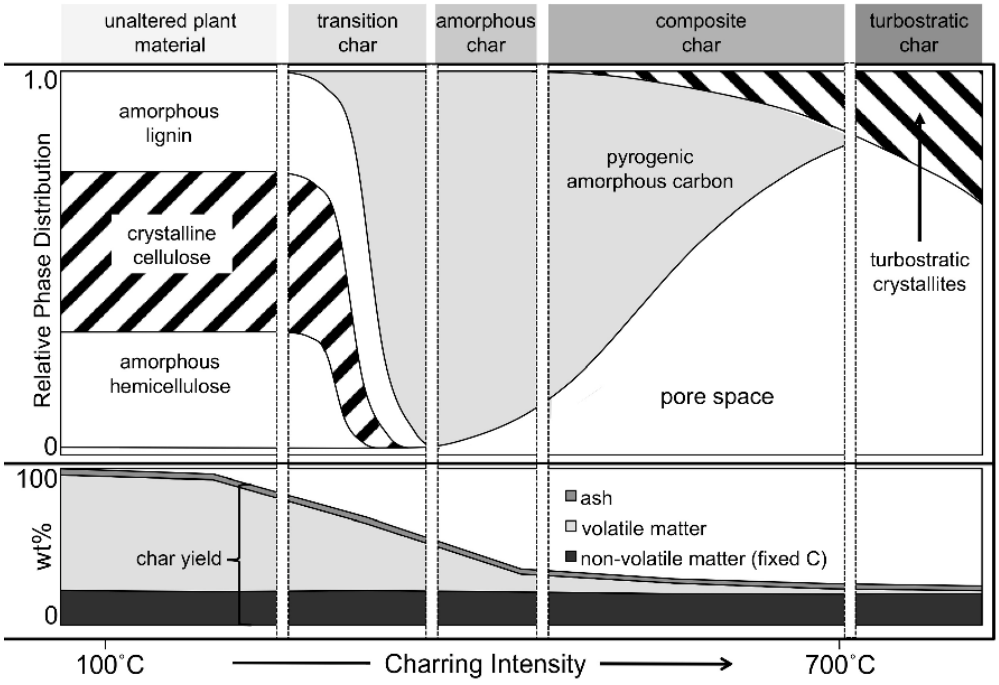


Figure 1.3. Transformation of biomass into BC. Lignin, cellulose and hemicellulose conversion is represented across a charring gradient. The relative phase distribution for each proposed phase in BC (turbostratic crystallites, amorphous C and pore space) is shown. (Keiluweit et al., 2010).

1.2.2. Biochar properties

BC has a wide range of physical, chemical and biological properties, provided by its complex composition and structure. As mentioned in the previous section, BC is composed by a web of

aromatic-aliphatic compounds arranged in graphite-like layers. This C-matrix creates BC's basic structure, i.e. its surface area and porosity, which depends on BC feedstock but, mainly, on its production temperature (Downie et al., 2009). BC porosity and pore size distribution is highly variable and ranges from nano- (<0.9 nm), micro- (<2 nm), meso (2-50 nm) to macro-pores (>50 nm) pores (Bornemann et al., 2007). At low temperatures of production, the pore structure of BC might be limited by the condensation of different compounds (known as tars). However, as the highest treatment temperature (HTT) increases, the mentioned tar components volatilize and the BC structure becomes more regular, the spacing between the graphene like-sheets decreases and the ordering and organization of the molecules increases. As a result, BC porosity is enhanced and it acquires large surface areas (Downie et al., 2009). These phenomena occur until the temperature reaches the limit at which deformation takes place (around 750˚C, Brown et al., 2006). Other factors influence BC structure, such as its production-heating rate, pressure, retention time, and ash percentage.

BC ash content refers to its mineral fraction, which is principally determined by the composition of the biomass and the temperature at which the BC is generated. High ash and low lignin feedstock result in BCs with a large percentage of mineral components. Likewise, its relative content increases due to a concentration effect with the pyrolysis temperature, as the ashes remain in the solid fraction of BC while the organic matter undergoes decomposition. The principal feature that characterizes high ash BCs is their drastic loss in porosity and surface area (Sánchez-García et al., 2019; Ronsse et al., 2013).

The major constituents of BC are C, H, N and O. During the heating process, the proportion of H, N and O lost is greater than the proportion of C. BC is characterized by having a high and recalcitrant C content, i.e. stable and resistant to microbial decomposition (Brassard et al., 2016). These heteroatoms are combined with the aromatic C structure, composing a wide range of different functional groups that grant BC basic/acidic properties, a hydrophilic/hydrophobic character and surface reactivity. The H/C, O/C molar ratios are also used to define the degree of aromaticity and stability of BC. In general, these ratios decrease with increasing HTT

(Amonette and Joseph, 2009). Values of molar H/C below 0.7 are required for a charcoal to be considered BC according to the International BC Initiative (IBI) guidelines. Apart from C, H, N and O, other atoms can be found in BC. During biomass thermal degradation, K and Cl vaporize at relatively low temperatures, while Ca, Si, Mg, P or S are only released at very high temperatures. Other minor elements such as Fe and Mn are largely retained during BC formation (Amonette and Joseph, 2009).

Surface functional groups provide BC with fundamental characteristics such as the cation exchange capacity (CEC), hydrophobicity and redox properties. The oxidation of BC's aromatic C and the formation of chemical groups with a negative charge (e.g. carboxylic groups), together with its high surface area, provides BC with numerous cation adsorption sites. BCs with greater CEC are those pyrolyzed at low temperatures and with high O/C ratios (Huff et al., 2018; Liang et al., 2006). BC hydrophobicity is also related to its surface functional groups, especially to the presence of alkyl functionalities, i.e. C-H (Kinney et al., 2012), and it decreases with increasing HTT (Sánchez-García et al., 2019).

BC also has an important redox activity, which defines its capacity to accept, donate and transport electrons. This ability derives from its degree of aromatization and its surface functional groups. The aromatic ring condensation forms a conjugated π-electron system that allows BC to transport electrons. The chemical groups responsible for BC's capacity to donate and accept electrons are principally quinone/hydroquinone and phenolic moieties, whose proportion varies principally with BC production temperature. Quinone/hydroquinone functionalities prevail at intermediate- and high-HTT BCs, whereas phenolic species reach their greatest proportion in BCs produced at relatively low temperatures, below 400°C (Prévoteau et al., 2017; Klüpfel et al., 2014). Additionally, BC redox activity can be physically and/or chemically modified by applying post or pre-pyrolysis treatments or by its weathering in soil (Chacón et al., 2017; Joseph et al., 2010).

The electrical conductivity (EC) and pH of BC are closely related to its mineral composition and surface functionalities. The EC varies between 0.04-54 dS m^{-1} and depends on its concentration of soluble salts, mainly driven by the feedstock utilized. It increases with increasing HTT (Singh et al., 2017). The pH of BC ranges largely from 3 to 12, although in most cases it is alkaline. It is correlated with the presence of oxygen functionalities and its HTT. At high pyrolysis temperatures, BC acidic groups (mainly carboxylic) are reduced and/or deprotonated, thereby increasing its pH. In addition, when the temperature increases, salts of alkali and alkaline elements increase, resulting in BCs that are more basic (Singh et al., 2017).

1.2.3. Effect of biochar on soil properties

The interest in BC for its application to soils is inspired by the properties of terra preta soils in the Amazon Basin (or most commonly known as Terra Preta de Indio or Amazonian Dark Earths). Terra preta soils originated from the addition of large amounts of charcoal, clay pottery, bones, manure and other organic wastes, with an origin dating to approximately 7000 years ago. At present, these Amazonian soils remain more fertile than their surrounding lands (Lehmann, 2009; Lehmann and Joseph, 2009). Through the following paragraphs, the reported effects of BC on soil will be outlined.

1.2.3.1. Soil physical properties

Some studies have demonstrated the capacity of BC to improve the soil's physical properties. The web of micro-pores make it capable of adsorbing small molecules (gases), whereas larger pores (macro and meso-pores) drive BC's ability to transport substances, supply aeration, texture and bulk density (Lal, 2006; Troeh et al, 2005). In addition, BC can promote soil aggregation and the retention of nutrients and low molecular weight organic compounds. It favours these processes by the creation of binding processes with soil organo-mineral complexes through its surface redox-active moieties (Gul et al., 2015). Another positive aftermath that has been observed after BC addition to soil is the enhancement of its hydraulic

parameters. For instance, soil available water content, saturated hydraulic conductivity (*Ks*), field capacity, permanent wilting point or its total porosity (Edeh et al., 2020).

However, these effects may be largely dependent on the type of soil, BC origin, dose and overall experimental conditions (Blanco-Canqui, 2017). Discrepancies have been reported between studies regarding BC's impact on soil bulk density, wilting point, field capacity (Razzaghi et al., 2020), *Ks* (Lim et al., 2016; Barnes et al., 2014) or, in general, water content (Atkinson, 2018). As a result of these findings, depending on the conditions, BC amendment of soils may be (or not) a plausible option for addressing drought episodes (Jačka et al., 2018), increasing water availability for plant uptake (Major et al., 2009), ameliorating soil compaction or promoting root growth and crop yields (Razzaghi et al., 2020).

1.2.3.2. Soil chemical properties

A soil's chemical properties are influenced by BC addition mainly due to its alkaline nature, high proportion of stable C mineral elements and the abundance and characteristics of the functional groups on its surface. BC has the potential to increase soil pH when applied to acidic or neutral soils due to the addition of mineral carbonates and basic-charged groups (Li et al., 2018). The increase in soil pH remediates the toxicity of acidic soils by decreasing exchangeable Al in soil (Dai et al., 2017). Furthermore, BC is capable of modifying soil CEC, which is a measure of how well some nutrients (cations) are bound to soil, thus preventing them from leaching and making them available for plant uptake. BCs pyrolyzed at low temperatures and with high O/C ratios result in high soil CEC due to their great proportion in hydroxyl groups and charge density (Wang et al., 2017; Mao et al., 2012; Lee at al., 2010; van Zwieten et al., 2010; Liang et al., 2006). Nevertheless, the long term effect of BC on soil's CEC still needs to be demonstrated across BCs and soils (Basso et al., 2015; Zhao et al., 2015).

BCs prepared from manures or sludge have a high proportion of inorganic constituents (see section 1.2.2.), and they can increase soil nutrient content and bioavailability. Levels of soil K,

P, Ca, Mg, Si or B have been reported to increase after BC application to various soils under different conditions (Sackett at al., 2015; Kloss et al., 2014; Liu et al., 2014b). Additionally, through its redox properties, BC can also alter soil functionalities and interact with its biogeochemical cycles. BC can act as an electron donor, an electron acceptor, a conductor or as an 'electron shuttle' (Joseph et al., 2010), affecting the activity of microorganisms. As an 'electron shuttle' (organic molecules that can serve as an electron carrier since it can be reversibly oxidized and reduced (Van der and Cervantes, 2009)), BC has been proven to likewise enhance bacteria metabolism in a vast variety of processes. For instance, the reduction of iron minerals by dissimilatory Fe(III)-reducing microorganisms or in CH_4 production by co-cultures of *Geobacter metallireducens* (Xu et al., 2016; Chen et al., 2014; Kappler et al., 2014).

1.2.3.3. Soil biological properties

The changes that BC produces in soil physico-chemical properties might affect soil microorganisms through a variety of processes. Firstly, BC may serve as a habitat for soil bacteria, fungi and protozoa (Jaafar et al., 2015; Quiliam et al., 2013; Ascough et al., 2010). Even though BC itself is not a source of nutrients, compared to bulk soil, it has the ability to enhance and facilitate nutrient transport, transformation and uptake (Yuan et al., 2017, Atkinson et al., 2010). Consequently, numerous studies have reported an increased microbial biomass in a variety of BC-amended soils (Liu et al., 2016). Nevertheless, since the impact of BC on soil microbiology is highly dependent on numerous factors (e.g. soil texture, vegetation and pH; BC application rate, origin and production) this effect cannot be extrapolated to all BCs, microorganisms and systems. In fact, several studies have shown no significant effects or even a decrease in soil microbial biomass after BC addition (e.g. Noyce et al., 2015; Dempster et al., 2012).

BC may indirectly affect plant growth by modifying the interactions of plants with soil microorganisms. Several mechanisms could be involved (Biederman and Harpole, 2013). For instance, BC can favour the association of mycorrhizas and earthworms with plant roots

(Verheijen et al., 2010), increase soil alkalinity, reducing the mobility of toxic elements for plants (Hass et al., 2012) or change soil nutrient cycles (Taghizadeh-Toosi et al., 2012).

1.2.4. Biochar as a tool to mitigate direct GHG emissions from soils

The modification of soil physical, chemical and biological properties can affect key biogeochemical processes such as those responsible for the production and emission of soil GHGs. In this section, the impacts of BC on the emission of two key greenhouse gases (N_2O and CH_4) are presented.

1.2.4.1. Biochar and nitrous oxide emissions

There is evidence that BC amendments to soil can modify nitrification and denitrification processes, altering N_2O emissions (Cayuela et al., 2014). Several mechanisms may be relevant, which will be outlined below.

Although BC pH varies from acidic to basic, the average pH values of BC is around 8.6 (Brassard et al., 2016). The liming effect of BC has been shown to promote the last step of denitrification and decrease N_2O product ratio of denitrification (N_2O/N_2O+N_2) (Cayuela et al., 2013, 2014). Hence, amending acidic soils with BC may mitigate its N_2O emissions. A low soil N_2O production has been related to changes in the abundance and activity of microorganisms involved in denitrification. For instance, Harter et al (2016) showed that microbial denitrifiers and N_2O reducers carrying genes which efficiently perform complete denitrification, were stimulated in BC-amended soils, resulting in lower ratios of $N_2O/(N_2O+N_2)$. Similarly, van Zwieten et al (2014) detected a significant increase in the *nosZ* gene (encoding N_2O reductase) abundance when their soil was treated with BC. Ji et al. (2020) and Xiao et al. (2019) also reported enriched *nirK* and *nirS* (nitrite reductase genes) abundances apart from *nosZ.* In addition, lower N_2O emissions can be produced as a result of BC hindering nitrification. For instance, by lowering the abundance of AOB (Liu et al., 2014a) or favouring the growth of AOA (Xiao et al., 2019), which would prevent N_2O emissions through nitrification. Other

researchers claim BC can decrease soil N_2O emissions via limiting substrates for nitrification and denitrification (van Zwieten et al. 2015, 2014; Kammann et al., 2012). However, this effect would be limited to BCs with high C/N and produced at low temperatures (<350˚C), as it is demonstrated that slow pyrolysis BCs produced at high temperatures do not immobilize N (Fiorentino et al., 2019)

Another hypothesis for explaining the suppression of N_2O emissions after BC amendment is a general decrease in soil microbial activity due to toxic compounds found in BC, especially polycyclic aromatic hydrocarbons (PAHs) (Cayuela et al., 2014; Kammann et al., 2012). Wang et al. (2013) attributed the lower productions of N_2O in a soil amended with low-temperature BCs to these compounds. However, Alburquerque et al (2015) rebutted this hypothesis and demonstrated that PAHs in BCs do not lead to lower N_2O emissions.

BC may mitigate soil N_2O emissions by enhancing soil aeration, porosity and gas diffusivity. Similarly, the changes that BC can generate on soil water dynamics can also have a substantial effect on N_2O emissions (Brassard et al., 2016). Additionally, abiotic mechanisms have been proposed as being able to play a significant role. For instance, Quin et al., (2015) suggested that BC could trigger soil N_2O mitigation by influencing the reduction of N_2O to N_2. However, this area of research has been barely investigated; hence, this abiotic theory has yet to be corroborated. Also, BC's active role in soil N transformations by means of its electrochemical properties has been considered lately. Cayuela et al. (2013) suggested that during denitrification, BC may act as a reducing agent, an electron shuttle, or as an electron sink. Although the research in the field is still limited, Chen et al. (2018) confirmed that redox-active components of BCs affected the denitrification processes and mitigated N_2O emission. They showed that BCs produced at low temperatures could function as electron donors to support N_2O reduction, whereas BCs generated at higher temperatures would serve as an electron sink or 'electron shuttle' (Fungo et al., 2019; Weldon et al., 2019; Yuan et al., 2019).

1.2.4.2. Biochar and methane emissions

Unlike N_2O, the role of BC in decreasing soil CH_4 emission is controversial. There is evidence that BC may hinder either methanogenesis or enhance methanotrophy; however, there is very limited literature available to support these phenomena. Although it is difficult to determine which BC characteristics are involved, several hypothesis have been postulated.

Improved soil aeration due to BC application may be an important factor in flooded and non-flooded soils. With methanogenesis being a nearly-exclusively anaerobic process and methanotrophy being an aerobic one, increasing soil O_2 content could boost CH_4 oxidation while outpacing its production (Brassard et al., 2016). Feng et al., (2012) reported that BC was able to decrease the methanogenic/methanotrophic ratio of paddy soils (Feng et al., 2012). In upland soils, the great net CH_4 uptake in BC-amended soils has also been explained by the increased soil porosity caused by BC addition (Song et al., 2015). Another result of the increased soil aeration is the enhancement of water holding capacity of BC amended-soils, which can result in low CH_4 emissions (Karhu et al., 2011). In addition, there is evidence of BC adsorbing not negligible quantities of CH_4 on its pores either in anhydrous or moist conditions (Sadasivam and Reddy, 2015)

BC's liming effect in acidic soils has been found to increase their CH_4 sink strength and decrease their production capacity. The potential explanation for this may be because methanotrophic communities are more sensitive to soil pH than that of methanogens, or due to the change in Al^{3+} solubility. Methanotrophs are very sensitive to Al^{3+}, whose availability strongly decreases at pH<5. Hence, by increasing soil pH, BC may reduce Al^{3+} release from cation exchange sites in the soil, thereby reducing toxicity levels for methanotrophs (Jeffery et al., 2016).

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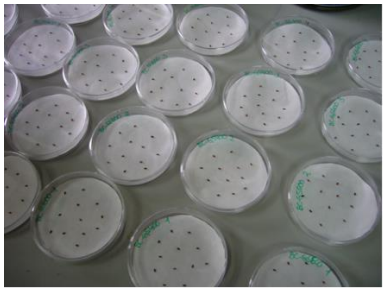
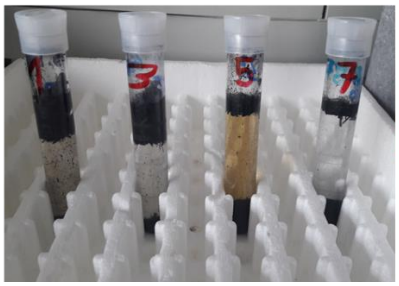
Chapter 2

Thesis hypothesis and objectives

Biochar (BC) is considered a promising negative emission technology (NET), as it is able to sequester C in soil and tackle non-CO_2 greenhouse gas emissions (GHG, e.g. N_2O and CH_4), properties that were recently highlighted in the last IPCC reports (2018, 2019). Additionally, BC can also be a used as a tool for improving soil properties. It is able to enhance the soil's physical characteristics, increase soil aeration, alter soil nutrient content and bioavailability, and promote the growth and activity of soil microorganisms. Consequently, BC is able to modify soil biogeochemical cycles, specifically C and N-cycles, which are relevant for GHG emissions. However, due to the wide variety of feedstocks and pyrolysis conditions that can be chosen and utilized for BC production, its resulting structure and composition can be particularly complex. Therefore, more research is needed in this field to understand how the properties of BCs drive their potential effects on soil and its biogeochemical cycles. In addition, Global Warming is becoming a real global threat. The alarming increase of N_2O and CH_4 emissions, two potent GHG caused by current agricultural practices, requires immediate mitigation actions. Novel, versatile and 'green' products, such as BC, may play a key role in achieving the reduction of agricultural GHG emissions.

The **main general objective** of the present thesis is to understand the interaction between BC and soil with respect to the biogeochemical processes involved in CH_4 and N_2O emissions. With this aim, a wide range of BCs were produced. By adjusting feedstock and pyrolysis conditions, the BCs had different physico-chemical characteristics and, consequently, their effects in soil also differed. Through their comprehensive analysis and posterior application in laboratory soil incubations, we were able to select and describe the characteristics that a BC should have in order to maximize soil CH_4 and N_2O mitigation. Thus, customizing BC properties seems to be a suitable approach that can be utilized to accomplish the objective of net zero emissions by 2050 (IPCC, 2019).

To achieve the general goal of this thesis, three key objectives were addressed:

Objective 1. To link biochars properties to their capacity to modify aerobic CH_4 oxidation in upland agricultural soils.

Soil CH_4 oxidation potential could be altered by its amendment with contrasting BCs. The rate of this impact would be dependent on the physicochemical and electrochemical properties of the BCs. Thus, the **hypothesis** was that BCs with a high surface area and high electron accepting capacity could result in enhanced CH_4 oxidation rates in oxic soils. This specific objective was addressed in Chapter 3, where 10 different BCs were generated from four different feedstocks at two different pyrolysis temperatures. In addition, two chemically-modified BCs with enhanced redox properties were tested. Through incubation experiments, the relationships between the physicochemical and redox properties of BCs and their impact on the CH_4 oxidation rates were analysed.

Objective 2. To link biochars properties to their capacity to modify N_2O fluxes from denitrification in soil, distinguishing them from other N_2O formation pathways.

Soil amendment with BC will affect soil N cycle dynamics involved in N_2O production, by modifying the soil's physical, physicochemical, and/or microbiological properties. According to the scientific literature, it is expected that most BCs would produce a decrease in soil denitrification rate and N_2O emissions, although this may depend on BC properties. Thus, the specific objective was to ascertain which physicochemical and redox characteristics of BC were linked to its capacity to interact with the soil N cycle under optimum denitrification conditions. The **hypothesis** was that BC mitigation potential would depend on its C/N ratio, content in polycyclic aromatic hydrocarbons (PAHs) or redox functional groups. This specific objective was addressed in Chapter 4, where different laboratory incubation experiments with ^{15}N were conducted to understand the impact of BC on soil N_2O emissions and soil mineral N dynamics (NH_4^+, NO_3^- or NO_2^-). The BCs were thoroughly analysed for a wide range of properties, including the presence of potentially phytotoxic substances such as their PAHs content.

Objective 3. To investigate the ability of biochar to directly donate electrons for the reduction of N_2O to N_2 with pure cultures of *Paracoccus denitrificans*

Paracoccus denitrificans is a gram negative bacterium, ubiquitous in soil and able to perform complete denitrification (i.e. from NO_3^- to N_2). For this reason, it has become one of the most popular model strains for studies of electron transfer during denitrification. The **hypothesis** was that the reduction of N_2O to N_2 by *Paracoccus denitrificans* may be amplified by BC addition. Biochar redox properties (capacity to donate and exchange electrons) would determine the extent of the reduction. Accordingly, oxidized BCs (i.e. aged in soil or chemically oxidised) could result in a lower potential to support N_2O reduction. In addition, other BCs characteristics could also affect the reduction process.

This specific objective was addressed in chapter 5, where the ability of different BCs to support N_2O reduction by *Paracoccus denitrificans* was evaluated by means of their redox properties in the absence of any other C or electron source. Anoxic and sterilized incubations of the bacterium and BC suspensions at a high N_2O atmosphere were established. During the experiment, changes in N_2O concentration were measured to understand the impact of the redox properties of the BCs on N_2O reduction by the bacterium. Two of the tested BCs were modified to enhance their redox properties, one through its ageing in soil (5 years) and the other through a chemical oxidation process.

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Chapter 3

Linking biochars properties to their capacity to modify aerobic CH_4 oxidation in an upland agricultural soil



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3.1. Abstract

Aerobic soils are the largest biotic sink for atmospheric methane (CH4). Although agricultural intensification is known to adversely impact soil CH_4 uptake, the application of organic amendments (e.g. composts, green residues) in agricultural soils has been found to stimulate the activity of CH_4 oxidizers. However, little is known about the influence of biochar (a carbonaceous by-product of biomass pyrolysis) on the soil CH_4 sink function. This study analyzes, through a series of laboratory incubation assays, how ten well-characterized biochars with contrasting properties influence CH_4 oxidation rate constants (k) in an aerobic high-pH agricultural soil. Through the use of ^{13}C-CH_4, we demonstrated that both CH_4 soil oxidation and CH_4 assimilation were responsible for the decrease in CH_4 concentration. A principal component regression (PCR) of the results suggested that the physico-chemical properties of biochars were directly linked to their ability to enhance or inhibit the oxidation of CH_4. Biochars from wood feedstocks and pyrolyzed at 600°C, characterized by a high pore area, led to the highest CH_4 oxidation rates whereas biochars with high ash concentrations and electrical conductivity significantly diminished CH_4 oxidation rates. Biochar redox properties were not found to be relevant for CH_4 oxidation in soil.

3.2. Introduction

Methane (CH_4) is considered one of the most powerful gases that contribute to the greenhouse effect; with a Global Warming Potential (GWP) of 28 over a 100-year timescale, compared to CO_2. The current agricultural input to global anthropogenic CH_4 emissions represents more than 50% (IPCC, 2014), with a prevision of a continuous increment for the next years. Nevertheless, in the global CH_4 budget, agricultural soils may represent a significant biotic sink for atmospheric CH_4 (Smith et al., 2000). Specifically, methanotrophic bacteria in upland soils remove about 9 to 47 Tg (CH_4) yr^{-1}, which represents around 5-10% of the total atmospheric

CH_4 sink (Rousk and Bengtson, 2014). However, anthropogenic factors related to agricultural activities can compromise the soil CH_4 sink potential. For instance, the use of fertilizers, pesticides and herbicides, or the pollution with metals has been found to reduce CH_4 oxidizers populations and activity (Tate, 2015; Contin et al., 2012). Even agricultural practices such as irrigation, tillage or grazing may cause a direct decrease in CH_4 oxidation by soils. For instance, the use of heavy machinery during tillage, causing soil compaction and intensive irrigation practices may both limit gas diffusion (Shukla et al., 2013).

Biochar (BC), a porous organic material produced by the pyrolysis of biomass at temperature of 400-900°C (Lehmann, 2007), has recently been suggested to play a role in modulating CH_4 emissions within agroecosystems (Kammann et al., 2017). Two biotic processes control the production and consumption of CH_4 between soils and the atmosphere. The production of CH_4 is driven by strict methanogenic bacteria and takes place exclusively in anoxic environments where organic C is microbially degraded. Biochar interaction with methanogens and the resulting CH_4 emissions are still not well understood as shown by the contrasting results reported in the literature (Jeffery et al., 2016; Song et al., 2016). Biochar effects on soil porosity, labile C and N pools, or soil pH are relatively well-known and may affect soil CH_4 production (Kammann et al., 2017). Methanotrophic bacteria in upland soils and in oxic/anoxic interfaces consume CH_4 (Cai et al., 2016). Biochar amendment has been reported to decrease CH_4 emissions by increasing methanotrophic abundances and/or decreasing the ratios of methanogenic to methanotrophic abundances in paddy and landfill cover soils (Feng et al., 2012; Sadasivam and Reddy, 2015a; Reddy et al., 2014). At the same time, BC addition may boost CH_4 and O_2 diffusion rates, which could decrease CH_4 production and/or increase CH_4 oxidation (Fang et al., 2016; Van Zwieten et al. 2015). However, a recent meta-analysis found that, on average, BC might decrease CH_4 uptake in upland soils (Ji et al., 2018).

One of the causes of this uncertainty is the high variability of BC properties. Biochar characteristics and functionality depend mainly on the original biomass, the pyrolysis conditions (temperature, residence time) and the post or pre-production processes (Harris et al., 2013;

Keiluweit et al., 2010). Pyrolysis temperature has a major impact on BC C stability, whereas the feedstock determines its surface functionality and pore size distribution (Sánchez-García et al., 2019). In addition, the impregnation of biomass or BC with chemical compounds (e.g. H_3PO_4, KOH, $KMnO_4$, $ZnCl_2$) may affect the end-product on a wide range of ways, such as improving its sorption capacity, or modifying the number of oxygen-containing moieties. As a result, each BC acquires a particular chemical and physical structure, with a variety of functional groups that lead to diverse functionalities, including contrasting redox properties (Chacón et al., 2017). Several recent studies have demonstrated the importance of the electrochemical properties of BC in biogeochemical redox reactions (Joseph et al., 2015). For instance, its role as electron shuttle has been demonstrated in the reduction of ferrihydrite by *Shewanella oneidensis* (Kappler et al., 2014) during anaerobic ammonium oxidation coupled to iron reduction (Zhou et al., 2016). Biochars with high electron accepting capacity (EAC) could play a key role as a sink of electrons, favouring oxidation reactions, e.g. from CH_4 to CO_2 (Prévoteau et al., 2016; Klüpfel et al., 2014).

To our knowledge, the effect of different types of BCs on CH_4 consumption in soil under oxic conditions has not been analysed thoroughly. In this study, ten BCs generated from four feedstocks and synthetized at two different temperatures were tested in incubation experiments with one upland agricultural soil at 40% of its water filled pore space (WFPS). Two of the tested BCs were modified to increase their EAC. Our research hypothesis was that BCs would alter soil CH_4 oxidation rate constants (k) differently, depending on BC physicochemical and electrochemical properties. We anticipated that BCs with high surface area and EAC, would lead to higher CH_4 oxidation rates in soil.

3.3. Materials and methods

3.3.1. Soil

Bulk soil was sampled from an organic olive crop field in Jumilla, Murcia, Spain (38°24' N, 1°22' W). Several subsamples were taken randomly from a depth of 0-0.25 m, mixed, homogenized, air-dried and sieved (<2 mm). Soil chemical properties were as follows: 16.8 g kg-1 of total organic C (TOC), 2.4 g kg^{-1} of total N, 300 g kg^{-1} $CaCO_3$, 1.62·10-2 g kg^{-1} NO_3^--N, 1.51·10^{-3} g kg^{-1} NO_2^--N, 6.75·10-4 g kg^{-1} NH_4^+-N and 7.73 g kg^{-1} PO_4^{-3}-P. Soil pH was 8.12 (1:2.5 g water extract), its electrical conductivity (EC) 518 µS cm^{-1} (1:2.5 g water extract) and its bulk density was 1.35 g cm^{-3}. The soil was classified as Haplic calcisol (USS Working Group WRB. 2015) and its texture was sandy loam (57% sand, 27% loam, 16% clay).

3.3.2. Production, basic properties and characterization of biochars

Eight BCs were produced from four different crop residues by slow pyrolysis at two highest treatment temperatures (HTT), 400 and 600°C. The feedstock was dried, chopped to small pieces and sieved (6 mm) before being introduced in a pyrolyzer consisting of a rotatory tube furnace with a quartz glass reactor (Nabertherm GmbH. RSR-B 80/500/11. Lilienthal, Germany). The process lasted a total of four hours. The samples were first heated to 105°C under a continuous flow of argon (Ar) (50 L h^{-1}). Subsequently, the temperature was increased at a rate of 5°C min^{-1} until the desired HTT (400 or 600°C) was reached and maintained for two hours (residence time). During this period, the flow of Ar was increased to 150 L h^{-1}. Finally, the BCs were left to cool down to ambient temperature inside the pyrolyzer, maintaining the inert atmosphere (50 L h^{-1}Ar). The selected feedstocks were residues from Mediterranean agriculture that have been recently characterized by Sánchez-García et al. (2019): (i) pruning residues from almond trees (Al), (ii) pruning residues from olive trees (Ol), (iii) post-harvest residues from tomato plants (To), and (iv) straw from a rice plantation (Ri). For each feedstock,

two BCs were produced, one at HTT of 400 and one at 600 °C: BC-Al400 and BC-Al600, BC-Ol400 and BC-Ol600, etc (Table A1, A2. Appendix).

Additionally, two modified BCs were produced from the same olive tree residues described previously with the objective of increasing BC oxygen-containing functional groups and therefore their EAC. The first modified BC, BC-O_2, was generated at 400°C following the pyrolysis process described above, but letting the BC cooling down without the Ar flow, i.e. allowing the air into the quartz reactor once the temperature had dropped to 250°C. The second modified BC, BC- $KMnO_4$, was generated from BC-Ol400 by a post-production treatment using a 3% $KMnO_4$ solution (EMSURE®ACS, Reag. Ph Eur. 99.0-100.5%. Merck KGaA. Darmstadt, Germany). BC-Ol400 was mixed with the $KMnO_4$ solution at a ratio of 0.1 g ml^{-1} and kept at 98°C for three hours. After that, the samples were washed with deionized water and centrifuged at 4400 rpm. Finally, the BC was oven-dried at 105°C for 24 hours (Li et al., 2014).

Before being used, the BCs were sieved below 0.5 mm (Retsch GmbH. Haan, Germany). A thorough characterisation of the physical and chemical properties of the BCs can be found in Sánchez-García et al. (2019). Physical characterisation included the measurement of total pore area, which was calculated through the mercury intrusion-extrusion technique (for pore size diameters from 0.003 to 600 μm, mesopores+macropores). Specific surface area (m^2 g^{-1} BC, mesopores around 0.01 μm) was obtained by Brunauer-Emmett-Teller (BET) technique by ASAP 2000 instrument (Micromeritics, U.S.A). Physicochemical and chemical characterization included proximate analysis (fixed C, volatile C, and ashes), pH, electrical conductivity and ultimate analysis (C, N, H) (ASTM-D1762-84 Chemical Analysis of Wood Charcoal, Hamer et al., 2014). The lignocellulosic composition of the original feedstock was also determined according to the American Society for Testing of Materials (ASTM) D1106-96 and Browning (1967).

In addition, the BCs electron exchange capacities (EEC) were determined using a three-electrode system following the method described in Klüpfel et al. (2015) with slight

modifications. Briefly, a 10 mM solution of 2,2′-azino- bis(3-ethylbenzthiazoline-6-sulfonic-acid) diammonium salt (ABTS) and 100 mM solution of neutral red (NR) were used as mediators for measuring the EDC and EAC, respectively. A buffer solution of 15 ml (1 M NaCl, 0.1 M phosphate, pH 7) and 100 µL of a mediator solution were added to the crucible and equilibrated to the desired redox potential (-0.49 V for EAC and +0.40 V for EDC, reported vs Ag/AgCl 3M KCl electrode). The integration of the reductive (EAC) and oxidative (EDC) current peaks produced after the addition of 100 µL of a BC suspension (4 g mL^{-1}) allows calculating the electron exchange capacities using Faraday's Law (Chacón el al., 2017).

3.3.3. CH_4 consumption experiment

Soil incubation experiments were carried out to study the evolution of a known initial headspace CH_4 concentration and to determine the CH_4 oxidation rates of soil and BC-amended soils. A total of 100 g (dry weight) of soil or BC-soil mixture were placed inside 250 ml polypropylene jars (Sarstedt. Nümbrecht, Germany) and sealed tight using screw cups fitted with rubber septa, which allowed headspace sampling with a gas syringe. The experiment lasted 21 days and consisted of 11 treatments: a control soil sample (soil) and the soil treated with each of the 10 BCs (each one named after the BC added) and it was performed at 40 % of the water filled pore space. The experiment was laid out as a randomized block design with four replicates per treatment (one replicate per block). Biochar was added at 2% rate (dry weight), equivalent to 68 tons Ha^{-1}. Soil and BC were mixed in the polypropylene jars manually prior to water addition. Then, the samples were wetted and homogenized with a spatula and carefully compacted to the same bulk density of 1.4 g cm^{-3} by tapping the surface. The jars were introduced in a 30°C incubator (Heraeus, Function Line. Thermo Fisher Scientific. Massachusetts, USA) to undergo a pre-conditioning period of one hour. Hereafter, 10 ml of 1000 ppmv CH_4 in N_2 (Abelló Linde, Barcelona, Spain), were injected to supply a concentration of CH_4 of, approximately, 40 ppmv. The syringe was flushed three times to allow for adequate mixing of the bottle headspace. Four blanks, comprising jars with the same CH_4 addition but without soil or BC were also included to test any potential leakage during the incubation. During the whole experiment, the incubation

units were kept closed in the dark at 30°C. A total of 10 gas samples (2 ml each) were taken (four the first day of incubation and the rest at days 2, 3, 5, 7, 14 and 21) and analysed in a gas chromatograph, VARIAN CP-4900 Micro-GC (Palo Alto, CA, USA).

3.3.4. Isotopic experiment

To confirm CH_4 oxidation, an additional incubation experiment, with identical environmental conditions as in section 2.3.3., was set up. Thus, if ^{13}C-CH_4 was oxidized, an increase of ^{13}C-CO_2 would be observed (Vicca et al., 2009). Given the calcareous nature of the soil used in this study, we traced the ^{13}C from ^{13}C-CH_4 in three pools. (i) as ^{13}C-CO_2 gas emitted from soil (distinguishing from the CO_2 from soil respiration); (ii) as soil inorganic C (^{13}C- CO_3^{2-} or ^{13}C-HCO_3^- , since some of the ^{13}C-CO_2 formed could be trapped in the soil inorganic C pool) and, (iii) in soil organic C fractions (^{13}C from CH_4 could be physically adsorbed to BC or assimilated by the soil microbial biomass).

In this case, only two of the BCs (BC-Ol400 and BC-To400) were tested, since they led to the highest and lowest oxidation rate constants (*k*) in the previous experiment. In this set up, 10 ml of a solution of 30% $^{13}CH_4$ (99.9%, enriched in 99% atom 13C. Campro Scientific GmbH. Berlin, Germany) were injected into the jars headspace. Three gas samples (2 ml) were taken 1, 24 and 96 hours after the addition of the labelled CH_4. Gas samples were collected with polypropylene syringes and stored in evacuated and subsequently He filled 12 ml vials (Labco Exetainer®. Lampeter, UK). The $^{13}CO_2$, product of $^{13}CH_4$ oxidation, was analysed at the Stable Isotope Facility (University of California, Davis, USA) with a Trace GC Ultra gas chromatograph (Thermo Electron Corp., Milan, Italy) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Electron Corp., Bremen, Germany).

In addition, two sets of soil samples were collected at the end of the incubation and oven-dried at 60 °C until constant weight. One set was treated with sulphurous acid (6% w/v) for carbonate removal (Bisutti et al., 2004), whereas the other set remained unaltered. Finally, approximately

35 mg of soil for each treatment (with and without carbonates) were weighed out in tin cups and sent to the Stable Isotope Facility of UC Davis for measurement of total C and ^{13}C atomic excess (PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer, Sercon Ltd., Cheshire, UK). Likewise, the original soil and BC samples (BC-Ol400 and BC-To400) were analysed for determining total C and ^{13}C.

3.3.5. Calculations and statistical analysis

The decrease in the concentration of CH_4 in the jars headspace for the first 96 hours was adjusted to a first order exponential function ($y=a \cdot e^{(-kt)}$) according to Hütsch et al., (1993). The log transformation of this function, Ln $CH_4=a+kt$, resulted in straight lines with one individual slope for each treatment. The slopes, *k*-values, can be interpreted as CH_4 oxidation rates and are characteristic values for the CH_4 uptake ability of a soil for a given treatment. Large negative values of *k* would indicate a rapid oxidation of CH_4 and positive values would mean net CH_4 production (Hütsch et al., 1994). As the solubility of CH_4 in water is low (24 mg L^{-1} at 20°C and ambient pressure; Schütz and Seiler, 1989), dissolved CH_4 was neglected under our experiment conditions (Born et al., 1990). The significance of differences between treatments was determined by one-way ANOVA. The Tukey's post hoc test ($P < 0.05$) was used to differentiate treatments within groups (IBM SPSS Statistics 23).

The influence of BC characteristics on the final CH_4 headspace concentrations was assessed by principal components regression (PCR) with IBM SPSS Statistics 23. When many variables are introduced into a regression model, multicollinearity might occur due to correlation among variables, often leading to biased model predictions. In order to analyse the structure and correlations between BC properties, a dimension reduction with Oblim rotation was performed over the data matrix, which resulted in a number or principal components and a subset of eigenvectors. Afterwards, the eigenvectors were used as independent variables in a linear regression model to quantify the effect of BC physico-chemical properties on CH_4 uptake rates (*k*-values) and CH_4 concentration at the end of the experiment (504 hours).

Regarding the isotopic experiment, values of [$^{13}CO_2$] below 167 ppmv were under the limit of quantification (LOQ) of the isotope ratio mass spectrometer, therefore, theses samples were considered to have no ^{13}C enrichment. For treatments with values above the LOQ, the average and the standard deviation (SD) were calculated for the four replicates. To analyse if the differences of $^{13}CO_2$ emissions and ^{13}C content in soil were significant between treatments, a one-way ANOVA and a Tukey's post hoc test ($P < 0.05$) were carried out with IBM SPSS Statistics 23.

3.4. Results

3.4.1. Biochar properties

Table A2 shows the main physico-chemical characteristics of the BCs used in this study. The concentration of organic C ranged between 42 and 84 % and all BCs (except BC-To400) had a high degree of aromaticity, as shown by their atomic H/C and O/C ratios. BC-To stand out for their substantially high electric conductivity (EC), ash concentration and their low C/N ratio. Almond wood residues had the highest lignin/cellulose (Lig/Cell) ratio from all the feedstocks used, whereas herbaceous wastes from tomato crops had the lowest. Total pore area (TporeA) and bulk density (Bulkρ) were highest in BC-Ol and lowest in BC-To. BET was generally low for all BC (1.6-13.5 m^2g^{-1}), except BC-Al600 (154.2 m^2g^{-1}). Regarding the redox properties, the overall capacity to exchange electrons (|EEC|) in non-treated BCs was higher for BCs pyrolyzed at 400˚C, ranging between 0.14 and 0.40 mmol $e^- g^{-1}$ BC. The EDC decreased with increasing temperature, whereas the EAC slightly increased, with exception of BC from rice straw (Figure 3.1).

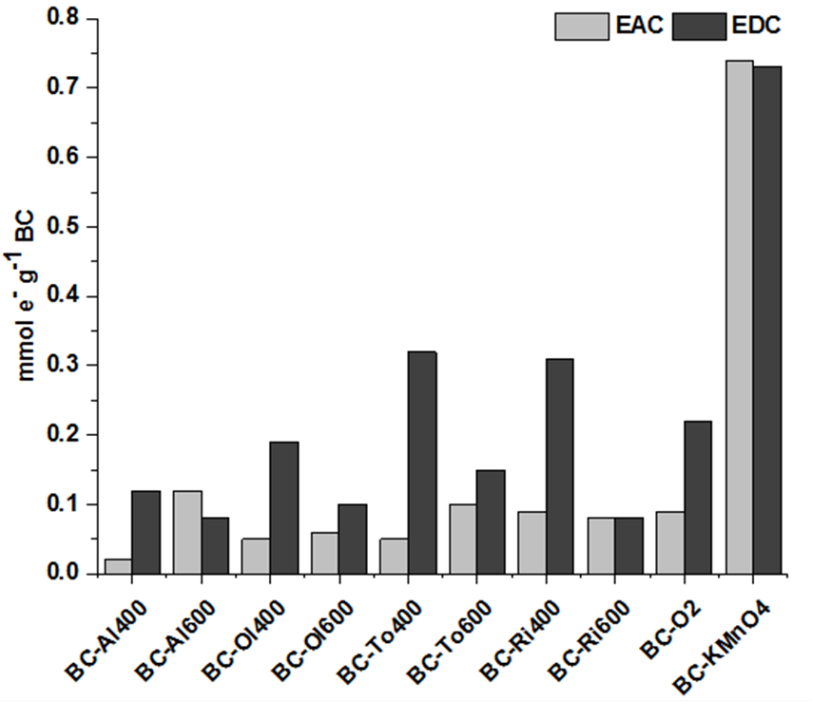

Figure 3.1. Redox properties (mmol $e^{-}g^{-1}$ BC) of the BCs: electron accepting capacity (EAC) and electron donating capacity (EDC).

The treatments applied to BC-Ol400 to generate BC-O_2 and BC-$KMnO_4$ enhanced its electrochemical characteristics (Table A2). Enabling the access of oxygen during the cooling-down phase doubled the EAC, whereas the EDC remained almost identical. Moreover, both processes decreased the pH, increased the ash concentration, and the atomic H/C ratio. The treatment with $KMnO_4$ had much greater impact, since the EAC was increased 16-fold, the EDC 4-fold, and the BET surface area decreased nearly 90% in comparison with BC-Ol400.

3.4.2. Methane oxidation rates

The straight lines resulting from the log-transformation of CH_4 concentrations with time (day 0 to 4 of the incubations) are depicted in Figure 3.2, whereas the numerical data for the slopes, (*k*-values)·100, and their R^2 are gathered in Table 3.1. Statistically significant differences were found between treatments at both pyrolysis temperatures. In general, BCs pyrolyzed at 600°C showed steeper slopes of CH_4 oxidation (lower *k* values) than BCs produced at 400°C (Figure 3.2; Table 3.1). Moreover, *k* values were strongly linked to BC original feedstock: BCs from olive tree pruning (BC-Ol400 and BC-Ol600) stood out with the steepest slope, which means

that these treatments led to the fastest CH_4 oxidation rates. Contrarily, BC-To400 and BC-To600 showed the lowest *k*-values, which were significantly different to BC-Ol treatments ($P< 0.05$). The remaining BC treatments led to *k* values similar to the control soil. Regarding the modified BCs (Figure. 3.1.a), the oxidation treatments applied to BC-Ol400 led to lower CH_4 oxidation rates compared to the untreated BC (Figure 3.2.a). The k-values were reduced by 40.5% for BC-$KMnO_4$ and by 29.8% for BC-O_2.

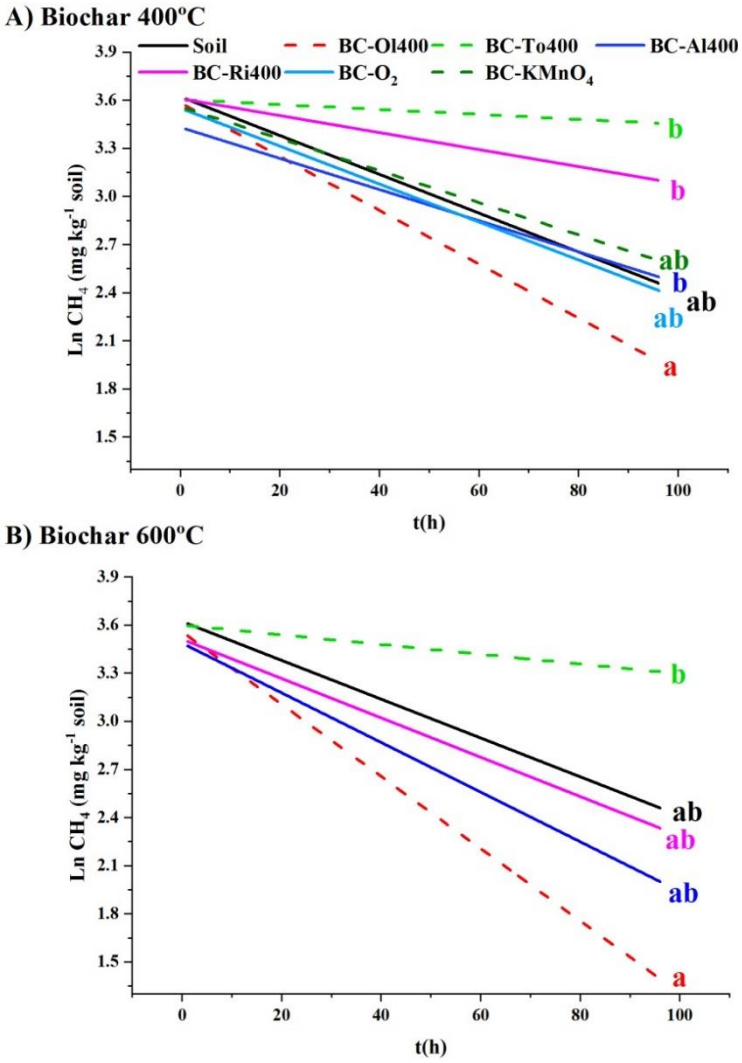

Figure 3.2. Fitted straight lines representation ($\text{Ln } CH_4\ (\text{mg C} \cdot \text{kg soil}^{-1}) = a + kt$) in the jars headspace during the first 4 days of the experiment. Figure 3.2 A) represents BCs at 400 °C and Figure 3.2 B) BCs at 600 °C. Different letters (a, b) express significant differences between the slopes (k) according to Tukey test ($P<0.05$).

Table 3.1. Values of the straight lines slopes, k-value·100 (h^{-1}), and their R^2, represented in Figure 3.2, for each BC treatment.

Treatments	k-values· 100 h^{-1}	R^2
Soil (Control)	-1.21	0.9927
BC-Al400	-0.97	0.8577
BC-Al600	-1.55	0.9684
BC-Ol400	-1.68	0.9972
BC-Ol600	-2.25	0.9912
BC-To400	-0.15	0.4836
BC-To600	-0.30	0.9616
BC-Ri400	-0.53	0.9851
BC-Ri600	-1.23	0.9459
BC-$KMnO_4$	-1.00	0.9438
BC-O_2	-1.18	0.9755

3.4.3. Methane evolution during the whole incubation experiment

Figure 3.3. shows the evolution of CH_4 concentrations (mg CH_4-C·kg^{-1}soil) in the jars headspace from day 0 to 21 for each BC treatment and the control soil.

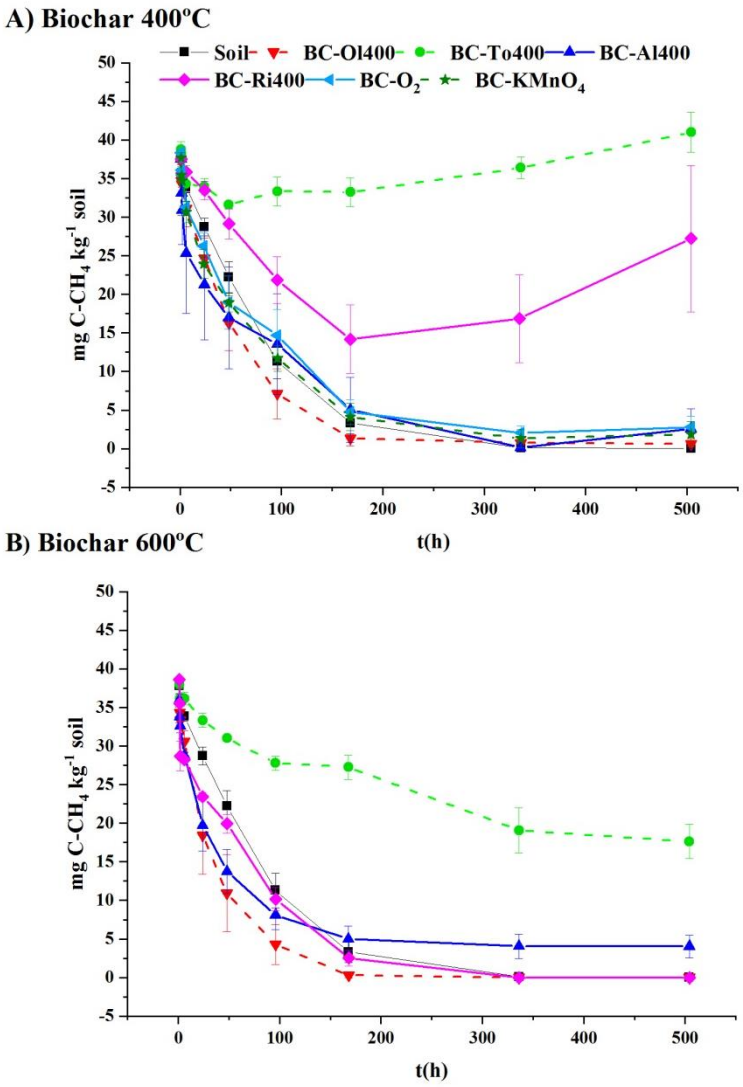

Figure 3.3. Evolution of CH_4 concentration (mg CH_4-C· kg^{-1}soil) in the samples headspace during the whole incubation period (500 hours) for BCs at 400 (A) and 600°C (B). The error bars represent the standard deviations (n=4).

For BCs produced at 400 °C (Figure 3.3.A), all treatments, except BC-To400 and BC-Ri400, reached 0.0 mg CH_4-C·kg^{-1} soil at around 340 hours, showing no differences with the control soil. Only BC-Ol400 reduced CH_4 concentration slightly faster than the other treatments. On the contrary, BC-To400 and BC-Ri400 treatments notably stimulated CH_4 production, although the turning point for BC-To400 took place earlier (before 100 h) than for BC-Ri400 (around 200 h). At the end of the incubation (504 h) their concentrations were 41 and 27 mg CH_4-C·kg^{-1} soil respectively. Regarding the modified BCs no differences were observed compared to the control, as was found in Section 2.4.2.

Results were different for BCs at 600˚C (Figure 3.3.B). In this case, all treatments exhibited a continuous decrease in the headspace CH_4 concentration. Similarly, to BC-Ol400, BC-Ol600 led to the fastest CH_4 depletion. In contrast, after 200 h, BC-To600 and BC-Ri600 did not increase the headspace CH_4 concentration as it was observed for BC-To400 and BC-Ri400.

3.4.4. Isotopic experiment: tracing the fate of $^{13}CH_4$ as emitted $^{13}CO_2$ and ^{13}C in soil organic/inorganic pools

Table 3.2 gathers ^{13}C-CO_2 concentrations (µg 13C·100 g-1 soil) in the jars headspace 1, 24 and 96 hours after the labelled $^{13}CH_4$ was injected. Initially, at t=1 h, both BC treatments led to slightly but significantly lower $^{13}CO_2$ concentration than the control soil. After 24 and 96 h BC-Ol400 showed a slightly higher, but statistically similar ^{13}C-CO_2 concentration, whereas BC-To400 led to significantly lower ^{13}C-CO_2 values.

Figure 3.4. displays the concentrations of ^{13}C in the organic and inorganic soil C pools at the end of the incubation. From the initial 1.97 mg of ^{13}C injected as $^{13}CH_4$ in each jar headspace, around 1 mg was found as inorganic C in all three treatments, and between 0.28 mg (BC-To400) and 0.40 mg (BC-Ol400) in the soil organic C pool at the end of the incubation. This implies that the majority of ^{13}C from CH_4 (between 73 and 79%) stayed in the soil matrix. No differences were observed in the concentration of ^{13}C as carbonates for the different treatments. However, BC-To400 led to a significantly lower ^{13}C signature in the organic C pool compared to the BC-Ol400 treatment.

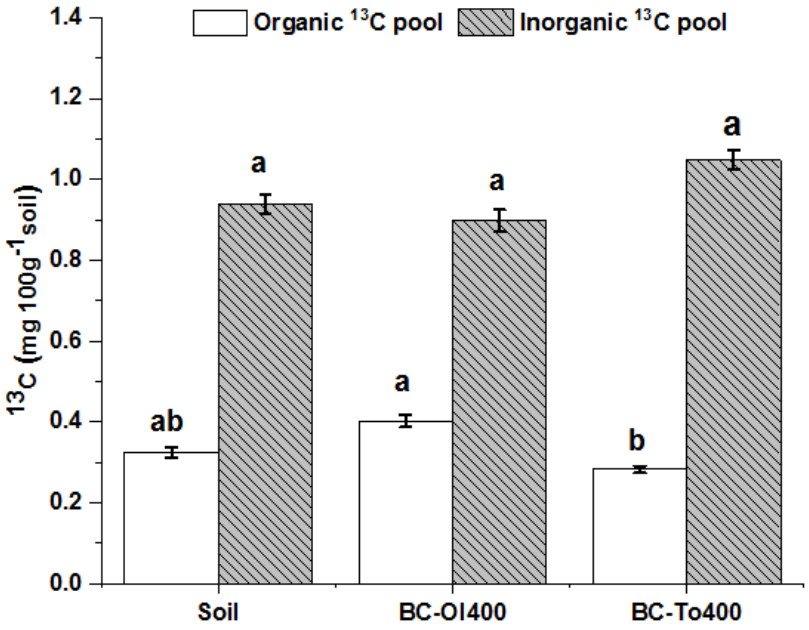

Figure 3.4. Concentration of ^{13}C in the organic and inorganic soil C pools (mg·100 g^{-1} soil) at the end of the isotopic experiment (500 h after $^{13}CH_4$ injection). Different letters show significant differences between treatments according to the Tukey test ($P<0.05$).

Table 3.2. Concentration of ^{13}C-CO_2 (µg ^{13}C·100 g^{-1} soil) in the jars headspace during the isotopic experiment 1, 24 and 96 hours after the addition of $^{13}CH_4$. The standard deviations (SD) and the results of the Tukey statistical test ($P<0.05$) are also shown. Different letters imply significant differences between treatments (n=4). Additionally, the ^{13}C natural enrichment ($\delta^{13}C$ (PDB)) of soil and BCs are included.

		t=1h			t=24h			t=96h		
Treatment	**$\delta^{13}C$ (PDB)**	**^{13}C-CO_2**	**SD**	**Tukey**	**^{13}C-CO_2**	**SD**	**Tukey**	**^{13}C-CO_2**	**SD**	**Tukey**
Soil	-20.8	0.41	0.06	a	14.18	0.13	a	42.41	0.53	a
BC-Ol400	-24.9	0.14	0.01	b	14.33	0.18	a	39.85	0.41	a
BC-To400	-28.4	<LOQ*	-	-	3.89	0.02	b	33.30	0.53	b

*below the limit of quantification (LOQ).

3.4.5. Relation between BC properties and their CH_4 oxidation potential. Principal Component Regression

The matrix from the PCR, in a 'step stair' form, can be visualized in Table 3.3. Biochar properties were grouped in three components, each one with a number (coefficient) that ranges from 1 to -1. The greater the coefficient, the greater the influence this particular BC property has in the component. These three components explained 47.4% of the variability.

Table 3.3. Three components matrix generated by the PCR with BCs physical and chemical properties. Coefficients <0.55 were deleted from the table.

	Component		
	1	**2**	**3**
EC	0.930		
Ash	0.890		
Lig/Cell	-0.863		
O/C	0.851		
TporeA	-0.806		
EAC		1.019	
EDC		0.880	
H/C			-0.927
pH			0.713

EC: electric conductivity; Lig/Cell = lignin/cellulose; TporeA=Total pore area; EAC=electron accepting capacity; EDC=electron donating capacity.

The Principal Components (PCs) that best explained the effect of BC variables on CH_4 oxidation rates and final CH_4 concentrations were PC1 and PC3 (Figure 3.5.; Table 3.4). PC1 was clearly the most determinant for both CH_4 *k*-values and concentrations. Therefore, the most relevant BC properties were: EC, ash concentration, the lignin/cellulose (Lig/Cell) ratio of the

original biomass, the atomic ratio O/C and the total pore area. Hence, considering these properties, high CH_4 oxidation rates (and low final CH_4 concentrations) would be favoured by BCs with low EC, ash concentration and O/C (positive coefficients, Table 3.3); and by BCs with high TporeA and produced from biomass with high Lig/Cell ratios (negative coefficients).

The linear regression model excluded PC2, which gathered BCs redox properties (EAC and EDC).

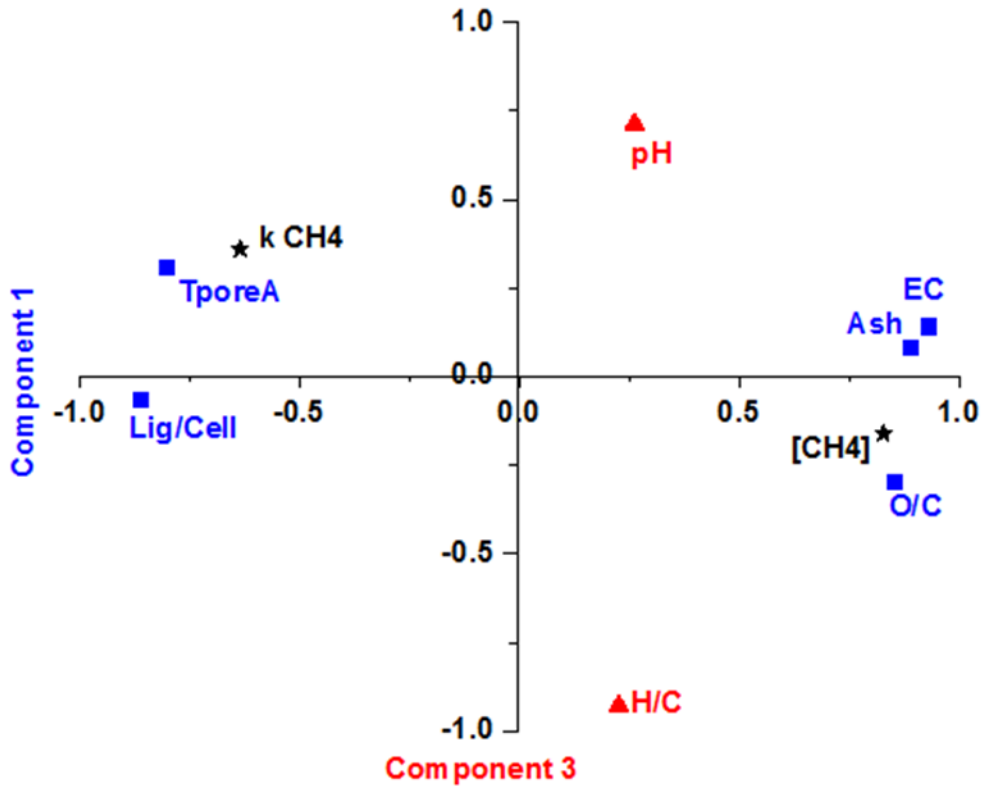

Figure 3.5. Arrangement of the two selected components by the PCR analysis (Table 3.3). The BC characteristics gathered in Component 1 are coloured in dark blue (■) and the ones belonging to Component 3 in red (▲). In addition, it appears the incubation outcome by CH_4 oxidation rates (k CH4) and CH_4 concentration at t=504 h ([CH4]), both in black (★).

Table 3.4. Linear regression results with both dependent variables tested: *k*-values of CH_4 oxidation until 96 hours of the experiment and CH_4 concentration at the end of the incubation, at 504 hours. The R^2 and the standard deviation are displayed together with the significance of the ANOVA (P).

	[CH_4] at t=504 h			**k-values at t=96 h**		
Component	R^2	Standard error	P	R^2	Standard error	P
1	0.547	10.238	0.000	0.237	0.011	0.000
2	**Excluded component**			**Excluded component**		
3	0.064	9.612	0.000	0.103	0.108	0.000
Total	0.611	-	-	0.340	-	-

3.5. Discussion

3.5.1. Soil methanotrophic activity and potential mechanisms of interaction with biochar

The calcareous soil used in our study showed high rates of CH_4 consumption (525 ng CH_4 g^{-1} soil d^{-1} for a CH_4 concentration of 40 ppm). This is five times higher than the oxidation rates found in an agricultural fluvisol with similar CH_4 concentration (Malghani et al., 2016) and, in general, in the upper range of values found for agricultural soils (Le Mer and Roger, 2001). There are several explanations for this result: (i) we set optimal conditions for methanotrophic activity i.e., 30 ºC, oxygen and water availability, and high CH_4 concentrations (Malghani et al., 2016), (ii) calcareous and sandy soils usually show higher rates of CH_4 oxidation than acidic or clay soils (Hutsch et al., 1994; Le Mer and Roger, 2001) and (iii) the selected soil had been under organic farming for more than 15 years, which implies that no pesticides or N mineral fertilizers were used and reduced tillage was applied. All these conditions are known to improve methanotrophy in soil (Ho et al., 2015; Hütsch, 2001; Boeckx et al., 1998). Some experiments have found a decrease in methanotrophic activity after soil drying (Syamsul Arif, et al., 1996). However, the drying-rewetting of our soil prior to the incubation did not compromise its methanotrophic activity. A possible explanation could be that the microbial communities in this

semiarid soil, being historically subjected to long dry periods and short rewetting pulses, became resilient to this disturbance (van Kruistum et al., 2018; Ho et al., 2016; Evans and Wallenstein, 2012).

The biological oxidation of CH_4 in our selected soil was confirmed in different ways. First, the decrease observed in CH_4 concentrations followed a first-order-kinetics function and its log transformation resulted in good-fit straight lines (Figure 3.2, Table 3.1), typical of biological CH_4 oxidation (Hütsch et al., 1993). Second, the increase of ^{13}C-CO_2 during the isotopic experiment shows direct evidence of ^{13}C-CH_4 oxidation (Vicca et al., 2009). Most of the oxidized CH_4 eventually became part of the inorganic C pool, given the calcareous nature of this soil (Figure 3.4.).

Our study demonstrates a limited positive impact of BC on CH_4 uptake in this soil, since, in general, slight (not statistically significant) increases in CH_4 oxidation rates were found for only two of the ten selected BCs. These BCs (BC-Ol400 and BC-Ol600) were characterized by a high total pore area, which suggests that they might enhance CH_4 consumption by (i) improving gas diffusivity in soil, facilitating O_2 and CH_4 exchange to microorganisms (Cong et al., 2018; Sun et al., 2013; Karhu et al., 2011) and/or (ii) simply by CH_4 physical adsorption in the BC surface area (Sadasivan and Reddy, 2015a; Sadasivan and Reddy, 2015b; Billemont et al., 2013). Our results suggest that physical adsorption of CH_4 to BC was not as a relevant mechanism, since the process takes less than two hours (Sadasivam and Reddy, 2015b). Thus, CH_4 adsorption might be the reason behind the significantly lower ^{13}C-CO_2 concentrations found in the BC treatments after 1h of incubation (Table 3.2), but cannot explain the continuous decrease in CH_4 observed (Figure 3.3). On the contrary, an increase in soil gas diffusivity might be directly related with a larger number of aerobic microsites (Joseph et al., 2010), improving methanotrophs activity (Wang et al., 2019; Reddy et al., 2014). This hypothesis is supported by the results of the isotopic experiment, since we found a slightly higher ^{13}C-CO_2 in BC-Ol400 treatment than in the control soil after 24 and 96 h. Moreover, the higher incorporation of ^{13}C in

the soil organic C fraction of the soil treated with BC-Ol400 (Figure 3.4.) suggests that a larger amount of ^{13}C-CH_4 was assimilated by methanotrophs (Knief et al., 2003).

Finally, a mechanism involving BC as terminal electron acceptor for CH_4 oxidation or as a mediator facilitating electron transfer in soil was discarded. Although electron transfer has been found to play a relevant role in many redox biogeochemical reactions (Yuan et al., 2019; Chen et al., 2017; Sun et al., 2017), we found no evidence of BC electrochemical properties playing a role in soil CH_4 oxidation. The physico-chemical modification of BC-Ol400 did not result in improved CH_4 oxidation rates (Figure 3.2. and 3.3.). The strong oxidation process applied to BC-$KMnO_4$ led to a large increase in both EDC and EAC with respect to BC-Ol400, but it also enormously increased its Mn concentration (8800 ppm) and modified its porous structure (e.g. see BET in Table A2). Regarding BC-O_2, the treatment increased its ash %, atomic H/C and EC (Table A2), which seem to be more determinant for modifying CH_4 uptake than redox properties.

3.5.2. Biochar properties relevant for aerobic CH_4 oxidation in soil

In general, we found that the impact of BC on CH_4 oxidation rates was modest, and strongly linked to BC physicochemical properties.

First, we found that for the same feedstock, BCs produced at 600 ºC had a stronger effect on CH_4 oxidation rates than BCs at 400 ºC (consistently lower *k*-values, Table 3.1). This was further supported by the principal components regression, which showed that high O/C molar ratios, typical of BCs with low degree of stabilization (Spokas, 2010), were related to low CH_4 oxidation rates. Second, the biomass used for BC production was found to strongly determine BCs ability to accelerate or decelerate CH_4 oxidation. The BCs properties gathered in PC1 and PC3 (Table 3.3) are mostly determined by the original feedstock (Sánchez-García et al., 2019). Thus, BCs with high EC and ash concentration inhibited aerobic CH_4 oxidation. This was corroborated by the isotopic experiment, where significantly lower ^{13}C-CO_2 was found in the

headspace of BC-To400 after 96 h, and significantly, lower ^{13}C was recovered in the soil organic carbon (SOC) fraction at the end of the experiment. The impact of salinity stress on soil methanotropic activity has been widely documented (Ho et al., 2018; Yang et al., 2018; King and Schnell, 1998). Methane uptake in upland non-saline soils is known to be highly sensitive to an increase in salt concentration, especially to Cl^-, with a strong inhibition by even small increases in salt content. Ammonium salts are also widely recognized methanotrophic inhibitors (Bodelier et a., 2004; King and Schnell, 1994). This implies that the use of BCs with high salt content (Domingues et al., 2017) might explain the inhibition of CH_4 uptake after BC amendment observed in some experiments (Ji et al., 2018). An additional inhibitory mechanism could be the increase of NH_4^+ concentration in soil after the addition of N-rich BCs (Schouten et al., 2012) or as a consequence of N priming effects (Fiorentino et al., 2019). Third, the electrochemical properties of BC (EDC and EAC) were found to be irrelevant under our experimental conditions in both cases: when O_2 was not limiting at the beginning of the incubation, and later on, when there was a shift from oxic to hypoxic conditions. Finally, BC total pore area (pores between 0.003µm and 600 µm) was highly correlated with oxidation rate constants. Our BCs had an unusually low BET surface area, which might indicate that some micro and mesopores were clogged. In this respect, future research is needed to study the impact of high BET area BCs on CH_4 uptake rate.

3.6. Conclusions

This study is a first attempt to link BCs properties to their effect on CH_4 consumption in well-aerated soils, with the aim of reconciling inconsistent results observed in previous studies. Our results indicate that the starting material is the most determinant factor influencing BC impact on CH_4 uptake. Woody BCs, preferentially pyrolysed at high temperatures, with low O/C ratios and high total pore area led to the greatest soil CH_4 oxidation. BCs with improved redox capacities (higher electron accepting capacity) seem to not particularly improve this process.

The knowledge is still far from complete and, given that our findings are based on one particular soil, further research is needed, including a variety of soils, to confirm and generalize these results. To date, most studies analyzing the impact of BC on CH_4 consumption in upland soils are short-term and do not focus on the mechanistic understanding of the processes. Our study opens new research questions, such us the role of ammonium inhibition for CH_4 consumption in BCs treated soils as well as the relevance of BCs physical structure to increase gas exchange. Moreover, the long-term response of the soil methanotrophic community composition after BC amendment should be evaluated. To our knowledge, there are no studies analyzing the impact of BC on the diversity and activity of methanotrophic bacteria in upland soils.

Acknowledgements

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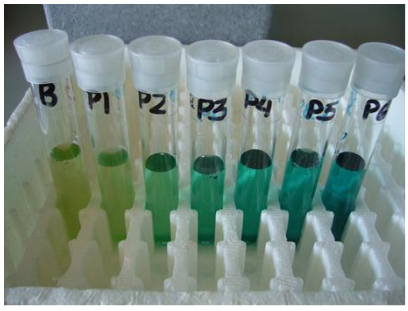
Chapter 4

Linking biochars properties to their capacity to mitigate N_2O emissions in an agricultural soil

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4.1. Abstract

The agricultural use of soil is one of the major sources of nitrous oxide (N_2O) emissions. The soil N-cycle includes a complex series of overlapping reactions involving numerous microorganisms that produce (or consume) N_2O under particular conditions. Biochar (BC) has been proposed as a useful tool for reducing N_2O emissions from soil; although its effect cannot be extrapolated to every type of soil and BC, and the exact mechanisms behind this effect remain unknown. This study analysed the impact of eight BCs on soil N_2O emissions in an incubation experiment under optimal conditions for denitrification. We demonstrated that the impact of BCs on soil denitrification and N_2O emissions was dependent on the physicochemical characteristics of the BCs. Most of the BCs used in this study were able to decrease soil N_2O production during the first days of incubation, but not the total emissions recorded at the end of the incubation. In contrast, a reduced group of BCs, characterised by a high amount of dissolved organic carbon and carboxylic functional groups in their surface, poor C/N and low germination index, increased N_2O emissions. The BC redox potential and content of polyciclic aromatic hydrocarbons (PAHs) did not affect, either positively or negatively, soil N_2O emissions by denitrification. The data presented open up novel lines of research on the coexistence of several soil N_2O emission pathways that would be differently affected by BC with contrasting physico-chemical and chemical properties.

4.2. Introduction

Nitrous oxide is the third most-important gas that contributes to global warming. Agriculture, Forestry and Other Land Use (AFOLU) accounts for approximately 82% of the total anthropogenic emissions (IPCC, 2018). The increase of human land use over the past decades has produced an on-going increase in N_2O release of 122% as compared to preindustrial times, with an annual increase rate of 0.24%, as recorded from 2015 to 2016 (WMO, 2017; Smith et al., 2008).

An important fraction of the N_2O released from agricultural practices comes from soil. The N-cycle is a complex network of overlapping biochemical reactions, which certain microorganisms follow, either completely or only to a certain middle step. The major processes that contribute to N_2O emissions are nitrification, denitrification and dissimilatory nitrate reduction to ammonia (DNRA). Autotrophic nitrification and heterotrophic denitrification are the most known N_2O formation mechanisms, but there are other less known pathways that should not be overlooked, such as nitrifier-denitrification and codenitrification (Kammann et al., 2017; Baggs, 2011). The main factors that determine the occurrence of a given mechanism and its rate of N_2O production are soil oxygen (O_2) concentration, soil pH and the availability of different C and N substrates (NH_4^+, NO_3^- or dissolved organic N). When the WFPS (water filled pore space) is below 60% and the partial pressure of O_2 is between 0.1-0.5%, nitrification is usually the dominant pathway, whereas at decreasing values of O_2 and greater WFPS, denitrification is the main source of N_2O (Bollmann and Conrad, 1998). At the highest WFPS (100%), under nearly anoxic conditions, N_2O production is considered negligible because of its reduction to N_2 by the enzyme nitrous oxide reductase (Kammann et al., 2017; Berger et al., 2013; Braker and Conrad, 2011). Nevertheless, there are soil microorganisms that lack the functional genes for synthetizing the aforementioned enzyme and, consequently, N_2O can be released in significant quantities (Harter et al., 2016).

Additionally, under most environmental conditions, several N_2O formation mechanisms overlap (Abbasi and Adams, 2000; Bonin et al., 1998) and their identification becomes extremely arduous. For instance, the detection of codenitrification is especially intricate. It comprises microbially-mediated N-nitrosation reactions that produce N_2O and/or N_2 when NO_2^- or NO are combined with one N atom from another N species, i.e. a nucleophile (Nu^-, e.g. primary amines, NH_2OH, NH_3). Co-denitrification can be idenfified with ^{15}N tracer studies. The key difference with denitrification is the hybrid character of its products due to the different origin of both N atoms at N_2O and N_2 (Spott et al., 2011a,b).

Biochar, a carbonaceous product of pyrolyzed organic materials (e.g. wastes from the agricultural industry) at temperatures <700°C in partial or total absence of O_2 (Lehmann et al., 2011), has arisen as a useful tool for reducing N_2O emissions. To date, the impact of BC on the N-cycle is still not well understood due to the contradictory outcomes from previous studies (Xie et al., 2020; Weldon et al., 2019; Yamamoto et al., 2019; Wu et al., 2019; Lan et al., 2019; Case et al., 2018; Thomazini et al., 2015; Sánchez-García et al., 2014; Li et al; 2013). Presently, growing evidence has shown that the influence that BC may have on N_2O emissions in a certain type of soil cannot be extrapolated to other soils. The final effect would lie on the interaction between both (BC and soil), which would be directly connected to their individual properties. Consequently, it would be advisable to analyse each BC/soil case separately (Meschewski et al., 2019; Van Zwieten et al., 2014).

Overall, BC can influence soil N dynamics through the following mechanisms: (i) improving soil aeration (Zhang et al., 2010; Lin et al., 2017); (ii) releasing toxic compounds that may decrease soil microbial activity, such as PAHs (Wang et al., 2019; Wang et al., 2013); (iii) immobilizing NO_3^- in soil (van Zwieten et al., 2015); (iv) decreasing the N_2O / N_2 ratio due to BC's alkalinizing effect (Wang et al., 2017; Cayuela et al., 2013); (v) taking part in N-cycle reactions thanks to BC's active redox properties (Harter et al., 2016; Quin et al., 2015; Cayuela et al., 2015); (vi) hindering dissolved organic C availability which controls the denitrification potential (Cayuela el al., 2014; Butterbach-Bahl et al., 2013); (vii) interacting with soil biota (Harter et al., 2017; Lehmann et al., 2011). However, the effects may be antagonistic as well (Weldon et al., 2019). Hence, due to the high number of variables that entangle this BC/soil interaction, opposing results are not uncommon. Biochar carbonization indices, pH, surface area, porosity, redox nature or C/N are among the main factors that have been identified to affect soil N_2O production (Dong et al., 2020, Borchard et al., 2019; Feng et al., 2018; Van Zwieten et al., 2014; Cayuela et al., 2013).

In the present study, eight BCs, generated by slow pyrolysis at two different highest treatment temperatures and from four distinct agriculture residues, were amended to a calcareous nitrate-fertilized soil under optimum denitrification conditions. The main goal of this study was to ascertain the impact of BC on denitrification and other possible co-occurring mechanisms through incubation experiments and the use of labelled and non-labelled NO_3^-. As a result, it will be possible to identify which type of BC is more efficient in achieving the largest N_2O reduction and to determine which of the BC characteristics were more effective (or detrimental) for soil N_2O mitigation. The initial hypothesis was that the physicochemical properties of BCs would drive its interaction with soil N_2O production coming from denitrification, anticipating that the BC's redox functional groups, concentration of PAHs or C/N would be key BCs properties that affect their N_2O mitigation potential.

4.3. Material and Methods

4.3.1. Soil and biochar description and characterisation.

The soil was sampled from the upper layer (0-0.25 m) of a table grape vineyard located in Totana, Murcia, Spain (N37°46'44" O1°33'53.24"). The texture was clayey-silty (5% sand, 44% clay and 51% silt). Soil chemical properties were as follows: 0.85 % organic C (C_{org}), 0.23 mg g^{-1} dissolved organic C (DOC), 0.16 mg g^{-1} dissolved inorganic C (DIC), 0.39 mg g^{-1} total dissolved C (TDC), 0.02 mg g^{-1} total dissolved N (TDN), 1.21 mg kg^{-1} $N\text{-}NO_3^-$, <0.1 mg kg^{-1} $N\text{-}NO_2^-$, <0.1 mg kg^{-1} $N\text{-}NH_4^+$, electrical conductivity (EC) of 0.17 mS cm-1 (1:2.5 g water extract), and pH 8.60 (1:2.5 g water extract). Prior to its use, the soil was air-dried and sieved (<2mm).

Eight different BCs (BC-Olv400, BC-Olv600, BC-To400, BC-To600, BC-Ri400, BC-Ri600, BC-GS400 and BC-GS600) were tested in the incubation experiments. These came from four feedstock types (Olv: olive tree pruning, To: tomato plants, Ri: rice straw, GS: grape stalks) and pyrolyzed at two different temperatures, 400 and 600°C. The description of the feedstocks and

pyrolysis conditions are shown in Table A1 (Appendix). The BCs were applied as they came out of the furnace, meaning that they were not milled or sieved. The particle size was in the range of 0.1 to 10 mm.

The BC's physicochemical characterization included proximate analysis (fixed C, volatile C, and ashes), pH, EC and ultimate analysis (C, N, H) (ASTM-D1762-84 Chemical Analysis of Wood Charcoal, Hamer et al., 2014). The extraction and determination of DOC, DIC, TDC and TDN was performed following the procedure described by Singh et al. (2017a). The BC's surface area was determined with a Brunauer-Emmett-Teller (BET) (ASAP 2000 instrument, Micromeritics, USA) and mercury intrusion porosimetry (AutoPore V 9600 (Micromeritics Instrument, Corp., USA) techniques. The superficial C bonding state was determined with X-ray photoelectron spectroscopy (XPS, K-Alpha Xray Thermo Scientific, UK) and surface mineral crystallographic structures by X-ray diffractometer (XRD, Bruker D8-Advance, Bruker Corp., USA). The lignocellulosic composition of the original feedstock was also determined according to the American Society for Testing of Materials (ASTM) D1106-96 and Browning (1967). More information about the techniques can be found in Sánchez-García et al. (2019).

Additionally, BCs electron exchange capacities (EEC) were determined using a three-electrode system. Briefly, a 10 mM solution of 2,2′-azino- bis(3-ethylbenzthiazoline-6-sulfonic-acid) diammonium salt (ABTS) and 100 mM solution of neutral red were used as mediators for measuring the electron donating capacity (EDC) and electron accepting capacity (EAC), respectively. A buffer solution of 1 M NaCl and 0.1 M phosphate (pH 7) together with a mediator solution were added and equilibrated to the desired redox potential. The integration of the reductive (electron accepting capacity - EAC) and oxidative (electron donating capacity - EDC) current peaks produced after the addition of 100 µL of a BC suspension (4 g mL^{-1}) allows calculating the electron exchange capacities using Faraday's Law (Chacón el al., 2017). Further details can be found in Pascual et al. (2020).

The germination index of BCs was determined according to Zucconi et al. (1981). In Petri plates covered with a sheet of filter paper, 12 seeds of Lepidium Sativum (Garden cress. Semillas BATLLE, S.A. Barcelona, Spain) were placed separately and 2 mL of the BCs extracts were added (1:20 w/v, except for a control with distilled water). The test was done in quadruplicate. The seeds were placed in an incubator under dark conditions and at 25 °C for 48 hours. At the end of this period, the germinated seeds were counted and the length of their roots measured.

4.3.2. Concentration of PAHs in biochars

The concentration of some of the most abundant PAHs in charcoal were analysed (Hilber et al., 2012). Firstly, each BC (in duplicate) was subjected to extraction with toluene (>99.5%, Sigma Aldrich. San Luis, MO, USA) in a rapid automatic soxhlet system (SOXTHERM® C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Two grams of each BC were placed in cellulose thimbles covered with cotton and immersed in 140 mL of toluene containing 4 boiling stones. The extraction temperature was fixed at 208 ˚C with a reduction interval of 4 min and a reduction pulse of 4 s. A series of extractions, evaporations and rising cycles were set, resulting in an extraction of 12-15 mL in 3 hours. This recovered volume was measured precisely and stored in amber glass bottles at 4 ˚C until analysis.

The PAHs determination was carried out in a gas chromatograph–mass spectrometer (GC-MS). The system consisted of an Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) equipped with an automatic liquid sampler (MPS2) (Gerstel, Mülheim, Germany) and coupled to an Agilent 5975C mass selective detector. The GC was fitted with a diphenyl analytical column Pursuit XRs measuring 100 mm x 3 mm and with a 3 µm particle size (Agilent Technologies, Palo Alto, CA, USA). The starting temperature was set at 100 ˚C, which was maintained for 1.5 min. Afterwards, a temperature gradient of 10 ˚C min^{-1} was applied until it increased to 250 ˚C, where it was maintained for 15 min. Solvent delay was 2.0 min. The injector temperature was 260 ˚C and its volume of injection was 1 µL. A calibration curve was done with naphthalene, phenanthrene, fluoranthene, anthracene and pyrene standards,

employing a mixture in toluene of 500 μg mL^{-1} and solid naphthalene at 99% (Sigma Aldrich. San Luis, MO, USA). Two methods were run, the first to scan and the second for quantification, in Selected Ion Monitoring (SIM) (LOD< 5 ppb) adding an internal standard, 1,4-dichlorobenzene-d_4 (98%, 98% atom D. Sigma Aldrich. San Luis, MO, USA).

4.3.3. Soil incubation experiments

Two parallel incubations were set up to accomplish the aim of the present study: Experiment 1 and Experiment 2. Both experiments were carried out in 250 mL polypropylene jars (Sarstedt. Nümbrecht, Germany), to which soil with and without BCs were added. As a result, nine treatments were tested. The control (BC0) consisted of 100 g of soil, and the rest of treatments consisted of 98 g of soil and 2 g (2% w:w) of the BCs described above (named after the BCs). In both cases, deionized water and the required concentration of the fertilizer was added to reach 90% WFPS, ensuring a complete moisture homogeneity and a bulk density of 1.4 g cm^{-2}. The jars were aerobically incubated for 11 days at 25 ˚C (Heraeus, Function Line. Thermo Fisher Scientific, Massachusetts, USA) under complete darkness, and covered with a wet cotton cloth for minimizing evapotranspiration. Even then, the moisture was gravimetrically adjusted in every jar every other day, always after the corresponding gas measurements. The experiments were laid out as a randomized block design.

Experiment 1. N_2O emissions from BC amended soils

Each treatment jar was fertilized with a solution of KNO_3 (200 kg N Ha^{-1}), which was added only once at the beginning of the experiment together with the deionized water to set the moisture conditions at 90% WFPS. Each treatment was set up with four replicates for measuring the headspace concentration of N_2O and CO_2 (ten times at 0, 24, 48, 72, 96, 120, 144, 168, 216, 258 hours). The gas measurement was carried out with a photo-acoustic monitor 1412i gas chromatograph (Lumasense Technologies A/S, Ballerup, Denmark). For doing so, 1 hour prior to the measurement, each jar was tightly sealed using screw lids fitted with two rubber septa.

A set of 12 extra replicate samples for destructive analysis were employed for measuring NO_3^- and NO_2^- (at 24, 48, 96 and 258 h). Additionally, NH_4^+ was also analysed but only in the last day of the incubation period (258 h). The extraction of NO_3^-, NO_2^- and NH_4^+ was carried out by shaking 0.8 g of moist soil 1:10 (dw/v) with water (for NO_3-, NO_2^-) or 2.0 M KCl (for NH_4^+) for 2 h. Afterwards, the extracts were centrifuged (2509 x g) and filtered (0.45 μm). The quantification of NO_3^-, NO_2^- in the extracts was performed with ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland). In the case of NH_4^+, a colorimetric method based on Berthelot's reaction (Sommer et al., 1992) was followed.

Experiment 2. Isotopic experiment

The previously-described incubation in Experiment 1 was reproduced under the same conditions but after the fertilization of each treatment with labeled $K^{15}NO_3$ (Potassium Nitrate-^{15}N. 99 atom% ^{15}N. CAMPRO Scientific GmbH, Germany). Headspace gas samples of 15 mL were withdrawn 7 times (at 0, 24, 48, 96, 144, 168, 258 hours) after closing the jars for 1 hour. Gas samples were stored in 12 mL glass vials, which were previously evacuated (Labco Exetainer®. Lampeter, UK).

The concentrations of ^{14}N-N_2O and ^{15}N-N_2O were analysed at the Stable Isotope Facility (University of California, Davis, USA) with a Trace GC Ultra gas chromatograph (Thermo Electron Corp., Milan, Italy) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Electron Corp., Bremen, Germany).

4.3.4. Calculations and statistical analyses

The calculations for the determination of the N_2O coming from denitrification at the Isotopic Experiment followed the equations reported by Stevens and Laughlin (2001), Stevens et al. (1993), Boast et al. (1988) and Mulvaney (1984):

$r\Delta^{45}R = (m/z\ 45\ /\ m/z\ 44)_{sample} - (m/z\ 45\ /\ m/z\ 44)_{atm}$; $\Delta^{46}R = (m/z\ 46\ /\ m/z\ 44)_{sample} - (m/z\ 46\ /\ m/z\ 44)_{atm}$

$^{15}X_N = 2 \cdot (\Delta^{46}R / \Delta^{r}\Delta^{46}R / \Delta^{45}R)$

$[N_2O]_{denitri} = [N_2O]_{total} \cdot atom\%^{15}N / {}^{15}X_N$

Where $^{15}X_N$ refers to the mol fraction of ^{15}N in the N pool from which the N_2O was derived, $atom\%^{15}N$ refers to the measured ^{15}N abundance in N_2O, *atm* refers to the reference atmospheric measurements, $[N_2O]_{denitri}$ to the N_2O concentration coming from soil denitrification, and $[N_2O]_{total}$ to the total N_2O concentration measured in the vial, coming from every possible soil pathway.

All the graphs included were drawn with the Origin 2018 64Bit software program. The differences between treatments were determined with a one-way analysis of variance (ANOVA) and Tukey-B's post hoc test ($P < 0.05$) with the use of IBM SPSS Statistics 23.

Additionally, to explore the relationship and correlation between the tested BC's physicochemical properties and their degree of effectiveness in reducing soil N_2O emissions, a principal component regression (PCR) was performed using IBM SPSS Statistics 23. When many variables are introduced into a regression model, multicollinearity may occur due to the correlation among variables, often leading to biased model predictions. In order to analyse the structure and correlations between BC properties, a dimension reduction with Oblim rotation was performed over the data matrix, which resulted in a number or principal components and a subset of eigenvectors. Afterwards, the eigenvectors were used as independent variables in a linear regression model to quantify the effect of the BC's physico-chemical properties on total accumulated N_2O (TotalN_2O) and the N_2O coming exclusively from denitrification (DenitrifN_2O) processes.

4.4. Results

4.4.1 Biochar properties

Table 4.1 shows the main properties of the BCs (for further data on BC characteristics, Table A2 in the Appendix can be consulted). The BCs showed contrasting properties mainly due to their different origins and pyrolysis temperatures. The origin of the feedstock determined the aromaticity and C concentration of the BCs. Two separate groups were formed according to the lignocellulosic composition of the original feedstock. While the C_{org}/N ratio of the BCs from olive tree pruning and rice straw obtained values between 95.4 and 60.4, BCs produced from tomato plants and grape stalks obtained lower values (C_{org}/N <39) at both production temperatures. The DOC values were also markedly affected by the feedstock. In general, DOC concentrations were low, and remained below 2.82 mg g^{-1} in all BCs, except for BC-To400 and BC-GS600, which obtained 7.54 and 6.86 mg g^{-1} respectively. The BCs also showed contrasting total pore areas (TporeA). BC-Olv had the greatest areas (50.4-93.1 m^2 g^{-1}) whereas BC-To had the lowest (11.9-12.6 m^2 g^{-1}). No correlation was found with the pyrolysis temperature.

The EC, ash concentration and pH of the BCs increased with the temperature of production. The EC values were generally low (0.59-4.52 mS cm^{-1}) with the exception of BC-To400 and BC-To600, which were 18.43 and 22.80 mS cm^{-1}, respectively. Regarding BC's ash content, BC-Olv and BC-GS showed concentrations below 14.5%, whereas BC-To and BC-Ri values were much higher (around 37.8%). The values of pH were in the range 9.65-12.10, with BC-To600 having the highest value. As expected, the increase in the production temperature from 400 to 600 ˚C also resulted in a decrease of the atomic O/C ratio and the oxygenated species in the BC surface (C-O, C=O/O-C-O and Carboxylic/carbonates). No substantial differences were found among surface functional groups in the BCs, except for the Carboxylic/carbonates relative atomic percentage. The outstanding values of BC-To400 and BC-GS600 are worth highlighting, which were about three and seven times higher than for the rest of BCs, respectively. None of the BCs were a relevant source of N, with negligible concentrations of nitrate or nitrite.

Conversely, they could contribute with small quantities of ammonium when added to the soil, especially in the case of BC-To400 (2.41 mg kg^{-1}).

Table 4.1. Main BC's physicochemical characteristics.

	BC-Olv400	**BC-Olv600**	**BC-To400**	**BC-To600**	**BC-Ri400**	**BC-Ri600**	**BC-GS400**	**BC-GS600**
Feedstock	**Olive tree pruning**		**Tomato plants**		**Rice straw**		**Grape stalks**	
Pyrolysis T (°C)	400	600	400	600	400	600	400	600
pH	9.90	11.05	9.65	12.10	9.73	10.21	10.3	10.7
EC (mS cm^{-1})	0.59	0.75	18.43	22.80	3.83	4.43	3.23	4.52
Ash (%)	4.9	4.8	34.4	38.2	36.6	41.9	12.6	14.4
C_{org} (%)	78.5	88.3	37.3	39.9	39.6	50.4	71.2	69.8
DOC (mg g^{-1})	0.68	0.83	7.54	0.93	2.82	1.68	7.97	6.86
DIC (mg g^{-1})	0.13	0.04	0.40	0.06	0.31	0.72	2.96	4.01
TDC (mg g^{-1})	0.81	0.87	7.94	0.99	3.13	2.40	10.9	10.9
TDN (mg g^{-1})	0.01	0.02	0.36	0.10	0.03	0.07	0.11	0.08
Atomic H/C_{org}	0.52	0.24	0.87	0.39	0.72	0.29	0.56	0.28
C_{org}/N	93.3	95.4	18.6	19.8	60.4	87.2	38.2	35.6
NO_3^- (mg kg^{-1})	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
NO_2^- (mg kg^{-1})	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
NH_4^+ (mg kg^{-1})	0.45	0.60	2.41	1.09	0.62	0.62	0.50	1.35
EDC/EAC	3.8	1.7	6.4	1.5	3.4	1.0	-	-
Lig/Cel	0.46	0.46	0.30	0.30	0.42	0.42	0.52	0.52
TporeA (m^2 g^{-1})	50.4	93.1	11.9	12.6	23.3	21.9	41.1	65.0
Carboxylic/carbonate *	3.7	3.4	10.1	3.2	3.2	2.7	4.0	21.8

Pyrolysis T=BC production pyrolysis temperature; EC= electric conductivity; EAC= electron accepting capacity; EDC= electron donating capacity; Lig/Cel=lignin/cellulose (feedstock); TporeA= total pore area; DOC: dissolved organic carbon; TDC: total dissolved carbon; TDN: total dissolved nitrogen. *Relative atomic percentage of C in BC surface.

The different feedstocks also affected the phytotoxicity of the BCs, as observed in the diverse germination indexes (Figure 4.1). BC-Olv and BC-Ri (at both 400 and 600 ˚C) did not show any significant phytotoxic effect according to the GI. However, four of the BCs did not surpass the 50% germination index (BCs from tomato plants and grape stalks), with BC-GS600 being the one with the lowest value (20%).

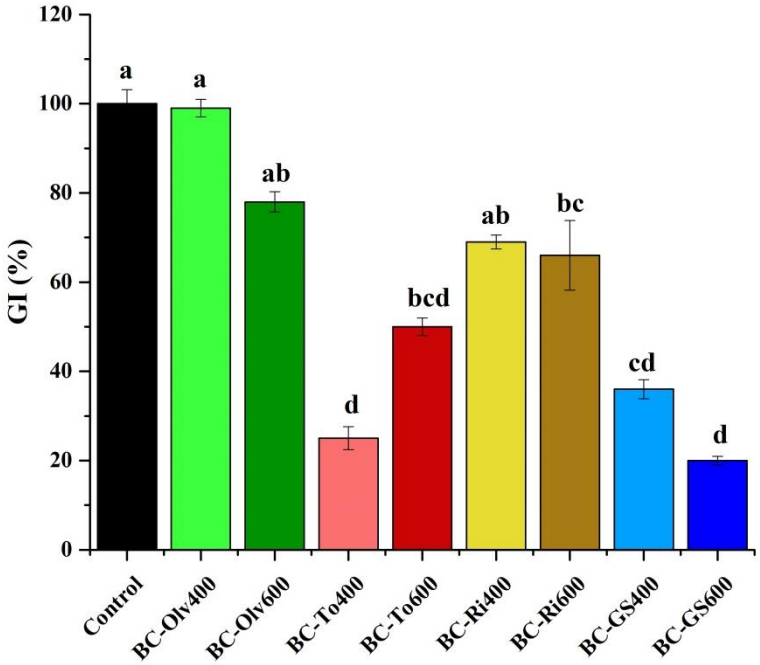

Figure 4.1. Germination index (GI, %) for each BC and the Control (water extract). Different letters (a, b, c, d) indicate significant differences between treatments according to the Tukey-B test ($P<0.05$).

The analysis of the PAHs in the BCs showed, in general, low concentrations in the range of ng g^{-1} BC (Table 4.2). Overall, BC-Olv600 and BC-Ri600 had the greatest concentrations of the measured PAHs, with total concentrations of 176.6 and 152.5 ng g^{-1} BC, respectively. On the other hand, BC-Olv400 (31.1 ng g^{-1} BC) and BC-GS600 (55.2 ng g^{-1} BC) were notable for being the BCs with the lowest concentrations of PAHs. Overall, the most abundant PAH in the BCs was naphthalene, followed by anthracene. The concentrations of phenanthrene and pyrene were close or below the limit of quantification in all the materials, with the only exception of pyrene in BC-Olv600, which showed a large experimental variability.

4.4.2. Experiment 1. N_2O emissions from biochar amended soils

Soil N_2O and CO_2 emissions were affected by the addition of the BCs (Figure 4.2). The unamended soil showed an initial CO_2 peak (528µg C-CO_2 h^{-1} kg^{-1} soil) followed by steady CO_2 emissions in the following days (around 200 µg C-CO_2 h^{-1} kg^{-1} soil).

Table 4.2. Concentration of selected PAHs (ng g^{-1} BC): naphthalene, anthracene, phenanthrene, fluoranthene and pyrene in the different BCs. The sum of all of them is also included.. The values appear with their standard deviation values (duplicates).

	PAHs					
Biochar	**Naphthalene**	**Anthracene**	**Phenanthrene**	**Fluoranthene**	**Pyrene**	**Total**
BC-Olv400	24.7 ± 4.8	4.0 ± 0.7	0.5 ± 0.0	0. 9 ± 0.1	1.1 ± 0.3	31.1 ± 4.9
BC-Olv600	130.3 ± 29.5	10.0 ± 0.9	0.9 ± 0.2	7.4 ± 2.7	28.0 ± 26.2	176.6 ± 29.6
BC-To400	46.0 ± 0.3	2.8 ± 0.7	2.1 ± 0.0	10.7 ± 1.7	3.4 ± 0.9	65.1 ± 1.8
BC-To600	65.8 ± 2.3	4.4± 0.3	1.1 ± 0.2	8.9 ± 7.3	9.0 ± 5.7	89.1 ± 7.7
BC-Ri400	50.5 ± 3.8	4.9 ± 1.1	1.1 ± 0.4	13.4 ± 2.7	1.9 ± 0.2	71.9 ± 4.8
BC-Ri600	118.0 ± 8.1	10.7 ± 2.3	0.7 ± 0.5	19.5 ± 17.3	3.6 ± 1.3	152.5 ± 19.2
BC-GS400	32.4 ± 2.8	2.4 ± 0.2	2.9 ± 0.7	29.1 ± 12.4	2.7 ± 0.4	69.3 ± 12.8
BC-GS600	35.5 ± 7.1	4.4 ± 0.0	2.0 ± 0.7	6.5 ± 0.9	6.9 ± 3.2	55.2 ± 7.1

The addition of BC-Ri400 and BC-Olv600 caused the highest CO_2 emissions with peaks at 96 h (777.1 and 593.6 µg C-CO_2 h^{-1} kg^{-1} soil respectively). The rest of the BCs caused a decrease in the production of CO_2 as compared to control soil. BC-To600 was noticeable for significantly decreasing soil CO_2 production for the entire period of incubation.

Regarding N_2O, the BC treatments could be separated into three groups depending on their effect on soil N_2O emission (Figure 4.2). In the first group, BC-To400 and BC-GS600

significantly increased soil N_2O emissions at 24 hours (162.7 and 164.8 µg N-N_2O h^{-1} kg^{-1} soil in comparison with the control (75.5 µg N-N_2O h^{-1} kg^{-1} soil). The second group, comprised by BC-Olv400, BC-Olv600, BC-Ri400, BC-Ri600 and BC-GS400, slightly decreased the peak of N_2O emissions registered at 24 hours. However, BC-GS400 and BC-Ri600 peaks were delayed to 72 and 96 hours, respectively. Finally, BC-To600 did not show any N_2O emissions, (between 0.8 and 5.1 µg N-N_2O h^{-1} kg^{-1} soil during the entire incubation period).

Figure 4.3 shows the concentration of water-extractable NO_3^- and NO_2^-, and KCl-extractable NH_4^+ during the incubation experiment. There were no differences between treatments except for the BCs coming from tomato plants, which reported significantly lower values of NO_3^- than the rest of the treatments at 24 and 48 hours (17.7-27.3 mg kg^{-1} soil). The concentrations of NO_3^- decreased during the incubation and only minor differences were observed among treatments at the end of the incubation (Figure 4.3 D). Regarding nitrite, BC-To600 treatment notably accumulated this anion in soil, which concentration rose to 8.5-10.0 mg kg^{-1} soil from 24 to 96 hours and was 5.4 mg kg^{-1} soil after 258 hours (Figure 4.3 D). The rest of the BCs showed low NO_2^- concentrations, always below BC-To600. Ammonium analysis (on the top right side of panel D in Figure 4.3) showed that treatments with BCs pyrolyzed at 600 ˚C presented less concentration in NH_4^+ in soil at the end of the incubation in comparison with BCs produced at 400 ˚C. The addition of BC-Olv600 and BC-Ri600 to soil led to a significantly lower concentration of NH_4^+ (4.35 and 4.37 mg kg^{-1} soil N- NH_4^+, respectively) compared to the control (7.40 mg kg^{-1} soil N- NH_4^+). On the other side, BC-To400 resulted in highest values (7.7 mg kg^{-1} soil).

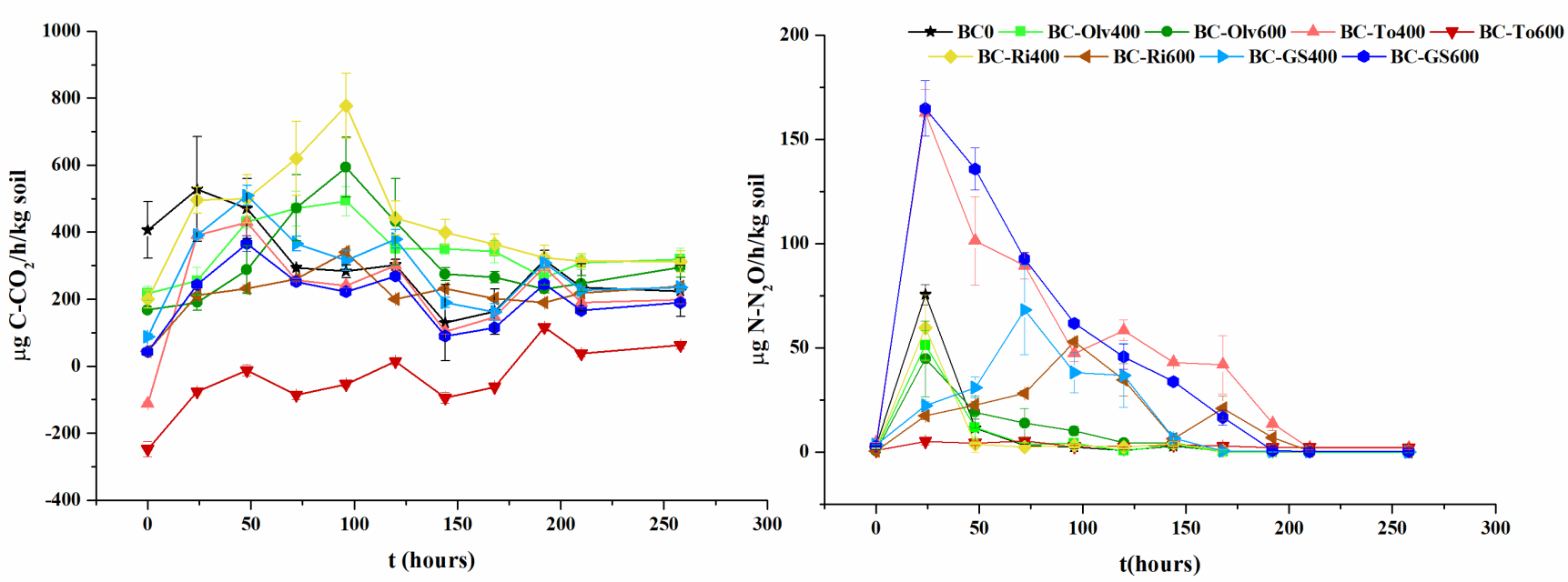

Figure 4.2. Fluxes of N_2O and CO_2 (μg h^{-1} kg^{-1} soil) in unamended soil (BC0) and soils amended with the different BCs (2%): Olv: olive tree pruning, To: tomato plants, Ri: rice straw, GS: grape stalks. Error bars represent the standard error of the mean (n=4).

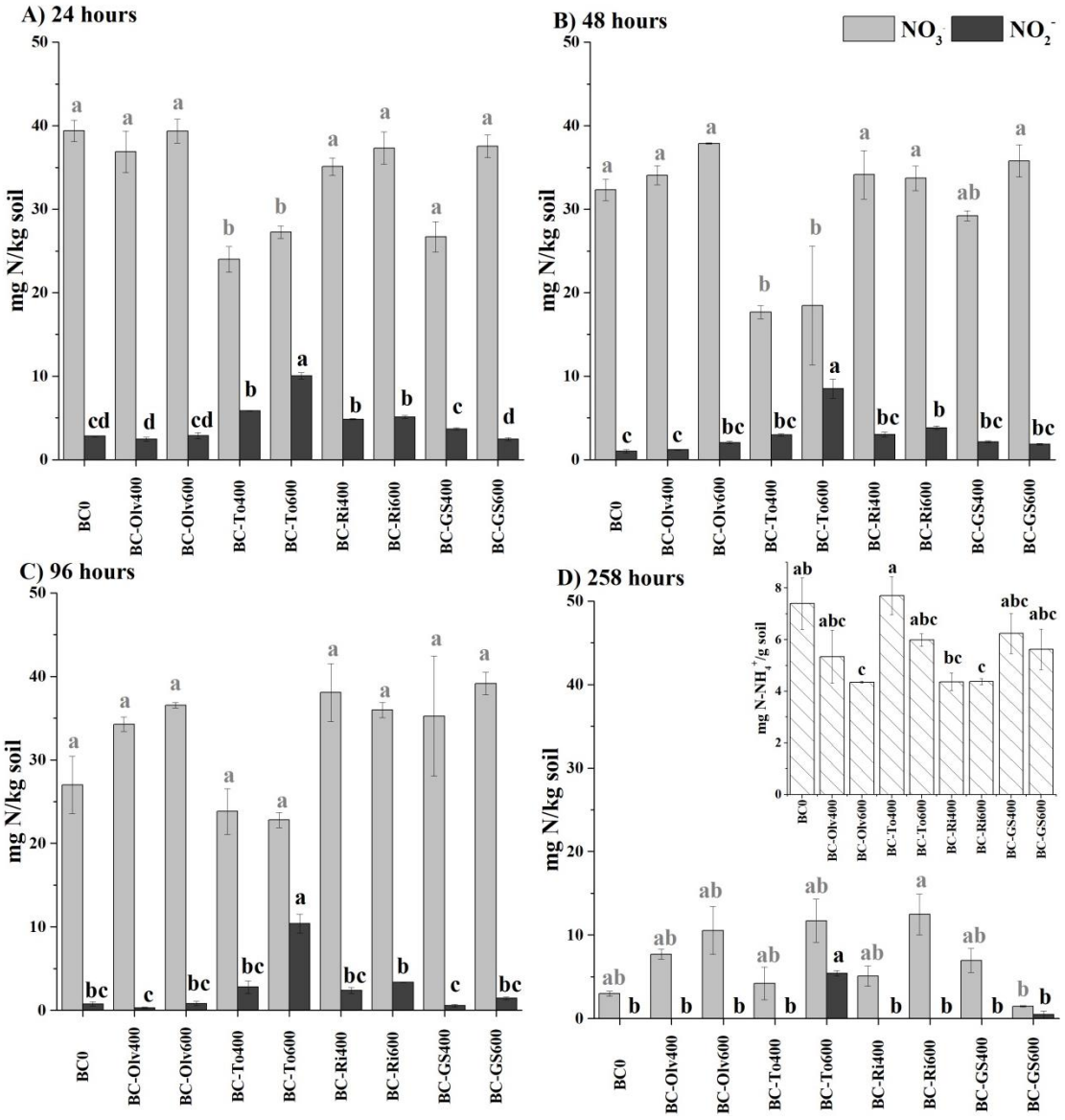

Figure 4.3. Water-extractable NO_3^- and NO_2^- concentrations in soil (mg N kg^{-1} soil) for the different treatments at 24 (A), 48 (B), 96 (C) and 258 (D) hours. The concentration of KCl extractable NH_4^+ at 258 hours is also shown in D. Error bars indicate standard error of the mean (n=3). Different letters (a, b, c) express significant differences between treatments according to Tukey-B test ($P<0.05$).

4.4.3. Experiment 2. Isotopic experiment

The addition of $^{15}N\text{-}NO_3^-$ in Experiment 2 allowed for the calculation of N_2O that was directly derived from denitrification, distinguishing it from N_2O produced by other pathways. Thus, Figure 4.4. shows total accumulated N_2O emissions ('Total N_2O', N_2O released from soil coming from every possible source. Experiment 1), and accumulated denitrification N_2O ('Denitrification N_2O'.

N_2O generated exclusively from denitrification. Experiment 2). BC-To400 and BC-GS600 generated the largest accumulated emissions of N_2O in comparison with the other treatments and the control. The rest of the BCs produced statistically similar N_2O emissions to BC0. Some BCs were able to slightly reduce total N_2O emissions (i.e. BC-Olv400 119.7 or BC-To600 131.1 µg N-N_2O h^{-1} kg^{-1} soil), although not significantly in comparison with the unamended soil (161.1 µg N-N_2O h^{-1} kg^{-1} soil). The percentage of N_2O derived exclusively from denitrification was 44.4% in the control soil. All BCs increased this percentage, except for BC-GS400, which clearly showed an increase in N_2O emissions through other pathways different from denitrification.

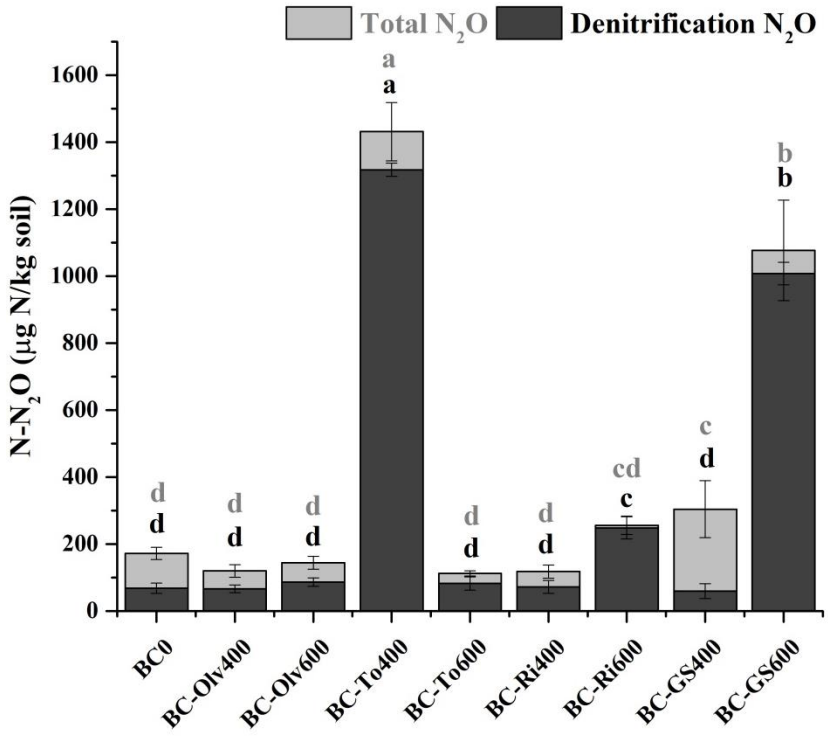

Figure 4.4. Comparison between the accumulated N_2O emitted (µg N kg^{-1} soil) in Experiment 1 ('Total N_2O', coming from all potential N_2O sources. Light gray bars) and Experiment 2 ('Denitrification N_2O', N_2O only from denitrification. Dark gray bars) for each treatment. Different letters (a, b, c, d) indicate significant differences between treatments according to the Tukey-B test ($P<0.05$) for total cumulative N_2O (light grey) and denitrification N_2O (dark grey).

4.4.4. Principal Component Regression

For the PCR, a wide range of BC properties were analyzed and subjected to a dimension reduction. After removing those with lower coefficients, three components were with nine BC chemical and physical properties (Table 4.3). The linear regression between the eigenvectors and TotalN_2O and DenitrifN_2O, resulted in both variables exclusively correlating with component 1 (Figure 4.5.), composed by BCs GI, C/N, DOC and carboxylic/carbonates surface abundance. The correlation was direct with DOC and carboxylic/carbonates (positive coefficients) indicating that BCs with higher values of these two properties would produce greater N_2O emissions. Conversely, BCs with the strongest N_2O mitigation potential would be those with high GI and C/N ratio (with negative coefficients, inverse correlation). The linear regression with both TotalN_2O and DenitrifN_2O resulted in similar statistical relevance (DenitrifN_2O, TotalN_2O): R^2=0.56, 0.64; ANOVA significance: 0.000, 0.000; ANOVA F: 37.4, 53.2.

The properties allocated to components 2 and 3 (ash, TporeA, EDC/EAC, H/C, PAHs) were discarded for being irrelevant or determinant for the N_2O emissions measured in the incubation experiments.

4.5. Discussion

4.5.1. Modification of soil N_2O fluxes through biochar amendment

The experiments included in the present chapter revealed that BC could decrease, increase or have no effect on N_2O emissions from denitrification in a calcareous fertilized soil, which confirms the initial hypothesis. The statistical analysis of the data allowed establishing a link between the mitigation potential and the BCs' physicochemical characteristics.

Table 4.4.Three component matrix generated by the PCR with the physical and chemical properties of the BCs. Coefficients <0.55 were deleted from the table.

	Component		
	1	**2**	**3**
GI	-0.925		
Carboxylic/carbonates	0.879		
DOC	0.778		
C_{org}/N	-0.738		
Ash		0.982	
TporeA		-0.901	
EDC/EAC			0.948
H/C			0.898
PAHs			-0.678

GI: germination index; DOC: dissolved organic carbons; Corg: organic C; EDC: electron donor capacity; EAC: electron accepting capacity; PAHs: policyclic aromatic hydrocarbons.

4.5.1.1. Biochars decreasing initial soil N_2O emissions

The most numerous group of BCs (BC-Olv400, BC-Olv600, BC-Ri400, BC-Ri600, BC-GS400), caused a reduction in soil's initial (24-48h) N_2O emission peak (Figure 4.2.). However, this pattern was not maintained during the rest of the incubation period, resulting in an overall lack of mitigation, as observed when the accumulated N_2O emissions were plotted in Figure 4.4. This outcome supports previous studies on calcareous soils (Van Zwieten et al., 2014), but contradicts other studies that reported significantly reduced emissions of N_2O in high pH soils amended with BC (Dong et al., 2020, Borchard et al., 2019). The group of BCs that had a temporal capacity to reduce N_2O emissions included BC-Olv400, BC-Olv600, BC-Ri400, BC-Ri600, and BC-GS400.

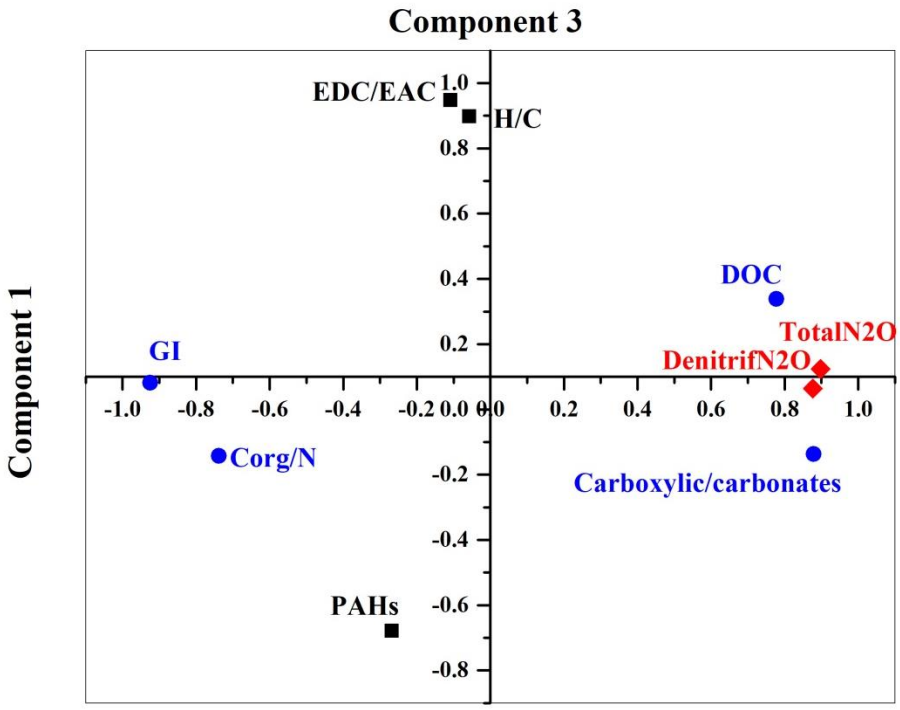

Figure 4.5. Principal Component Analysis (PCA) results. Arrangement of two components by the PCR analysis, including Component 1, the selected variables as correlated with the N_2O results from the incubations (Table 4.3). The BC characteristics collected in Component 1 are colored in dark blue (●) and the ones belonging to Component 3 in black (■). In addition, accumulated N_2O (TotalN2O) and N_2O emissions coming from denitrification (DenitrifN2O) are shown in red (♦).

All these BCs were characterized by a high C /N ratio and no phytoxicity (GI>60%) (Table 4.1 and Figure 4.1).

As predicted, C/N was a confirmed factor that influenced N_2O emissions. Biochars with a low C/N ratio may provide significant amounts of N sources for nitrification and denitrification and therefore increase N_2O emissions (Cayuela et al., 2014; Steiner et al., 2008). However, in contrast to previous findings in the literature (Weldon et al., 2019; Cayuela el al., 2015), the atomic H/C ratio, another important ratio linked to BCs mitigation potential, did not influence N_2O soil emissions from denitrification in this particular soil (Figure 4.5). This may be related to other BCs properties having a stronger influence (e.g DOC or toxicity).

4.5.1.2. Biochars increasing soil N_2O emissions

Two of the BCs, BC-To400 and BC-GS600, clearly increased soil N_2O emissions (Figure 4.2.). These BCs showed high values of DOC, superficial atomic abundance of carboxylic groups and low values of GI. Dissolved organic carbon, which is easily degradable and available for microorganisms, usually represents a small proportion of the total organic C in slow-pyrolysis BCs (Liu et al., 2019). Considering the low initial DOC in the soil (0.23 mg g^{-1}), the addition of readily-available organic C within these BCs could stimulate heterotrophic denitrification in soil and therefore N_2O emissions. Biochar surface functional groups have been reported to play a decisive role in controlling the BC's effect on N_2O production and reduction (Sumaraj and Padhye, 2017; Quin et al., 2015). Specifically, oxygen-containing functional groups with the important functionality of accepting electrons, such as carboxylic, can compete with N_2O and inhibit or limit the last step of denitrification, and consequently, increase soil emissions of N_2O (Yuan et al., 2019; Chen et al., 2018). In this study, the electron accepting capacity of the BCs did not correlated with N_2O emissions. This may indicate the presence of other complex interactions with the soil that mask this effect. Further research is needed to discard confounding factors related to the soil matrix.

BC-To400 and BC-GS600 showed the lowest GI (25 and 20%, respectively) of all the BC treatments (Figure 4.1). When used as a soil amendment for agronomic purposes, it is important to ensure that the BC does not exhibit any type of toxic effect (Gascó et al., 2016). Apart from the PAHs, BC may contain many other undesirable compounds such as crystalline silica, dioxins, phenolic compounds, or heavy metals (Thies et al., 2015). The relationship between the toxicity of the BCs (measured by their GI), and their observed N_2O emissions may be a proof that microorganisms involved in the N cycle and capable of reducing N_2O to N_2 are, to some extent, sensitive to the presence of toxic compounds. The concentration of PAHs in BCs can be substantially high, depending on the BC feedstock, production conditions, and method (De la Rosa et al., 2019; Weidemann et al., 2018). The exclusion of the PAHs by the PCR, contrarily to our

initial hypothesis, together with the comparison of their concentration with other BCs employed in the literature, suggests that the low concentrations of PAHs measured in the BCs tested here had a minimal impact on N_2O emissions. For instance, Keiluweit et al. (2012) showed that biomass-based BCs had 840-986 ng g^{-1} of phenanthrene or 296-689 ng g^{-1} of pyrene. However, none of our materials used in this experiment obtained such high values (Table 4.2.) or surpassed the EC50 (half-maximal inhibitory concentration values considered toxic for N transformations mediated by microorganisms (Guo et al., 2011; Maliszewska-Kordybach et al., 2007). Hence, BC-To400 and BC-GS600 toxicity may be a consequence of compounds that were not analyzed, as neither PAHs, nor heavy metal (measured, Table A2) concentrations were high enough to represent a toxicity risk.

4.5.1.3. The singular effect of BC-To600

BC-To600 showed a different behavior from the rest of the BCs, decreasing both N_2O and CO_2 emissions (Figure 4.2.). Additionally, BC-To600 led to a significant higher soil nitrite concentration during the entire incubation period alongside with low nitrate contents (Figure 4.3.), which is characteristic of soil conditions that inhibit nitrification. This BC obtained low values for all properties included in component 1 (Table 4.1. and 4.3.), which would mean that it has the potential to increase (due to its low GI and C/N) and decrease (caused by its low DOC and low carboxylic/carbonates) N_2O soil emissions at the same time. As observed in Figure 4.4 and 4.3, BC-To600 has a prevailing tendency to block N_2O production from denitrification, as observed by the flat evolution of N_2O and high accumulation of nitrite in the soil. A similar result was obtained by other studies, for instance, by Wang et al. (2013). They observed that, at the end of the incubations, the N content in BC-amended soils was clearly higher than that of the control, which they related to a depressed denitrification process. Consequently, a toxic effect cannot be discarded in this specific BC, which may also be supported by its low CO_2 emissions (Chintala et al., 2014), high pH (Bakken et al., 2012) or high concentration of some heavy metals such as Fe, Cu, Zn or Pb (Bollag

and Barabasz, 1979) (Table A1). Conversely, it could not be related to its concentration of PAHs, as this BC has low and similar values as compared to the rest of the BCs (Table 4.2.).

4.5.2. Potential N_2O formation pathways in addition to denitrification.

The use of labelled $^{15}N\text{-}NO_3^-$ (Experiment 2) allowed studying the occurrence of alternative N_2O formation pathways, apart from denitrification (Figure 4.4). Due to the complexity of N reactions in soil, there are multiple processes that produce N_2O (Hu et al., 2015; Wrage et al., 2001), and hence, the identification of the actual mechanisms occurring alongside denitrification is intricate.

In this experiment, all the treatments showed N_2O emissions from pathways different to denitrification, representing between 80.6-3.1% of total N_2O emissions. Among the processes that may be relevant for future research are nitrifier denitrification and codenitrification. Codenitrification occurs under the same conditions and utilizes the same set of enzymes as denitrification. Although it is still a rather unknown process, with conditions that remain to be described precisely, Spott et al., (2011a,b) were able to contribute with important information about this mechanism. In the present study, it was not possible to prove that codenitrification took place. Future assays that focus on codenitrification or experiments including the analisis of the expression and activity of genes involved in each of the N_2O production mechanisms in the N cycle, would be nedded to complete and expand the results presented here. In addition, specific redox studies that directly assess the interaction BC/denitrification microorganisms would contribute with information to discard or support the redox theories.

4.6. Conclusions

This study allowed assessing the impact that the contrasting physico-chemical and chemical properties of the BCs had on soil N_2O emissions under optimum conditions for denitrification. The BCs selected had different physical and chemical characteristics, with the aim of finding patterns of

behavior and key determinant properties linked to changes in N_2O emission patterns. The addition of BC to a calcareous soil at 90% WFPS led to contrasting impacts (decrease, increase or no alteration) of N_2O emissions, and the mechanisms through which it was released. The majority of the BCs showed an overall limited capability to decrease N_2O emissions in this soil. Conversely, BCs with a susbtantial DOC, the presence of oxygenated functional groups on their surface, and low GI, resulted in clear increases in soil N_2O emissions. The BC PAH content and potential to donate, accept or transfer electrons were discarded as reasons for altering soil denitrification rates, as no correlation was found with N_2O emissions. Denitrification was the most important process leading to N_2O emissions for most of the BC treatments, as shown by a ^{15}N tracer experiment, although other mechanisms were also present.

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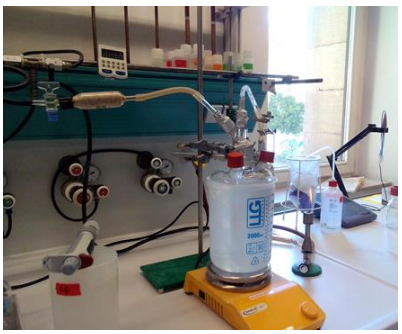
Chapter 5

Biochar as electron donor for reduction of N_2O by *Paracoccus denitrificans*

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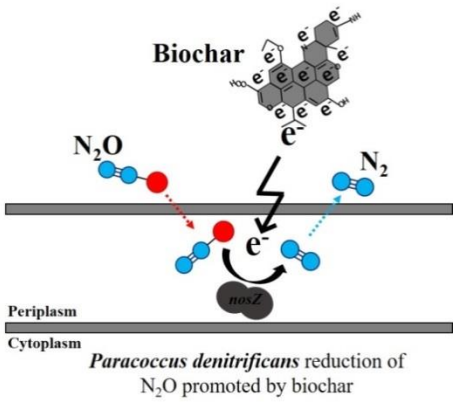
5.1. Abstract

Biochar has been shown to influence microbial denitrification and mitigate soil N_2O emissions. However, it is unclear if biochar is able to directly stimulate the microbial reduction of N_2O to N_2. We hypothesized that the ability of biochar to lower N_2O emissions could be related not only to its ability to store electrons, but to donate them to bacteria that enzymatically reduce N_2O. Therefore, we carried out anoxic incubations with *Paracoccus denitrificans*, known amounts of N_2O, and nine contrasting biochars, in the absence of any other electron donor or acceptor. We found a strong and direct correlation between the extent and rates of N_2O reduction with biochar's EDC/EEC (electron donating capacity/electron exchange capacity). Apart from the redox capacity, other biochar properties were found to regulate the biochar's ability to increase N_2O reduction by *Paracoccus denitrificans*. For this specific biochar series, we found that a high H/C and ash content, low surface area and poor lignin feedstocks favoured N_2O reduction.. This provides valuable information for producing tailored biochars with the potential to assist and promote the reduction of N_2O in the pursuit of reducing this greenhouse gas emissions.

Keywords: denitrification; charcoal; electron shuttle; redox; nitrous oxide; electron donating capacity



Graphical abstract

5.2. Introduction

Nitrous oxide (N_2O) is one of the main contributors to global warming among the greenhouse gases released by agricultural activities (IPCC, 2014). Soils are the primary source of atmospheric N_2O and their contribution has been constantly increasing since pre-industrial times (Butterbach-Bahl et al., 2013). Recently, biochar soil amendments were proposed as an effective approach to tackle N_2O emissions in agro-ecosystems (Kammann et al., 2017; Clough et al., 2013). Biochar (BC) is created through the pyrolysis of biomass under high-temperature and low-oxygen conditions (Lehmann, 2007). When added to soil, BC affects its microbial activity, physical structure and chemical properties, and is more recalcitrant to microbial decomposition than the original feedstock (Keith et al., 2011; Zhang et al., 2010). Several meta-analyses have demonstrated that BC mitigates nitrogen (N) losses and reduces nitrous oxide emissions from soils (Borchard et al., 2019; Liu et al., 2019; Cayuela et al., 2014). Specifically, it changes the microbial community of nitrous oxide reducers (Krause et al., 2018; Harter et al., 2017). However, despite this research, the exact mechanisms of action are not fully understood (Weldon et al., 2019).

Paracoccus denitrificans is a model soil microorganism that is widely employed for bioenergetic studies. It is capable of denitrification down to its last step and rapidly reduces N_2O to N_2 with nitrate or nitrite as the electron acceptor and succinate, NADH, glucose, acetate or methanol as the electron donor (Olaya-Abril et al., 2018; Hahnke et al., 2014; Felgate et al., 2012; Baumann et al., 1996; Kučera et al., 1983; Stouthamer, 1980). This denitrification reaction is catalyzed by the multicopper enzyme nitrous oxide reductase (N_2OR). This enzyme is encoded by a *nor*/*nos* gene cluster that drives the synthesis of the essential proteins required for its activity. The periplasmic *nosZ* protein is not always present in every denitrifying bacterium, which causes the blocking of N_2 production and the subsequent release of N_2O (Carreira et al., 2017; Torres at al., 2016).

Several recent studies have demonstrated that BC stimulates the last step of denitrification, where N_2O is reduced to N_2. The suggested mechanisms have included the 'electron shuttle' theory (Fungo et al., 2019; Cayuela et al., 2013), the effect BC has over soil pH and N_2O residence time (Weldon et al., 2019), the involvement of BC redox active components (Chen et al., 2018) or its N_2O adsorption potential (Quin et al., 2015). Nevertheless, this last step is highly variable across BC types (Weldon et al., 2019; Yuan et al., 2019). When applied to soils, BC can affect N_2O dynamics through abiotic and/or biotic mechanisms. Biochar has been found to adsorb and abiotically reduce N_2O injected in sterilized soil columns (Quin et al., 2015), but also stores N_2O and stimulates microbial N_2O reduction (Harter et al., 2016). In addition, BC has been suggested to mediate redox reactions during biological denitrification, acting as a reducing agent (i.e. an electron donor) for denitrifying bacteria or as an electron shuttle. When functioning as an electron donor, reduced functional groups in BC are biologically oxidized and the electrons are donated to N-species that function as electron acceptors (Chen et al., 2018). When acting as an electron shuttle, BC is reversibly reduced and oxidized by both accepting and donating electrons. BC reduction proceeds by abiotic reductants or by reducing bacteria. Meanwhile, BC oxidation occurs either by abiotic electron acceptors or microorganisms that use these electrons for energy generation and/or CO_2 fixation (Xu et al., 2016; Yu et al., 2016). Both electron donor or electron shuttle functions are based on the presence of redox-active functional groups (quinones/hydroquinones) and redox-active aromatic structures that allow the presence of delocalized π-electrons in BC (Sun et al., 2017; Yuan et al., 2017; Chen et al., 2014), a property that differentiate them from other redox-active carbon rich materials such as humic substances (Wu et al., 2017). Nevertheless, the role of BC redox reactions for influencing N_2O emissions has not been experimentally verified with pure cultures of denitrifying bacteria or soil matrices yet (Yuan et al., 2019). The main difficulty in determining the role of BC during denitrification processes lies in its complex properties. These properties hinder the distinction between redox and other BC properties that could also be involved (e.g. its sorption capacity). The origin of BC's intricate properties is mainly controlled by the ratios of lignin,

cellulose and hemicellulose in the feedstock as well as its pyrolysis production parameters (Prévoteau et al., 2016; Zhao et al., 2013). Based on previous studies, a link between BC characteristics and its ability to donate, accept, or in general, to transfer electrons can be envisaged. For instance, the presence of redox-active functional groups at the BC surface is the primary cause for the electron donation ability at low temperature possessed by BCs (Sun et al., 2018; Yu et al., 2016; Kappler et al., 2014). However, the conductivity of electrons through polycondensed aromatic structures dominates the transfer of electrons in BCs pyrolyzed at the highest treatment temperature (HTT)>650°C, as these have a high degree of aromaticity (molar hydrogen/carbon, H/C, ratios <0.3) (Sun et al., 2018; Sun et al., 2017; Chen et al., 2014).

Previous studies have been carried out with a limited number of BCs, which do not allow their conclusions to be generalized. Consequently, the effect of BCs with contrasting redox properties on the microbe-catalyzed reduction of N_2O to N_2 remains unknown. It is therefore necessary to study the effect of different BC characteristics on microbial N_2O reduction. This would allow producing BC on demand by adjusting the feedstock utilized and the pyrolysis conditions to enhance their redox potential and boost N_2O-reducing bacteria activity.

Consequently, the main objective of our work was to evaluate the ability of dissimilar BCs to support N_2O reduction by *Paracoccus denitrificans* by means of their redox properties in the absence of any other C or electron source. Moreover, the effect of BCs modified by both ageing in soil and post-pyrolysis chemical treatment was evaluated. Our two hypotheses were (i) the use of BCs will lead to the reduction of N_2O to N_2 by *Paracoccus denitrificans* with an extent that depends on BC redox properties (i.e. their electron exchange capacity, EEC, and electron donating capacity, EDC); (ii) The oxidation of BC (either by biological aging in soil or after chemical treatment) will decrease its potential to donate electrons and support N_2O reduction by *Paracoccus denitrificans.*

5.3. Material and methods

5.3.1. Biochars

Nine BCs were tested stemming from a variety of feedstocks (Apendix, Table A1 and A2): BC-Olv400, BC-Olv600, BC-To400, BC-To600, BC-Ri400, BC-Ri600, BC-OlvM, BC-Oak650, BC-Oak650A. Six BCs were produced from tree-plant residues pyrolyzed at two different temperatures. More information about their origin can be found in Sánchez-García et al. (2019). A company (PROININSO, S.A. Málaga, SPAIN) supplied another woody BC. This BC was used i) fresh, as commercially acquired (BC-Oak650), and ii) aged (BC-Oak650A), after five years in a soil field experiment (Sánchez-García et al., 2016). This aged BC was recovered by collecting the particles by hand from the field, followed by several milliQ water washes. Lastly, a modified BC from olive trees (BC-OlvM) was synthesized by a post-pyrolysis chemical treatment (Lima et al. 2017). Briefly, the char was subjected to a series of oxidative steps using $NaNO_3$, H_2SO_4, $KMnO_4$ and H_2O_2. The objective of synthetizing this BC was to obtain a material with greater electron exchange capacity by increasing the number of surface redox-active functional groups. Stock suspensions of all BCs were prepared by adding 10 g of BC powder to 100 mL of anoxic milliQ water inside a glovebox (MBraun UniLab-2000, Germany). The suspensions were sonicated for 10 minutes and sterilized in an autoclave (Yang et al., 2020; Kappler at al., 2014).

5.3.2. Biochar characterization

A detailed characterization of all BCs can be found in Sánchez-García et al. (2019) and a summary in Table A2. Additionally, the Brunauer-Emmett-Teller (BET) surface area (m^2 g^{-1} BC) was analyzed (ASAP 2000 instrument, Micromeritics, USA). The BCs' electron donating capacity (EDC) and electron accepting capacity (EAC) were determined for the BC suspensions by mediated electrochemical reduction and oxidation (1000C Multi-potentiostat, CH Instruments, USA) using

the electron transfer mediators 4,4'-bipyridinium-1,1-bis(2-ethylsulfonate) (*ZiV)* and 2,2,'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), respectively. The resulting reductive (MER) and oxidative (MEO) current peaks were integrated to yield EAC and EDC (mmol e^- g^{-1} BC) (Klüpfel et al., 2014)

$$EAC = \frac{\int {}^{I_{red}}/_{F} dt}{m_{BC}} ; EDC = \frac{\int {}^{I_{ox}}/_{F} dt}{m_{BC}}.$$

In these equations, the reductive and oxidative baseline-corrected currents in MER and MEO are represented by I_{red} and I_{ox} respectively, F is the Faraday constant (96485.34 s A mol e^{-1}) and m_{BC} is the added mass (g) of biochar (BC).

5.3.3. Microorganism used and cultivation conditions

Paracoccus denitrificans ATCC 19367 (provided by Sebastian Kopf, California Institute of Technology) was used as a typical denitrifying bacterium. The culture medium was prepared with 22 mM $NaHCO_3$ buffer (adjusted to pH 7) with all the compounds listed in Table S5.1. This culture medium was divided into flasks containing 25 mL of each of them. To each of these 25 mL-flasks, the following were added: a 1.25 mL aliquot from the initial stock of *Paracoccus denitrificans* ATCC 19367 with $3.6 \cdot 10^7$ cells m L^{-1}, 500 µL of the electron donor $NaNO_3$ (1 M) and 250 µL of the electron acceptor succinate (1 M). The cultures were incubated anoxically in the dark at 28˚C without any shaking.

During pre-cultivation, *Paracoccus denitrificans* growth was followed by quantifying cell numbers every day for five days with a Flow Cytometer (Attune *NxT* acoustic focusing cytometer and auto sampler, Life technologies. ThermoFisher Scientific, USA) using a commercial LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes. L-7012). Its growth rate is shown in Figure 5.1 (final cell number: $2.15 \cdot 10^9$ cells mL^{-1}).

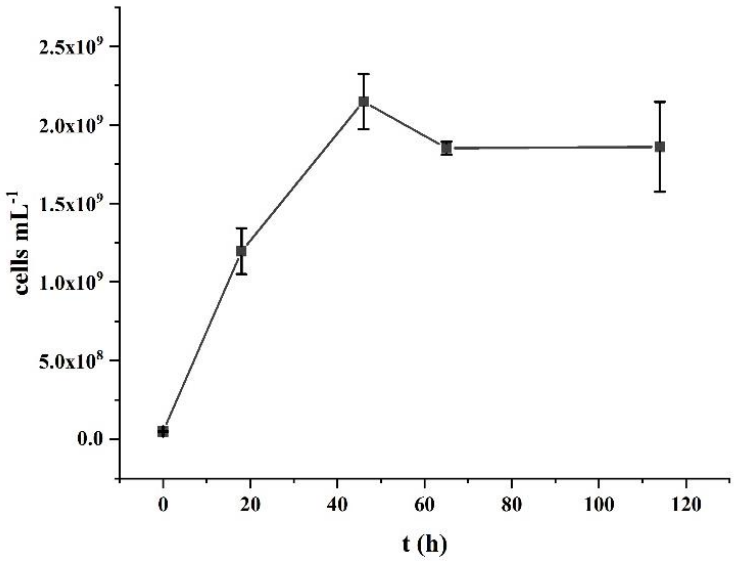


Figure 5.1. Growth of *Paracoccus denitrificans* ATCC 19367 when incubated with the substrates shown in Table S5.1. The incubation was done for 120 hours in the dark at 28˚C.

5.3.4. Nitrous oxide reduction setups

A succession of vacuum and N_2 flushes were applied to 58 mL serum bottles (HCl-washed and sterilized by autoclaving). Afterwards, anoxic conditions were established by filling the bottle with a mixture of N_2/CO_2 (80/20 %vol). Twenty mL of $NaHCO_3$ (22 mM) buffer solution were added to each bottle as well as the required volumes of the 0.1 g mL^{-1} suspensions of the BCs. Thirty-six different treatments were set up depending on the BCs, their concentrations, 1 (BC1) and 5 (BC5) g L^{-1}, and the conditions, abiotic (A) or biotic (B). In addition, two controls were prepared, BC0_A and BC0_B, both of them with 0 g L^{-1} of BC. In case of the biotic samples, *Paracoccus denitrificans* ATCC 19367 cells were injected (750 μL of the initial stock (previous section) at an initial cell number of $1.1 \cdot 10^6$ cells mL^{-1}). Lastly, $NaHCO_3$ (22 mM) was used for equating the volumes to 25 mL and the resulting overpressure was released. No electron donor or acceptor aside from BC was added. After two days of equilibration (at which the small residual $NaNO_3$ and

succinate that could have remained from the growth culture was assumed to be consumed), 0.6 mL of sterilized N_2O (99%) were injected into the headspace (initial N_2O concentration around 2.3% vol) after which the overpressure was released. Gas samples were taken for one week from the bottles at regular intervals. Throughout the entire procedure, the pureness of the culture inside the samples jars was ensured by operating close to a Bunsen burner, using sterilized material and employing 90% ethanol constantly. The incubations were carried out in the dark, at 28˚C and on a shaker at 100 Mot 1/min (Edmund Bühler GmbH SM-30C, Germany).

The nomenclature for the different treatments was as follows: BCX_YT_Z, where X indicates the concentration of BC (0, 1 or 5 g L^{-1}), Y stands for the original feedstock (Olv, To, Ri, Oak), T denotes the highest temperature of pyrolysis (400, 600 or 650 °C) and Z the biotic (B) or abiotic (A) nature of the experiment. For the aged and modified BCs, an extra letter was added after the one referring to the feedstock, A and M respectively (BCX_Oak650A_Z, BCX_OlvM_Z)

5.3.5. Nitrous oxide measurements

Gas samples of 100 µL were taken using a gastight syringe and transferred into N_2-filled 22.4 mL glass vials. Nitrous oxide concentrations were determined using a gas chromatograph (GC 450 Greenhouse Gas Analyzer, Bruker Daltonic, Bremen, Germany) coupled to an autosampler (GX-271 LH, Gilson, Limburg, Germany). The separation of the trace gases was carried out using a Hayesep D column (80-10 mesh), with the oven temperature set at 80°C. N_2O concentration was analyzed with a ^{63}Ni electron capture detector at 300°C, which employs N_2 as the carrier gas and a mixture of 90% argon and 10% methane as the makeup gas. Standard gases with 25, 50, 75 and 100 ppm N_2O in N_2 were used for calibration (nonlinear). Chromatograms were integrated using Bruker Compass CDSTM 2012 software.

5.3.6. EDC evolution measurements

Samples volumes of 500 µL were taken from each bottle at the beginning (before the N_2O injection) and at the end of the experiment (162 hours after the N_2O injection). Sampling was carried out inside a glovebox where the aliquots were centrifuged for 5 min at 20000 *x g* and washed with anoxic milliQ H_2O until reaching a neutral pH (this step was not necessary in our case as every sample was already at pH 7). Afterwards, 1 mL anoxic milliQ H_2O was added to each sample and, additionally, those with 5 g L^{-1} of BC were diluted 1:5. After being thoroughly shaken with a Vortex, every sample was immediately frozen at -20˚C until measured. EDC determinations were performed electrochemically as described previously (Klüpfel et al., 2014).

5.3.7. Qualitative microscopy assays

The bacteria-BC attachment was analyzed by fluorescence microscopy (Leica DM 5500B, Leica, Germany). The living cells were visualized at 488 nm in green after being stained by DNA dye Syto 9 combined with propidium iodide (LIVE/DEAD BacLight Bacterial Viability Kits, Molecular Probes. L-7007).

5.3.8. Calculations and statistical analyses

Biochar redox characteristics, EDC and EAC, were calculated in duplicate as described by Klüpfel et al. (2014). The electron exchange capacity, which describes a BC total capacity to donate and accept electrons, was obtained by adding the value of EDC to EAC (EEC= EAC + EDC). In addition, the EDC/EEC ratio or reduction index (RI) of the BCs was determined, which is a direct measurement of its relative extent of reduction (or oxidation) (Klüpfel et al., 2014).

The N_2O concentration at time=*n* hours ($[N_2O]_n$ in mg N-N_2O $flask^{-1}$) was plotted normalized in respect with the concentration at time 0 hours ($[N_2O]_0$) for facilitating the comparison between treatments. It was represented as C/C_0 and calculated:

$$C/C_0 = [N_2O]_n/[N_2O]_0$$

Soil reduction of N_2O and formation of N_2 follows a competitive Michaelis-Menten type kinetics but can be simplified to first order kinetics at low nitrate concentrations (Cho and Mills, 1979; Van Cleempt et al., 1975). Values of C/C_0 against time was calculated (for the first 45 hours of incubation) and the data fitted to a straight line with slope *k* and intercept *a*.

$$t = k \cdot C/C_0 + a$$

As a way to assess the rate of change in the N_2O concentration with time, the 'N_2O reduction extent' was defined as:

$$N_2O\ reduction\ extent = ([N_2O]_0 - [N_2O]_n)\ x\ 100$$

To estimate the differences among treatments, the standard deviation was calculated from triplicate or duplicate replicates. Additionally, when possible, the significant differences were determined by a one-way ANOVA. The Tukey's post hoc test ($P < 0.05$) was used to discriminate treatments within groups. For significant differences, different letters were assigned. Additionally, an electron balance was performed by calculating the number of electrons needed for the N_2O reduction observed from the start to the end of the incubations (165 hours). For these calculations, both the N_2O in the headspace and the one dissolved were considered, for which the N_2O solubility at 28°C ($4.032 \cdot 10^{-4}$) and its Ostwald Coefficient (0.5553) were used (Geventman, 1992; Wilhelm et al., 1977). A Hierarchical Cluster Analysis was carried out to group the treatments as a function of BCs

RI and the N_2O reduction extent they produced considering the whole period of incubation (*n*=162 hours in the above equation). The Ward method was applied with the aim of grouping cases so as to minimize the variance within clusters.

A Principal Component Regression (PCR) was also performed. A dimension reduction with Oblim rotation was applied over a selection of BC properties. It resulted in a number of principal components arranged in a matrix and composed by positive and negative coefficients assigned to each BC property. Values lower than +0.52 or -0.52 were not considered and deleted from the matrix. Afterwards, a linear regression was carried out with the mentioned matrix as the independent factor and the N_2O reduction extent (t 0 to t 165 hours) for the biotic samples with BC 5 g L^{-1} as the dependent factor. The higher the coefficient assigned to a certain BC property in the matrix, the stronger the influence it has over the dependent variable. Those negative values will have an inverse relation with dependent value whereas positive numbers will indicate a direct relation.

All the mentioned statistical analyses were developed with IBM SPSS Statistics v.23 and every graph included was drawn with Origin 2018 64Bit software.

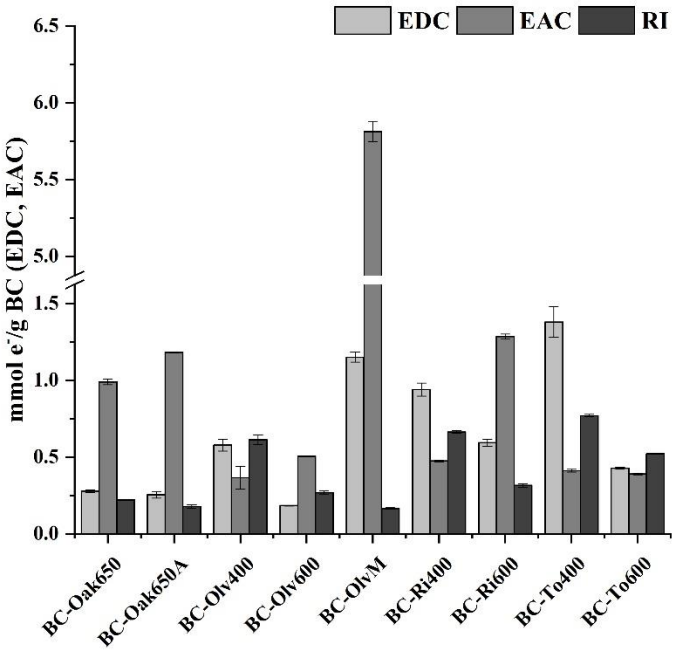


Figure 5.2. Electron donating capacity (EDC), electron accepting capacity (EAC) (mmol e- g^{-1} BC) and reduction index (RI, i.e. EDC/EEC) measured in BC suspensions of 10 g per 100 mL.

5.4. Results and discussion

5.4.1. Biochar characterization

The quantification of BC redox properties is depicted in Figure 5.2. The EDC values were lower for BCs pyrolyzed at 600°C as compared to 400°C while the trend for the EAC was exactly the contrary. This is in agreement with previous studies (Zhang et al., 2018; Prévoteau et al., 2016; Klüpfel et al., 2014). Biochars generated at intermediate temperatures (300-400°C) preferentially function as electron donors due to the presence of surface functional groups, as their amorphous carbon structure limits electron transport (Sun et al., 2017; Klüpfel et al., 2014). In contrast, BCs with a high proportion of graphitic structures (high HTT BCs) donate fewer electrons due to a lower

content of hydroxyl groups induced by the dehydration of lignin-derived phenols and alcohols, coupled with the onset of aromatization while the temperature rises (Harvey et al., 2012).

The post-pyrolysis oxidation treatment applied to BC-Olv400 largely increased both the EDC and EAC of BC-OlvM by creating additional redox-active functional groups (Lima et al., 2017). This increment was especially substantial for the EAC, which was nearly 15 times (5.81 mmol of e^- g^{-1} BC) the one of BC-Olv400 (0.39 mmol of e^- g^{-1} BC). The slight increase in the EAC of BC-Oak650A in comparison to BC-Oak650 reflects the oxidation processes related to aging in soil. These processes affect BC surface and redox properties due to the formation of heterogeneous coatings (Hagemann et al., 2017; Archanjo et al., 2017; Wiedner et al., 2015; Cheng et al., 2006) by precipitation of organic molecules and inorganic mineral compounds (Prévoteau et al., 2016).

The reduction index (RI), varies widely for our BCs (Figure 5.2), with values ranging from 0.17 (the most oxidized BC, BC-OlvM) to 0.77 (the most reduced BC, BC-To400). Without taking into consideration other parameters, based on our hypothesis and on these RI values, those BCs that would promote the microbial reduction of N_2O concentration to a greater extent would be (from the strongest reducer to the least): BC-To400 > BC-Ri400 > BC-Olv400 > BC-To600 > BC-Ri600 > BC-Olv600 > BC-Oak650 > BC-Oak650A > BC-OlvM.

All BCs generally exhibited low surfaces areas (Figure 5.3), with values ranging from 1.16 to 20 m^2 g^{-1}, with the exception of BC-Oak650 (175.2 m^2 g^{-1}). A clear trend in relation to BCs pyrolysis temperature was not observed. After the bio-physico-chemical weathering in soil for four years, the BET surface area of BC-Oak650 decreased, significantly but was still the BC with the second highest BET value. A decrease of surface area of BC after aging has been previously documented and has been related to pore clogging by the formation of heterogeneous coatings consisting of different mineral compounds associated with organic compounds (Archanjo et al., 2017).

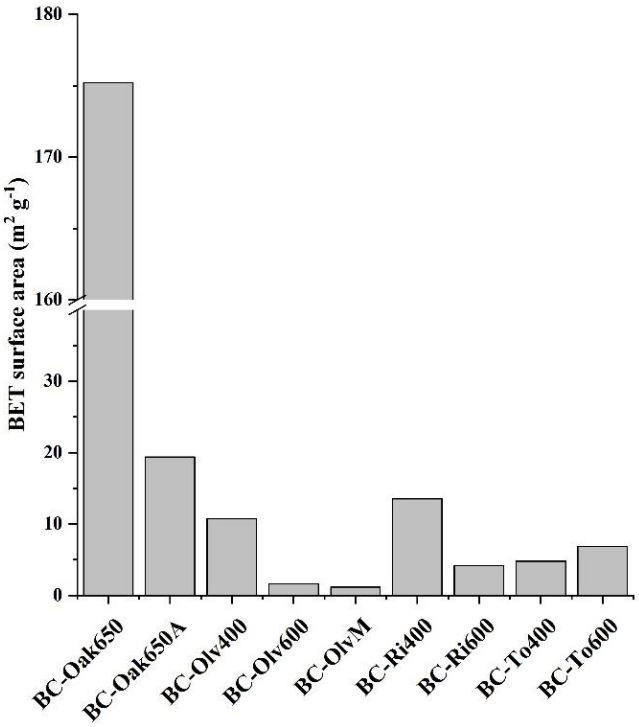


Figure 5.3. Brunauer−Emmett−Teller surface area (BET; $m^2\ g^{-1}$) of all BCs used at the incubations.

5.4.2. Microbial N_2O reduction

The evolution of N_2O in the biotic treatments over incubation time is shown in Figure 5.4. Its concentration decreased significantly for the majority of the BC-amended treatments. In some cases, N_2O was even completely removed (BC5_Ol400_B and BC5_To400_B) or diminished to a final concentration of 7.2% of the initial value (BC5_Ri400_B). Nevertheless, when the bacterium was in contact with N_2O without the presence of any BC (BC0_B, Figure 5.4) or with BC-OlvM, nearly no change in its concentration was detected. A very similar pattern was observed for abiotic samples (Figure S5.1), in which C/C_0 did not vary more than 20%. These results suggest that the BCs promote microbiological N_2O reduction (as part of the denitrification pathway).

Nonetheless, it was observed that especially for samples BC1_Oak_B, BC5_Oak_B, BC5_OakA_B or some abiotic setups (e.g. BC1_Ri400_A), there was a slight increase in the N_2O concentration.

Although no N source was been added to the sample flasks, these minor increases could have been attributed to N_2O production by *Paracoccus denitrificans* with either the remaining electron donor $NaNO_3$ used for bacterial growth or some nitrogen component in BC. The ability of *Paracoccus denitrificans* to generate N_2O is well known (Gaimster et al., 2018). However, under an anoxic atmosphere and NO_3^--limited conditions, its major denitrification product is N_2 (Felgate et al., 2012). Hence, as the increase of N_2O content in the flasks is not steady but fluctuating and the mentioned BC causing these increments are the ones with greater BET surface areas (Figure 5.3), we speculate that adsorption-desorption mechanisms might be responsible for this fluctuation in N_2O concentration. Although research studies focusing on BC potential to adsorb N_2O in solution were not found, some experiments have proved that preparations of anhydrous BC sorbs up to 2-16 mg N_2O per g BC (Cornelissen et al., 2013). In addition, under high levels of moisture (75%, water holding capacity, WHC), it was demonstrated that BC is able to absorb and transport CH_4 (Sadasivam and Reddy, 2015). The reason why samples BC1_OlvM_A and BC5_OlvM_A (Figure S5.1) produced a slight reduction of N_2O in spite of having a very low BET surface area (Figure 5.3), is currently unknown.

The change of BC concentration from 1 g L^{-1} to 5 g L^{-1} had a substantial impact for the N_2O reduction observed across the biotic incubations if the results of the first 45 hours of incubation are considered. During this period, most decreases in N_2O occurred in our incubations (Figure 5.4) and the major denitrification activity of *Paracoccus denitrificans* takes place (Baumann et al., 1996; Thomsen et al., 1994). Table S5.2 shows the slope and R^2 of the fitting lines that resulted from adjusting the decrease of N_2O during the period mentioned to a straight line. In addition, the relation of slopes at both BC concentrations is included (k5/k1). It is clearly noticeable that this decrease is more pronounced with 5 g L^{-1} BC than with 1 g L^{-1} BC. At the lowest BC concentration, only BC1_Ri600_B and BC1_To400_B slopes differed significantly from the rest of the treatments. Whereas, at the highest concentration (5 g L^{-1}), significant differences were observed between the

control and every BC pyrolyzed at 400°C as well as BC5_Ri600_B. The extent of the change (k5/k1) varied depending on the BC. The greatest shift was achieved by BC-Oak650 and BC-Ri400 (5.4 and 4.6 respectively). Only BC-Oak650A and BC-OlvM k5/k1 were close to zero, and these were precisely the BCs showing nearly no reduction of N_2O (Figure 5.4). Increasing effects following an increment in the dose of BC have been previously detected in other BC experiments (Weldon et al., 2019; Kappler et al., 2014).

To ascertain if a relationship existed between the evolution of the N_2O concentration caused by the BC and their redox potential, the BC's reduction index (RI) was plotted against the extent of N_2O reduction (% of $[N_2O]_0$-$[N_2O]_{162}$ for the biotic samples with 5 g L^{-1} BC), which results are shown in Figure 5.5. All BCs pyrolyzed at 600°C showed less than 45% removal of N_2O and no more than a 50% change of RI, whereas the BCs pyrolyzed at 400°C showed higher values of both (>65%). The only exception to this rule was BC-Ri600, which showed one of the highest N_2O reduction extents (100%) for its modest RI (31.6%). The Hierarchical Cluster Analysis applied confirmed the two groups separated in Figure 5.5 are statistically different (ANOVA F value=34.676; Prob>F=3.822 10^{-6}): one composed by all BCs pyrolyzed at 600-650°C together with BC-OlvM and the other by BCs produced at 400°C plus BC-Ri600.

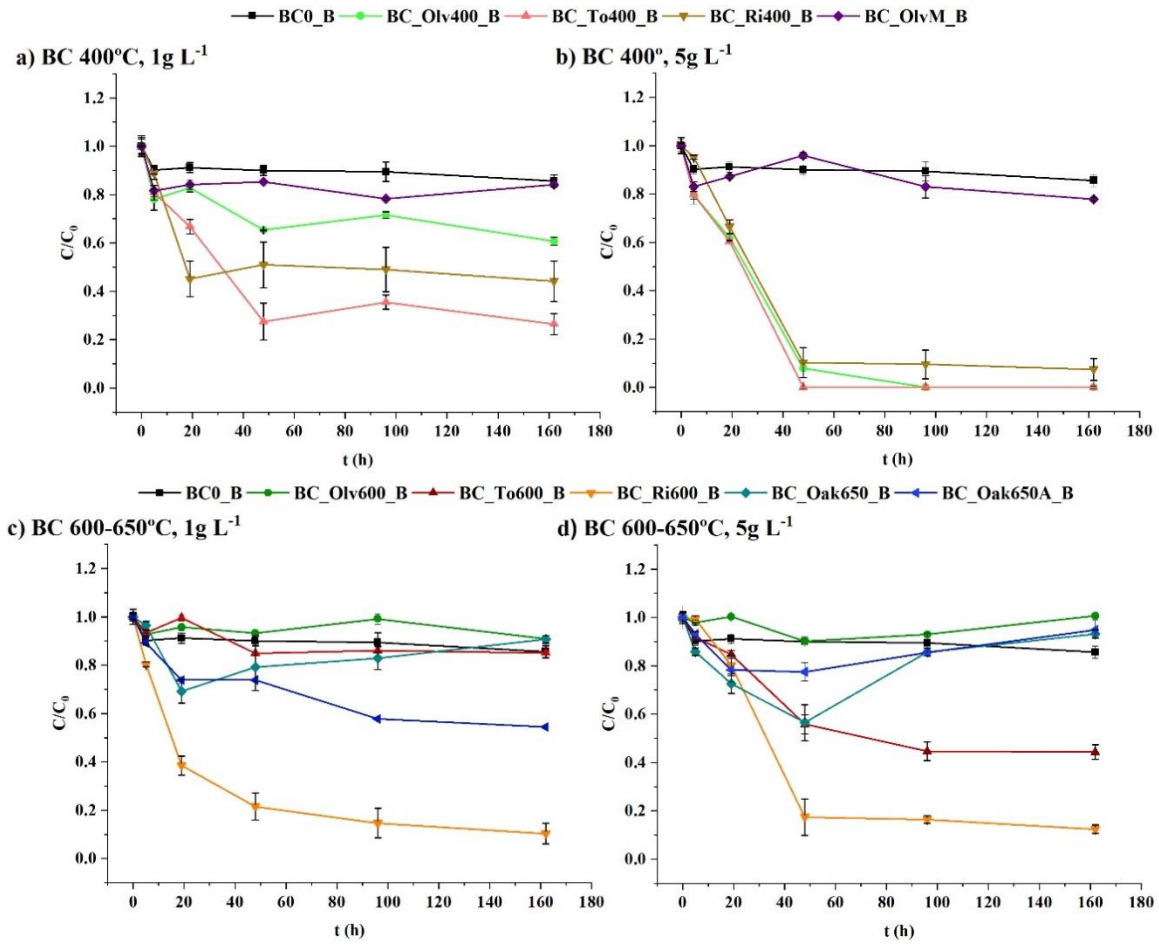


Figure 5.4. Change in relative N_2O concentrations shown as C/C_0, where C refers to the N_2O concentration (mg N in N_2O) per bottle at a specific time point and C_0 refers to the N_2O concentration at time 0. All samples contained *Paracoccus denitrificans* (biotic samples). Setups a) to d) vary regarding BC concentration and temperature of BC pyrolysis.

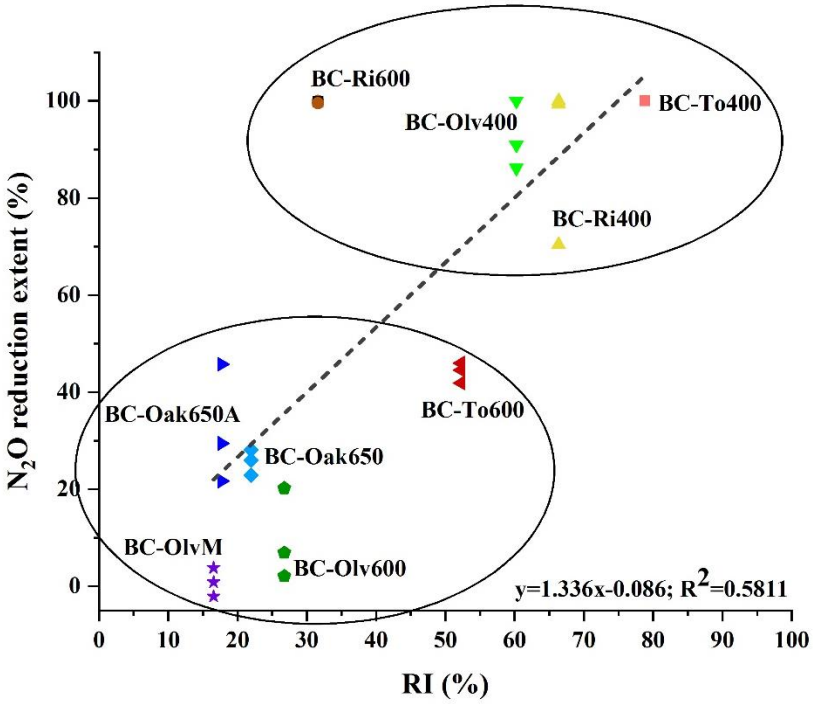


Figure 5.5. Correlation between the microbial N_2O reduction (% of initial N_2O reduced after 162 h) versus BCs RI. The values of the extent of N_2O reduction were calculated for biotic setups with a BC concentration of 5 g L^{-1}. The data was adjusted to a line which equation and R^2 appears at the bottom right corner of the graph (Pearson's r=0.7623, ANOVA F value=34.676, Prob>F=3.822 10^{-6}). The two groups marked with circles are the result of the application of the Hierarchical cluster analysis.

Chen et al. (2108) studied in detail which redox-active compounds of BC contributed to N_2O reduction in an experiment with denitrifying bacteria. They concluded that BC enhances denitrification through different mechanisms depending on its temperature of production. Biochar produced at low temperatures will enhance the process through its role as electron donor, and BC created at high temperatures through its ability to accept electrons together with its electrical conductive structure. This is in agreement with what we obtained in Figure 5.5;the mechanism through which the tested BC promoted a greater extent of N_2O reduction (all the ones pyrolyzed at 400˚C) would mainly be thanks to their potential to donate electrons (specifically their RI), which confirms our first hypothesis. However, the non-perfect adjustment of the fitting line depicted in

Figure 5.5 (R^2=0.5811), together with the fact that samples with BC-Ri600 produced a high N_2O reduction with no connection to its RI, suggest that other factors exist aside from the BCs' redox properties that could be involved and that actively determine the outcome of the incubation.

This hypothesis is supported by the position of the chemically-modified BC (BC-OlvM) at the bottom left of the graph in Figure 5.5. It seems to demonstrate that an extraordinary enhancement on both EAC and EDC is counter-productive if it results in a low RI. Moreover, it might show that having an ordered and condensed aromatic structure is necessary. The harsh chemical oxidation procedure applied to BC-OlvM (which was initially also produced at 400°C) consumed reducing equivalents (electrons) from its structure (Lima et al., 2017). Therefore, this ability of BC to donate electrons was decreased by a near-complete destruction of its prevailing aromatic ring structures and chemical functional groups (Chacón et al., 2017). Furthermore, BC-OlvM had a very high concentration of Mn (88157 ppm, Table A2) that could potentially be toxic to the bacteria (Kaur et al. 2017). Consequently, the basis of our second hypothesis 'the oxidation of BC will decrease its potential to donate electrons and support N_2O reduction by *Paracoccus denitrificans'* has been demonstrated, although the reasons are much more intricate, as explained above.

Biochar BC-Oak650 and its weathered variant BC-Oak650A showed a very similar behavior in the N_2O reduction assays leading to a modest microbial reduction of N_2O. This suggests that five years of field ageing did not decrease the ability of BC to stimulate the reduction of N_2O by *Paracoccus denitrificans*. At a concentration of 1 g L^{-1}, the BC-Oak650A even resulted in N_2O reduction that was four times higher as compared to the fresh BC, resulting in the rejection of part of our second hypothesis: soil ageing would decrease BC's ability to support microbial N_2O reduction. Nevertheless, our findings agree with the results by Hagemann et al. (2017). In their research, a BC placed in soil for three years was shown to have the same potential to reduce N_2O emission in comparison with its fresh version. They attributed this activity to a combination of processes that take place during BC ageing, including its breaking down into smaller particles, higher amounts of

oxidized functional groups on its surface, and a lower pH. We observed that during ageing of BC-Oak650A, its EAC slightly increased while its pH, BET surface area and C/N ratio decreased substantially in comparison with BC-Oak650 (Figure 5.2 and Table A2). Our study showed substantial differences between field-aged BCs and chemically-oxidized BCs; whereas field aging did not affect N_2O reduction significantly, chemical oxidation invalidated the effect of BC-Olv400. During soil weathering, BC undergoes a wide range of physical and chemical transformations that differ from those obtained by chemical treatments (such as our modified BC (BC-OlvM) or H_2O_2-treated BCs (Yuan et al., 2019). Therefore, caution needs to be taken when extrapolating the results from chemically-altered BCs to aged BCs. In summary, we have proved that BC soil weathering would not necessarily imply a decrease in its ability to reduce N_2O to N_2. A mix of factors could be involved, as BC-Oak650A and BC-Oak650 had nearly the same incubation outcome, having similar redox properties but substantially differing in other characteristics.

5.4.3. Change of biochars redox state and electron balance

In addition to changes in N_2O concentration during the incubations in the presence/absence of *Paracoccus denitrificans*, we also followed the changes of the BCs' EDC values. The measurements were made at the beginning of the experiment, before N_2O was injected into the headspace, and at the end of the incubation, after 162 h. Table S5.3 summarizes these results for samples with BC 5 g L^{-1} under biotic and abiotic conditions. In addition, it shows the theoretical amount of e^- required to explain the observed decrease in N_2O concentration during that period (0-162 h). Unfortunately, the EDC values did not show any significant differences among treatments due to the variability in the replicates. In addition, the electron balance for the biotic samples showed that only BC-Olv600, BC-To400, BC-Oak650 and BC-Oak650A provided enough electrons for the reduction of N_2O undergone in their samples. As shown previously (Figure 5.5), the RI (EDC/EEC) of the BC is directly correlated to N_2O reduction by *Paracoccus denitrificans*, but not the EDC of the BC. Hence, not having measured the changes in EAC throughout the

incubation period may be one of the reasons why we cannot see a correct balance of electrons. The influence of other BC characteristics, which may be affecting this outcome, needs to be considered as well. In addition, we do not discard *Paracoccus denitrificans*' stored C as being an extra source of electrons, a process that has been proven to occur for other bacteria (Jiang and Kappler, 2008). This variability on the connection between the mmol e^- needed for N_2O reduction and the ones missing in the BCs is showcased by the representation of Table S5.3 with replicates (n=3) in Figure 5.6. Nevertheless, what this figure demonstrates is what it was also observed in Figure S5.1: the underlying process driving N_2O reduction was mostly biotic.

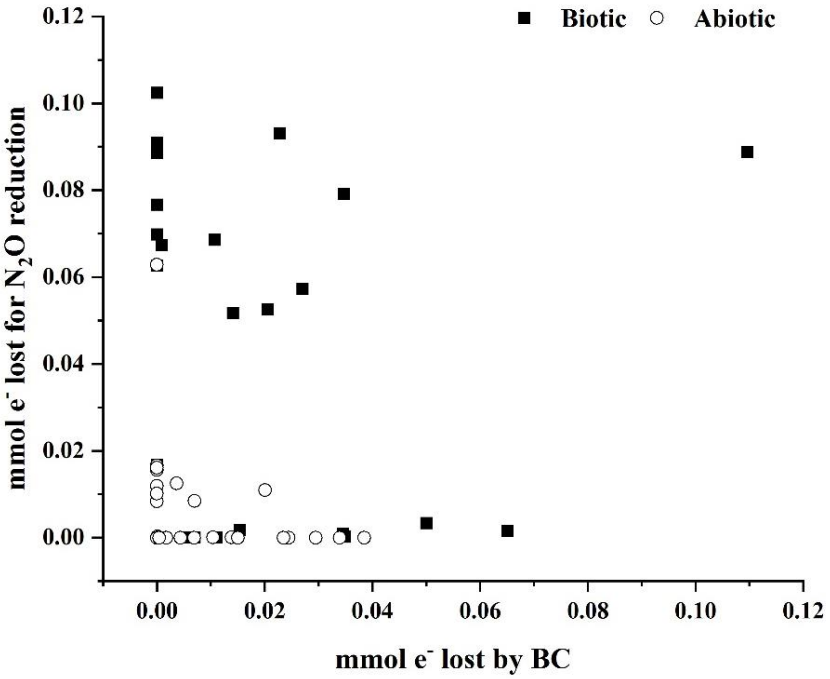


Figure 5.6. Amount of electrons (mmol e^- $flask^{-1}$) needed for the decrease in the N_2O concentration throughout the incubation period (162h) versus the electrons lost by BC. Filled black squares represent the biotic samples and the open circles the abiotic ones, both including every BC treatment (5 g L^{-1}) sample with their triplicates.

Support for microbiological N_2O reduction in connection with BC also comes from microscopic analyses. Microscopy images taken at the end of the incubations (Figure S5.2) showed that most bacterial cells were associated with BC particles. Although further conclusive analyses or

techniques would be needed, these observations do not rule out the possibility that BC indeed serves as an electron donor for N_2O reduction by *Paracoccus denitrificans*. This process of electron donation by BC for N_2O reduction does not require the formation of conductive structures such as pili. Instead, it seems sufficient for the cells to be tightly attached to the BC (Chen et al., 2014; Yu et al., 2016; Baek et al., 2018; Zhang et al., 2018).

5.4.4. Correlation of biochar properties with N_2O reduction by *Paracoccus denitrificans*

To further investigate the potential role of BC redox properties and to elucidate which others could significantly influence N_2O reduction, a Principal Component Regression (PCR) was carried out for the samples containing 5 g L^{-1} BC. From the wide range of BC's physical and chemical characteristics that were subjected to the PCR, the ten included in Table 5.1 were selected for being the most relevant. The reduction of variables applied separated them into three components (Bartlett's Sphericity test significance p-value= $p<0.05$). The linear regression results showed component 1 and 3 as being relevant to a strong extent of N_2O reduction with a combined value of 56.0% (21.5 and 34.5%. respectively). Therefore, it is confirmed that the redox properties of BC (EAC, EDC and RI) have a substantial influence over *Paracoccus denitrificans* N_2O reduction. High values of EDC (coefficient of +0.895) and RI (+0.729) together with low values of EAC (-0.937) favor a strong extent of N_2O reduction. In addition, a low proportion of lignin over cellulose (lig/cell) on the feedstock, a lower BET surface area and a high H/C and ash content affected the reduction of N_2O by *Paracoccus denitrifcans*.

The H/C ratio is regarded as an indicator of the BCs' aromatic index, i.e. aromatic structures that have been found to increase BC's redox activity (Yuan et al,. 2017; Klüpfel et al., 2014). High values of H/C are generally found in BCs produced at low temperatures (Sánchez-García et al., 2019; Suliman et al., 2016). Moreover, the proportion of lignin and cellulose on the feedstock determine, to a great extent, the amount and nature of electron-accepting and donating moieties

found on BC (Prévoteau et al., 2016). The PCR outcome suggests a low-lignin biomass, thus no woody materials, would be more appropriate for BC feedstock in order to assist *Paracoccus denitrificans*' N_2O reduction. The BC surface area is an indicator of its capacity to absorb molecules as gases (Lehmann and Joseph, 2009) and is inversely related with its ash content (Ronsse et al., 2013). Both their appearance with negative and positive coefficients, respectively, in Table 5.1, suggest that the abiotic process of N_2O absorption is not a major mechanism under our experimental conditions.

Therefore, without taking into consideration the redox properties, this statistical analysis points out that straw and crop residues (i.e. BC-Ri and BC-To) pyrolyzed at low temperatures (400°C) had the greatest number of the characteristics needed to achieve a greater and more efficient N_2O reduction by *Paracoccus denitrificans* than BCs produced from wood pruning (BC-Olv and BC-Oak). However, comparing BCs is very intricate, due to the high variability of their physical and chemical characteristics, and the complexity increases when linking these properties to their production feedstock and effects, suggested to be independent (Ronsse et al., 2013) and antagonistic (Weldon et al., 2019), respectively.

Table 5.1. Principal Component Regression (PCR) matrix and outcome. The 3-component rotation matrix is shown into which the BC characteristics were ordered. The coefficients for each of them were sorted according to value and the ones <0.52 were deleted. The result of the contribution of the components to the observed extent of N_2O reduction (in % for the biotic samples with BC 5 g L^{-1}) analyzed by a linear regression, were included on the bottom of the table (R^2, ANOVA F and Sig. ANOVA).

	Component		
	1	2	3
EAC	-0.937		
Lig/cell	-0.918		
BET	-0.801		
Ash	0.529		
Fe		0.962	
C/N		-0.931	
EC		0.780	
EDC			0.895
H/C			0.885
RI			0.729
Variance explained (%)	45.7	26.5	16.0
R^2	0.215	-	0.345
ANOVA F	18.45	-	15.82
Sig. ANOVA	0.000	-	0.000

EAC: electron accepting capacity; Lig/cell: proportion of lignin and cellulose in the feedstock; BET: Brunauer−Emmett−Teller surface area; EC: electric conductivity; EDC: electron donor capacity; RI: reduction index; ANOVA F: value of F; Sig. ANOVA: F statistical significance.

5.5. Conclusions

Our results demonstrate that BC aids *Paracoccus denitrificans*, facilitating the reduction of N_2O to N_2, mainly by means of its reduction state. Nevertheless, the extent of this reduction is significantly affected by other BC physical and chemical properties, in particular those ruled by their feedstock and pyrolysis temperature. For this specific BCs series, we found that BCs produced at a low temperature (400 ºC) and coming from non-woody biomass (high H/C and ash percentage together with low surface area and poor lignin feedstock content) promoted larger extent of N_2O reduction in the presence of *Paracoccus denitrificans*. In addition, we have proved the biotic character of the process, as no N_2O reduction was achieved in the absence of the bacterium. This study provides information for generating on demand and tailor-made BCs with the specific mentioned goal in mind. However, research in this field is still at an early stage, and further research is needed. More specifically, isotopic experiments and molecular biology analysis of DNA and RNA focused on the expression of the functional genes encoding the reduction of N_2O to N_2, would be important. Additionally, incubation experiments including an electron donor/acceptor aside from BC would be essential to prove the role and extent of the latter to support N_2O reduction in laboratory set-ups that closely mimic natural soil conditions.

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Supplementary Data

Table S5.1. Composition of the culture medium for *Paracoccus denitrificans* ATCC 19367.

Chemical	Concentration	Chemical	Concentration
KH_2PO_4	0.6 g L^{-1}	SL-10 solution (Widdel 1983)	
NH_4Cl	0.3 g L^{-1}	$FeCl_2 \cdot 4\ H_2O$	1500 mg L^{-1}
$MgSO_4 \cdot 7\ H_2O$	0.5 g L^{-1}	$ZnCl_2$	70 mg L^{-1}
$CaCl_2 \cdot 2\ H_2O$	0.1 g L^{-1}	$MnCl_2 \cdot 4\ H_2O$	100 mg L^{-1}
$NaHCO_3$	1.85 g L^{-1}	$CoCl_2 \cdot 6\ H_2O$	190 mg L^{-1}
Selenite-Tungstate solution		$NiCl_2 \cdot 6\ H_2O$	24 mg L^{-1}
NaOH	400 mg L^{-1}	$Na_2MoO_4 \cdot 2\ H_2O$	36 mg L^{-1}
$Na_2SeO_3 \cdot 5\ H_2O$	6 mg L^{-1}	H_3BO_3	6 mg L^{-1}
$Na_2WO_4 \cdot 2\ H_2O$	8 mg L^{-1}	HCl (25%)	10 mL $/L^{-1}$
Vitamins: B_{12}	100 mg L^{-1}	$CuCl_2 \cdot 2\ H_2O$	2 mg L^{-1}

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Table S5.2. Slope (k) and R^2 resulting of adjusting to a line the reduction of N_2O concentration for the first 45 hours of incubation. The relation between the slopes when the BC was added in a concentration of 5 g L^{-1} to 1 g L^{-1} is also included (k5/k1). Each value appears with its standard deviation (SD). In addition, the slopes were analyzed with a one-way ANOVA Tukey's post hoc test ($P < 0.05$) for comparing each BC treatment with the control, BC0, separately for 1 g L^{-1} and 5 g $L^{-}1$. Different letters imply significant differences between treatments (n=3).

	k		R^2			k		R^2		k5/k1
	Average	SD	Average	SD		Average	SD	Average	SD	
BC0_B	-0.0030 b	0.0057	0.823	0.0793	**BC0_B**	-0.0030 d	0.0057	0.823	0.0793	-
BC1_Olv400_B	-0.0054 b	0.0024	0.575	0.2585	**BC5_Olv400_B**	-0.0183 ab	0.0017	0.963	0.0267	3.4
BC1_Olv600_B	-0.0008 b	0.0016	0.435	0.1121	**BC5_Olv600_B**	-0.0010 d	0.0011	0.402	0.0984	1.3
BC1_To400_B	-0.0140 a	0.0057	0.890	0.1793	**BC5_To400_B**	-0.0200 ab	0.0013	0.973	0.0175	1.4
BC1_To600_B	-0.0026 b	0.0006	0.455	0.1663	**BC5_To600_B**	-0.0088 cd	0.0006	0.971	0.3180	3.4
BC1_Ri400_B	-0.0042 b	0.0022	0.646	0.2997	**BC5_Ri400_B**	-0.0192 bc	0.0046	0.986	0.0041	4.6
BC1_Ri600_B	-0.0157 a	0.0049	0.739	0.1582	**BC5_Ri600_B**	-0.0217 a	0.0011	0.941	0.0176	1.4
BC1_Oak650_B	-0.0043 b	0.0043	0.835	0.1846	**BC5_Oak650_B**	-0.0231 cd	0.0337	0.479	0.3558	5.4
BC1_Oak650A_B	-0.0048 b	0.0003	0.663	0.0649	**BC5_Oak650A_B**	-0.0007 d	0.0033	0.661	0.1121	0.1
BC1_OlvM_B	-0.0015 b	0.0010	0.575	0.2585	**BC5_OlvM_B**	0.0006 d	0.0019	0.963	0.0267	-0.4

Table S5.3. Electron balance (mmol e^- $flask^{-1}$) for each BC treatment at 5 g L^{-1}. The columns labeled 'N_2O' refer to the mmol e^- needed for the reduction of N_2O observed at the incubation experiments (Figure 5.4) and the columns with 'EDC BC' to the mmol e^- each BC lost (measured over the incubation samples). Both variables were calculated subtracting their value at t=0h to the one at t=162h (beginning and end of the incubation). Different letters imply significant differences between treatments (n=3) according to one-way ANOVA Tukey's post hoc test ($P < 0.05$).

	Biotic samples		**Abiotic samples**	
mmol e-	N_2O	EDC BC	N_2O	EDC BC
BC5-Olv400	0.093 a	0.002 a	0.012 a	0.000 a
BC5-Olv600	0.002 d	0.050 a	0.010 a	0.000 a
BC5-OlvM	0.017 d	0.000 a	0.016 a	0.000 a
BC5-To400	0.087 ab	0.152 a	0.000 a	0.000 a
BC5-To600	0.054 c	0.021 a	0.010 a	0.007 a
BC5-Oak650	0.001 d	0.020 a	0.000 a	0.022 a
BC5-Oak650A	0.000 d	0.006 a	0.000 a	0.021 a
BC5-Ri400	0.080 ab	0.023 a	0.000 a	0.000 a
BC5-Ri600	0.069 bc	0.000 a	0.000 a	0.009 a

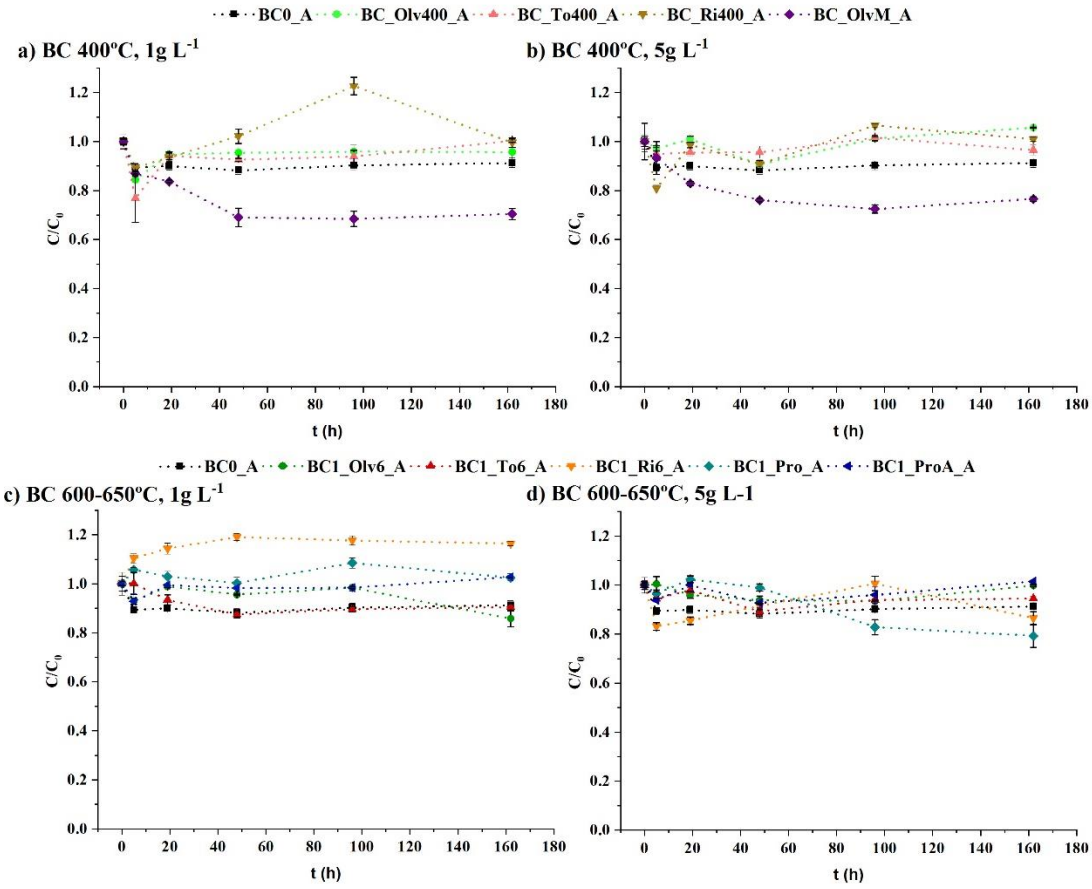

Figure S5.1. Concentration of N_2O over time shown as change in the ratio C/C_0 where C refers to the N_2O concentration (mg N in N_2O) per bottle through the incubation and C_0 to the N_2O concentration at time 0. All samples included are abiotic. Setups a) to d) vary regarding BC concentration (1 or 5 g L^{-1}) and temperature of pyrolysis (400 or 600˚C).

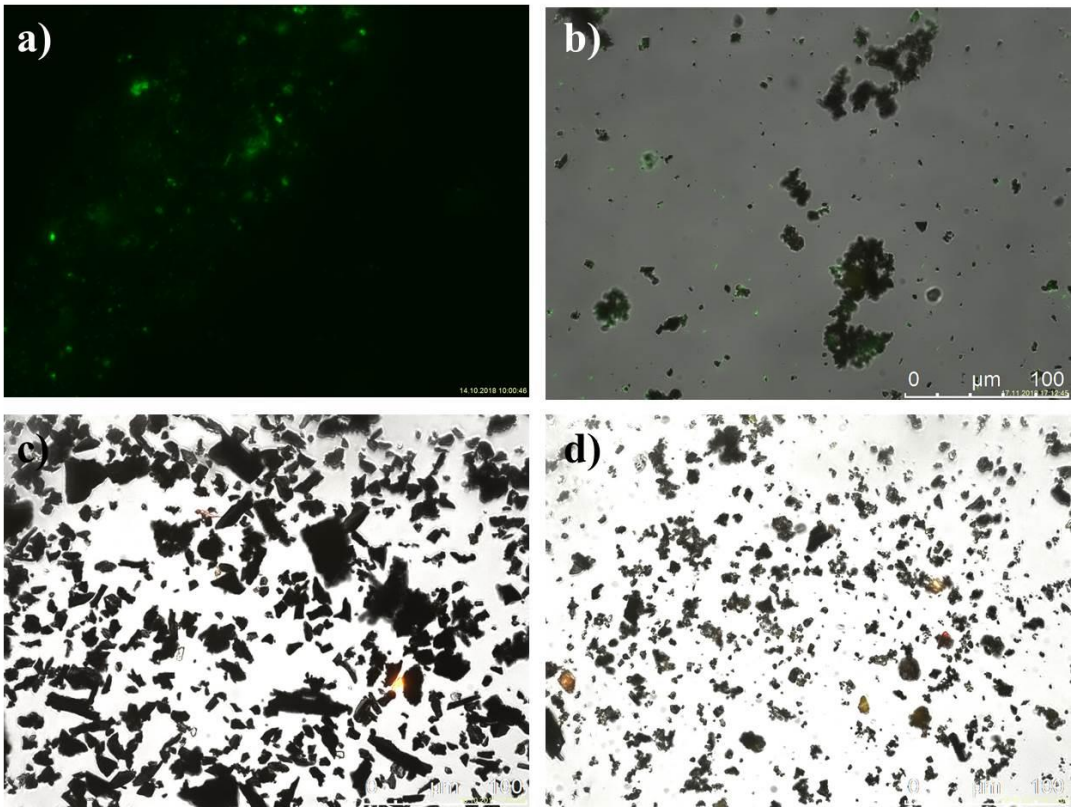

Figure S5.2. Association of *Paracoccus denitrificans* cells with BC at the end of the incubation (160 hours). In photos a) and b), fluorescence microscopy is used to show the living (green) cells associated with (black) BC-To400 particles. The size and shape of BC particles of BC-Olv400 are shown in panel c) and the ones of BC-To400 in panel d).

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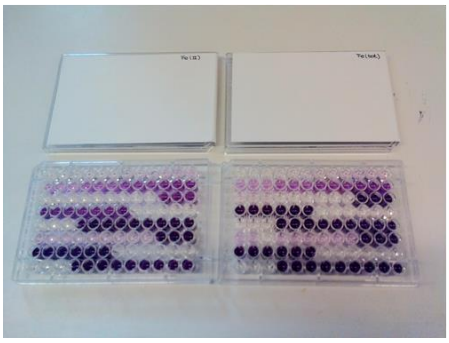
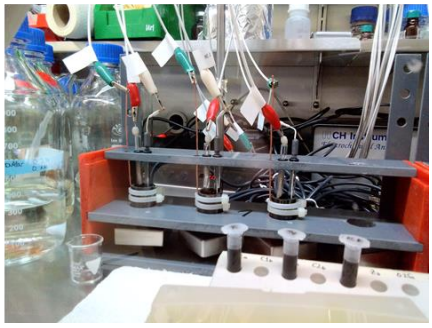
Chapter 6

General Discussion





In the past few decades, there has been a growing concern about climate change and its related future alarming scenarios, such as the increase in the global average temperature, the acceleration in the rise of the sea level, or the increase in extreme climatic events, with most of them related to the rise in GHG emissions due to human-related activities. Although some governments have made valuable efforts, there is still a need to strengthen the global ambition to tackle climate change, especially by the AFOLU sector, which represents a unique challenge. After years of research, BC addition to soil is currently considered as a promising option for soil GHG mitigation (IPCC, 2018, 2019). Nevertheless, its potential use is still up to debate. The general objective of this thesis was to cast a light over the benefits of BC as a soil amendment, specifically as a tool to reduce soil CH_4 and N_2O emissions. The primary goal was to identify the characteristics that BC should possess to achieve the most efficient mitigation rates. In addition, a secondary objective was to obtain a deeper understanding on the mechanisms involved in such mitigation. With these purposes, a series of laboratory experiments were carried out, where a number of BCs with contrasting physicochemical properties were tested. In Chapter 3 and 4, soil incubation experiments of two agricultural soils amended with different BCs were set up to study their influence on CH_4 oxidation potential and N_2O emissions. In Chapter 5, the complexity of the soil matrix was eliminated, and the interaction of BC and denitrifying bacteria was examined in culture media.

6.1. Biochar impact on soil CH_4 oxidation

As several meta-analyses and reviews have pointed out (Cong et al., 2018; Ji et al., 2018; Kammann et al., 2017; Jeffery et al., 2016; Song et al., 2016), the effect of BC on soil CH_4 emissions is still not well understood. Specifically, only a few studies have focused on the ability of BC to modify soil CH_4 oxidation in aerated upland soils, i.e. under environmental conditions favoring methanotrophy. Moreover, the scant available literature has reported contradictory results, which were strongly dependent on a wide variety of factors (e.g. type of soil, BC, environmental conditions). Considering the previous findings and the lack of thorough research, the first

hypothesis of the present dissertation is outlined: BCs differing in both their origin and production conditions would differently impact CH_4 oxidation potential in an aerated soil. The extent of the effect will be a function of the BC's physicochemical properties.

Firstly, the work described in Chapter 3 demonstrated that the observed changes in soil CH_4 oxidation were mediated by microorganisms and not due to abiotic processes such as adsorption, as suggested by other researchers (Sadadivam and Reddy, 2015). Secondly, the results also proved that most BCs had a moderate impact on soil CH_4 oxidation rates. Previous studies found larger changes under similar conditions (Ji et al., 2018; van Zwieten et al., 2015), whereas others showed similar outcomes (Jeffery et al., 2016; Song et al., 2016). However, in this study, important differences in the rate of CH_4 oxidation were reported across the BCs tested, which were strongly connected with some of their properties. Although the essential role of BCs characteristics on soil CH_4 oxidation had been suggested in previous studies, most of them were limited by the low number of BCs tested.

Biochar feedstock and temperature of production were found to be two variables that were directly connected with its ability to improve/hinder soil CH_4 oxidation. It was observed that the BCs that promoted soil CH_4 oxidation were those produced from woody biomasses at high temperatures (600˚C). This result supports the findings from Jeffery et al. (2016), who found that BCs pyrolyzed at a high temperature favored the CH_4 sink capacity of soils. Specifically, two characteristics were highlighted in these BCs: their low O/C ratios (Spokas, 2010), and large surface areas (Sánchez-García et al., 2019; Downie et al., 2009). Additionally, it was observed that BCs with low EC and ash content were also described to promote soil CH_4 oxidation rates (Singh et al., 2017; Ronsse et al., 2013). The mechanism by which this type of BC might trigger CH_4 oxidation could be linked to improvements in soil aeration or gas diffusivity exchange capacity, which is in agreement with the conclusions from Fang et al. (2016). These changes would ultimately influence methanotroph

activity and population (Reddy et al., 2014; Wu et al., 2020), although this was not proved by our experiment.

Conversely, the BC's capacity to donate/accept electrons was discarded as being determinant in modifying soil CH_4 oxidation rates. No correlation was found between both variables, although two BCs with significantly improved capacities to donate electrons were tested. No previous research has been found that focused on the role of the BC's electrochemical properties on CH_4 soil oxidation dynamics. However, it has been proven that BC redox capacity can change and modulate other biogeochemical processes (e.g. Yang et al., 2020; Saquin et al., 2016; Quin et al., 2015; Joseph et al., 2015) and, most recently, methanogenesis (Wang et al., 2020) or anaerobic oxidation of CH_4 (Zhang et al., 2019). Therefore, the analysis of its possible involvement on the aerobic CH_4 oxidation to CO_2 was necessary and justified.

6.2. Biochar impact on soil N_2O production

The study of the impact of BC on soil N_2O production is complex when compared with other biogeochemical cycles. Apart from the considerable complexity and variety of BCs, numerous N_2O formation pathways in soil exist (nitrification, denitrification, nitrifier-denitrification, codenitrification, DNRA …). Recent research in the field has specified some properties of BCs as decisive for promoting/blocking the different pathways of soil N_2O formation (Weldon et al., 2019; Feng et al., 2018; Van Zwieten et al., 2014; Cayuela et al., 2013). The link between BC physicochemical properties and their impact on soil N_2O emissions and consumption was the main driver of Chapters 4 and 5.

The second objective of the present thesis, carried out in Chapter 4, focused on linking the BC's physicochemical characteristics to its capacity to interact with the soil N cycle under denitrification conditions. We hypothesized that the C/N ratio, concentration of PAHs, and redox functional

groups would be the key. Chapter 4 proved that BC is able to interact with soil, modifying its fluxes of denitrification N_2O emissions. The effect of BCs on N_2O emissions was directly related with the BCs' physicochemical properties. BCs could be separated into two groups. The first group, which gathered the majority of the BCs, showed a very limited effect on N_2O mitigation. Their addition to soil did not change the total cumulative N_2O emissions. Nevertheless, they were able to decrease N_2O emissions during the first stages (24-48 h). The second group of BCs promoted a significant increase in soil N_2O emissions, both in the short and long term, with total cumulative N_2O emissions several times higher than the unamended soil. One BC could not be included in any of the described groups. This BC decreased N_2O and CO_2 emissions as compared to the control and led to a sustained increase in NO_2^- concentration in soil.

To achieve the objective of the present chapter, a Principal Component Analysis (PCA) was performed to link the BCs' response in the incubations with their physical and chemical characteristics. The PCA revealed that the key BC properties were its C/N ratio, germination index, content in dissolved organic C (DOC), and the presence of oxygenated functional groups on its surface (carboxylic/carbonates). Specifically, BCs with a high C/N ratio and no phytoxicity (GI>60%) would decrease soil denitrification N_2O emissions, whereas BCs with high carboxylic/carbonates groups, low GI and DOC would promote soil N_2O production.

These findings are in contradiction with previous results from the literature, which mostly showed a significant decrease of N_2O emissions after BC soil amendment. The prime cause of this discrepancy may be attributed to the peculiarities of the soil used in the present study (with a high $CaCO_3$ concentration and pH, and very low DOC) (Weldon et al., 2019; Cayuela et al., 2013). Previous research has shown that one particular BC may be very effective in reducing N_2O emissions in one soil while increasing emissions in another soil under the same exact environmental conditions (Sanchez-García et al., 2014). This has been associated to different N reactions (e.g. ammonification, nitrification, denitrification) operating in different soils, which would be

differently affected by the BC. Further experiments would be needed to determine the reasons behind this observation. In addition, this study demonstrated that BC redox properties and the concentration of PAHs (naphthalene, anthracene, phenanthrene, fluoranthene and pyrene) were not related to N_2O emissions. These properties have often been associated to the BCs' mitigation potential (Wang et al., 2019; Harter et al., 2016; Cayuela et al., 2015; Wang et al., 2013), but this study refutes their relevance.

Apart from denitrification, other N_2O formation pathways were noticed to be occurring in the present study. Considering the experimental conditions, codenitrification and nitrifier-denitrification were the two most probable mechanisms taking place.

As shown in Chapter 4 and by previous experiments in the scientific literature (Weldon et al., 2019; Lan et al., 2019; Sánchez-García et al., 2014), BC is able to induce changes in soil denitrification. Numerous theories have been suggested to explain this phenomenon, including the ability of BC to stimulate the last step of denitrification, i.e. the reduction of N_2O to N_2, through different mechanisms (Harter et al., 2016; Fungo et al., 2019; Cayuela et al., 2013). Recently, the redox active components of BC have been suggested to play a key role in denitrification (Yuan et al., 2019; Chen et al., 2018). To further improve the knowledge on how BC characteristics, and in particular its electrochemical properties, influence denitrification, the third study of this thesis was performed. The hypothesis was that the rate of reduction of N_2O to N_2 by a denitrifier bacterium (*Paracoccus denitrificans*) would be promoted by BC, and that the degree of N_2O reduction would be linked to BC redox properties. To broaden the research, two BCs with improved redox capacities (oxidized chemically at the laboratory and naturally aged in the field) were subjected to the experiments.

Chapter 5 demonstrated that under anoxic conditions, and in the absence of any other electron donor/acceptor, BC was able to support *Paracoccus denitrificans* reduction of N_2O to N_2.The study

proved that the main mechanism utilized for the reduction of N_2O was biotic and dependent on the presence of both the bacteria and the BC.

Biochar promotion of N_2O reduction was linked with its capacity to donate electrons and its overall electron exchange capacity (i.e. reduction Index, RI=EDC/EEC). Previous works have observed that electrochemical properties of BC are actively involved in N_2O reduction to N_2 (Yuan et al., 2019; Chen et al., 2018). However, previous studies did not focus on a particular bacterium. One of the primary conclusions was that low-HTT (400˚C) BCs significantly promoted N_2O reduction by *Paracoccus denitrificans*. Additionally, it was demonstrated that this effect was directly related with BCs redox properties: greater N_2O reductions were achieved by BCs with a high RI, generally found in BCs pyrolyzed at low temperatures. This is in agreement with the theory from Chen et al., (2018), who stated that these BCs (low-HTT) are able to act as electron donors thanks to their large content in reduced phenolic moieties. Nevertheless, there were some exceptions to this general pattern. Ultimately, it was found that the explanation was that other BCs characteristics were also significantly involved.

Apart from the BCs' electrochemical properties, their H/C ratio, surface area, ash content and their feedstock portion of lignin and cellulose, also contributed to the rate of N_2O reduced by the bacterium. Particularly, in the presence of BCs with low rates of carbonization, high specific surface area and low-lignin starting materials (non-woody), the extent of the reduction was higher. These results agree with Zhang et al. (2018), who also observed a link between BC surface and redox properties and the increasing activity of two different *Geobacter* co-cultures. Similarly, Saquing et al. (2016) reported that a woody BC efficiently enhanced nitrate reduction by the bacterium *Geobacter metallireducens* thanks to the BCs' electron donor capacity. To date, most of the available literature is based on experiments and meta-analyses covering the different steps of denitrification, not distinguishing between N_2O formed and consumed in the soil or even covering

N_2O emissions from different N_2O production pathways, where an enormous variety of factors are involved.

The natural weathering or oxidation that BC undergoes when applied to the field, did not affect its potential to support the reduction of N_2O by *Paracoccus denitrificans*. These results support previous findings in the literature (He et al., 2019), which observed that field-aged BCs did not lose their capacity to reduce N_2O to N_2. Despite the transformations undergone by BC aging in soil (Hagemann et al., 2017) weathered and fresh BCs have similar RI values, and both BCs produce similar effects when promoting N_2O reduction. This result backs the importance of BC's RI over other BC properties regarding the last step of denitrification.

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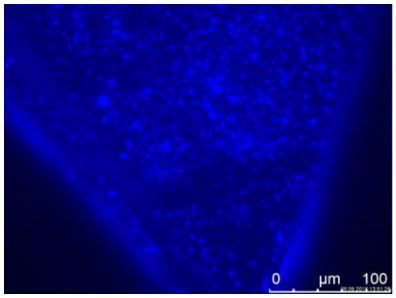
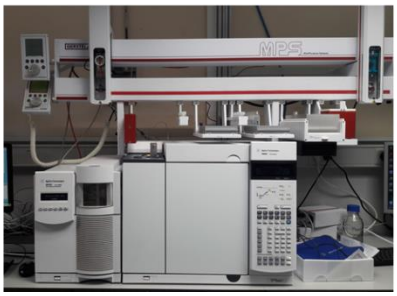
Chapter 7

Conclusions





The conclusions of this thesis can be summarized as follows:

1. In agricultural soils under optimum conditions for metanotrophy, biochar is able to modify soil CH_4 oxidation potential, by either increasing or decreasing their CH4 oxidation rates.

2. The biochar's impact on soil CH_4 oxidation rate is related to the biochar's physicochemical properties.

3. Biochars achieving the highest rates of CH_4 oxidation are those produced from woody feedstocks at high temperatures, and are characterized by a low ash content, moderate electrical conductivity, and high total pore area.

4. Biochar is capable of changing soil N_2O emissions under optimum conditions for denitrification in a calcareous soil. Most of the biochars did not have a significant impact on the cumulative N_2O emission, whereas two of the studied biochars caused a substantial increase in soil N_2O emissions.

5. Biochars characterised by a low C/N ratio, high dissolved organic carbon concentration, and high toxicity, drove the highest soil N_2O emissions. Biochar redox properties, atomic H/C ratio, and concentration of PAHs were found not to be relevant for N_2O emissions.

6. The isotopic analysis of the N_2O demonstrated that denitrification was the predominant N_2O formation pathway. However, other processes were found to also significantly contribute to total N_2O emissions, such as nitrifier-denitrification, and codenitrification.

7. Biochar promotes *Paracoccus denitrificans* microbiological N_2O reduction to N_2 under strict anoxic conditions. A strong and direct correlation was found between the rates of N_2O

reduction by the bacterium and the reduction index (RI) of the biochar, defined as the "electron donating capacity/electron exchange capacity" ratio. Apart from the redox properties of biochar, a combination of other properties also affected N_2O reduction by *Paracoccus denitrificans*. Greater reductions rates of N_2O are achieved with biochars produced at low temperatures, coming from non-woody feedstock, and characterized by high H/C, ash percentage, and low surface area.

Appendix. Biochar production and main characteristics

A wide range of biochars was employed in this thesis. Most of them were produced at the CEBAS-CSIC (Murcia, SPAIN) facilities by using a rotatory tube furnace (RSR-B 80/500/11; Nabertherm, Germany) equipped with a quartz tube (80 mm × 500 mm) (Figure A1). Argon was used as the inert gas to purge O_2 and to maintain the anoxic conditions during the whole pyrolysis process. The flow of Ar was kept between 150 and 50 L h^{-1} depending on the temperature and phase of the process. After air-drying and milling the feedstock to a particle size lower than 6 mm, a volume of approximately 0.6 dm^3 was introduced in the quartz tube. Each of the feedstock selected was pyrolyzed at two temperatures, 400 and 600˚C. The process lasted 4 hours and comprised five phases, which are depicted in Figure A2.

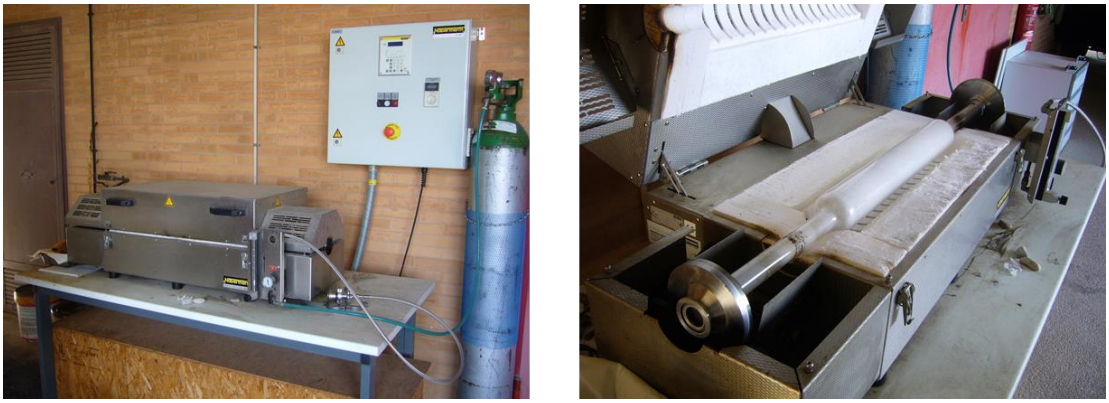


Figure A1. Pyrolyzer RSR-B 80/500/11, Nabertherm (Germany) used for the production of biochar at CEBAS-CSIC.

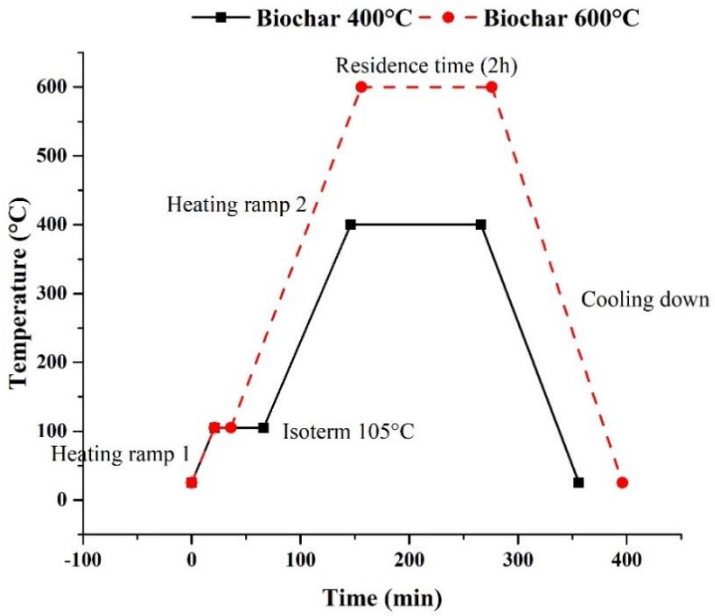


Figure A2. Description of the temperature ramps used for the pyrolysis process for biochars produced at 400˚C (black solid line) and 600˚C (red dashed line). The starting and endpoint was ambient temperature (25˚C). Atmospheric conditions determined the cooling down period that varied between 60 and 120 min.

The biochars were stored until their use in glass bottles tightly sealed using screw cups fitted with rubber septa. A cycle of vacuum and helium (He) flushes was applied to ensure an inert atmosphere in the glass bottles. Table A1 gathers general information of all biochars including a description of the feedstock biomasses and their origin, temperature of pyrolysis and abbreviation used to identify them in the present document.

Table A1. Biochars employed throughout the thesis, with their feedstock origin and temperature (T) of pyrolysis (˚C).

Biochar	T of pyrolysis (˚C)	Feedstock	Origin
BC-Olv400	400	Olive tree	Olive tree pruning branches from a commercial organic farm (SAT Casa Pareja, Jumilla, Murcia, Spain)
BC-Olv600	600		
BC-KMnO4 (or BC-OlvM)*	400		
BC-O2*	400		
BC-To400	400	Tomato plant	Tomatoes plants stems, branches and fruits discarded after the greenhouse production campaign (Mazarrón, Murcia, Spain)
BC-To600	600		
BC-Ri400	400	Rice straw	Straw after harvesting in a commercial exploitation (Coop. Virgen de la Esperanza, Calasparra, Murcia, Spain).
BC-Ri600	600		
BC-Al400	400	Almond tree	Almond tree pruning branches from a farmers' cooperative (COATO, Totana, Murcia, Spain)
BC-Al600	600		
BC-GS400	400	Grape stalks	Wastes from the viticulture industry (Bodegas Luzón, Jumilla, Murcia, Spain)
BC-GS600	600		
BC-Oak650	650	Oak tree	Private company: PROININSO, S.A. Málaga, SPAIN
BC-Oak650A**	650		

*Chemically or physically modified biochar; **Aged biochar

Two modified biochars were produced applying post-pyrolysis treatments to BC-Olv400. These are BC-O2 and BC-KMnO4 (BC-OlvM), which detailed processes of synthesis are explained in chapter 3 section 3.3.2. Additionally a commercial biochar (BC-Oak650) was obtained from a private company (PROININSO). This biochar was generated by slow pyrolysis at 650 °C, at atmospheric pressure and with a residence time in the reactor chamber of 12-18 hours. BC-Oak650A represents the aged biochar of BC-Oak650 after being in the field for 5 years (Sánchez-García et al., 2016). Their main physical and chemical properties are include in Table A2.

Table A2. Biochars physical and chemical properties.

	BC-Olv400	BC-Olv600	BC-To400	BC-To600	BC-Ri400	BC-Ri600	BC-O2
Feedstock	**Olive oil tree**		**Tomato plants**		**Rice straw**		**Olive oil tree**
T pyrolysis (°C)	400	600	400	600	400	600	400
pH	9.90	11.05	9.65	12.10	9.73	10.21	8.53
EC (mS cm^{-1})	0.59	0.75	18.43	22.80	3.83	4.43	0.61
VM (%)	35.4	37.5	44.5	40.5	30.6	26.5	-
Ash (%)	4.9	4.8	34.4	38.2	36.6	41.9	10.9
C_{org} (%)	78.5	88.3	37.3	39.9	39.6	50.4	72.7
Fixed C (%)	59.7	57.7	21.0	21.3	32.8	31.6	-
DOC (mg g^{-1})	0.68	0.83	7.54	0.93	2.82	1.68	-
DIC (mg g^{-1})	0.13	0.04	0.40	0.06	0.31	0.72	-
TDC (mg g^{-1})	0.81	0.87	7.94	0.99	3.13	2.40	-
TDN (mg g^{-1})	0.01	0.02	0.36	0.10	0.03	0.07	-
N (%)	0.84	0.93	2.00	2.01	0.66	0.58	0.84
O (%)	13.5	8.08	19.1	14.9	12.8	8.18	12.3
H (%)	3.38	1.72	2.78	1.23	2.39	1.22	3.26
Atomic H/C_{org}	0.52	0.24	0.87	0.39	0.72	0.29	0.54
Atomic O/C_{org}	0.13	0.07	0.31	0.28	0.24	0.12	0.13
C_{org}/N	93.3	95.4	18.6	19.8	60.4	87.2	86.5
NO_3^- (mg kg^{-1})	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	-
NO_2^- (mg kg^{-1})	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	-
NH_4^+ (mg kg^{-1})	0.45	0.60	2.41	1.09	0.62	0.62	-
Ca (%)	1.80	2.60	8.28	9.45	0.79	0.92	11.01
Mg (%)	0.32	0.47	2.10	2.40	0.42	0.47	1.30
K (%)	0.69	0.76	5.48	6.30	4.13	4.63	8.54
P (%)	0.07	0.08	0.37	0.40	0.07	0.08	0.75
Fe (ppm)	212.3	307.7	668.0	938.5	150.8	201.0	0.11
Mn (ppm)	25.7	29.8	197.7	224.4	387.5	440.0	31.74
Cu (ppm)	3.8	4.8	135.2	173.4	2.2	3.3	6.49
Zn (ppm)	15.1	12.4	147.9	168.9	36.2	38.2	0.00
Pb (ppm)	0.40	0.36	15.3	17.6	0.45	1.26	0.00
EAC (mmol e^- g^{-1} BC)	0.05	0.06	0.05	0.10	0.09	0.08	0.09

EDC (mmol e^- g^{-1} BC)	0.19	0.10	0.32	0.15	0.31	0.08	0.22
EEC (mmol e^- g^{-1} BC)	0.24	0.16	0.37	0.25	0.40	0.16	0.31
EDC/EAC	3.8	1.7	6.4	1.5	3.4	1.0	2.44
Lig/Cel (feedstock)	0.46	0.46	0.30	0.30	0.42	0.42	0.46
TporeA (m^2 g^{-1})	50.4	93.1	11.9	12.6	23.3	21.9	50.4
Porosity (%)	59.0	60.3	68.8	72.2	76.4	75.3	-
BET (m^2 g^{-1})	10.7	1.6	4.8	6.8	13.5	4.1	-
Bulk density (g mL^{-1})	0.46	0.48	0.38	0.39	0.28	0.28	0.46
C-C/C-H*	53.1	59.7	57.7	77.6	53.1	53.2	-
C-O*	29.0	28.0	22.5	16.2	30.5	30.6	-
C=O/O-C-O*	12.1	8.9	9.8	2.9	13.3	13.5	-
Carboxylic/carbontate *	3.7	3.4	10.1	3.2	3.2	2.7	-

	BC-Al400	**BC-Al600**	**BC-GS400**	**BC-GS600**	**BC-Oak650**	**BC-Oak650A**	**BC-KMnO4 (BC-OlvM)**
Feedstock	**Almond tree**		**Grape stalks**		**Oak tree**		**Olive oil tree**
T pyrolysis (°C)	400	600	400	600	650	650	400
pH	9.31	12.29	10.3	10.7	9.4	7.1	8.0
EC (mS cm^{-1})	0.46	3.30	3.23	4.52	0.40	0.97	0.25
VM (%)	31.3	19.4	33.3	33.2	30.7	42.0	79.0
Ash (%)	10.4	7.9	12.6	14.4	13.0	12.0	16.0
C_{org} (%)	68.2	81.5	71.2	69.8	66.8	65.3	63.2
Fixed C (%)	58.3	72.6	54.1	52.4	-	-	-
DOC (mg g^{-1})	-	-	7.97	6.86	-	-	-
DIC (mg g^{-1})	-	-	2.96	4.01	-	-	-
TDC (mg g^{-1})	-	-	10.9	10.9	-	-	-
TDN (mg g^{-1})	-	-	0.11	0.08	-	-	-
N (%)	1.07	1.19	1.87	1.96	0.70	0.91	0.71
O (%)	22.2	17.1	12.30	8.41	31.1	32.2	16.7
H (%)	3.34	1.60	3.31	1.60	1.40	1.59	3.32
Atomic H/C_{org}	0.59	0.24	0.56	0.28	0.25	0.29	0.63
Atomic O/C_{org}	0.24	0.16	0.13	0.09	0.35	0.37	0.20
C_{org}/N	63.7	68.5	38.2	35.6	95.4	71.8	89.0
NO_3^- (mg kg^{-1})	-	-	<0.5	<0.5	-	-	-

NO_2^- (mg kg^{-1})	-	-	<0.1	<0.1	-	-	-
NH_4^+ (mg kg^{-1})	-	-	0.50	1.35	-	-	-
Ca (%)	2.69	3.25	1.52	2.02	0.38	5.10	15.13
Mg (%)	0.40	0.47	0.56	0.69	0.25	0.41	2.05
K (%)	0.99	1.18	5.14	6.44	0.67	0.25	4.38
P (%)	0.10	0.12	0.17	0.21	0.20	0.18	0.58
Fe (ppm)	211.8	199.2	357.0	450.8	542.0	739.5	0.18
Mn (ppm)	24.0	28.8	21.5	27.0	463.3	366.0	88157.74
Cu (ppm)	5.53	6.68	17.7	22.3	9.9	11.5	5.03
Zn (ppm)	14.1	20.7	18.3	22.3	11.9	12.1	15.3
Pb (ppm)	7.6	14.3	0.51	0.59	0.1	0.07	0.14
EAC (mmol e^- g^{-1} BC)	0.02	0.12	-	-	0.99	1.18	0.47
EDC (mmol e^- g^{-1} BC)	0.12	0.08	-	-	0.28	0.26	0.73
EEC (mmol e^- g^{-1} BC)	0.14	0.20	-	-	1.27	1.44	1.20
EDC/EAC	6.0	0.67	-	-	0.28	0.22	1.55
Lig/Cel (feedstock)	0.67	0.67	0.52	0.52	0.70	0.70	0.46
TporeA (m^2 g^{-1})	-	-	41.1	65.0	-	-	-
Porosity (%)	-	-	58.7	63.7	-	-	-
BET (m^2 g^{-1})	8.5	154.2	-	-	175.2	19.38	1.2
Bulk density (g mL^{-1})	-	-	0.46	0.46	-	-	-
C-C/C-H*	47.2	55.1	47.6	44.5	-	-	-
C-O*	35.0	31.2	34.2	21.7	-	-	-
C=O/O-C-O*	16.0	10.2	14.2	12.0	-	-	-
Carbox/carbont *	1.87	3.51	4.0	21.8	-	-	-

T pyrolysis=BC production pyrolysis temperature; EC= electric conductivity; VM= volatile matter; EAC= electron accepting capacity; EDC= electron donating capacity; EEC= electron exchange capacity; Lig/Cel=lignin/cellulose; TporeA= total pore area; BET= Brunauer–Emmett–Teller. DOC: dissolved organic carbon; DIC: dissolved inorganic carbon; TDC: total dissolved carbon; TDN: total dissolved nitrogen. *Relative atomic percentage in BC surface.

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