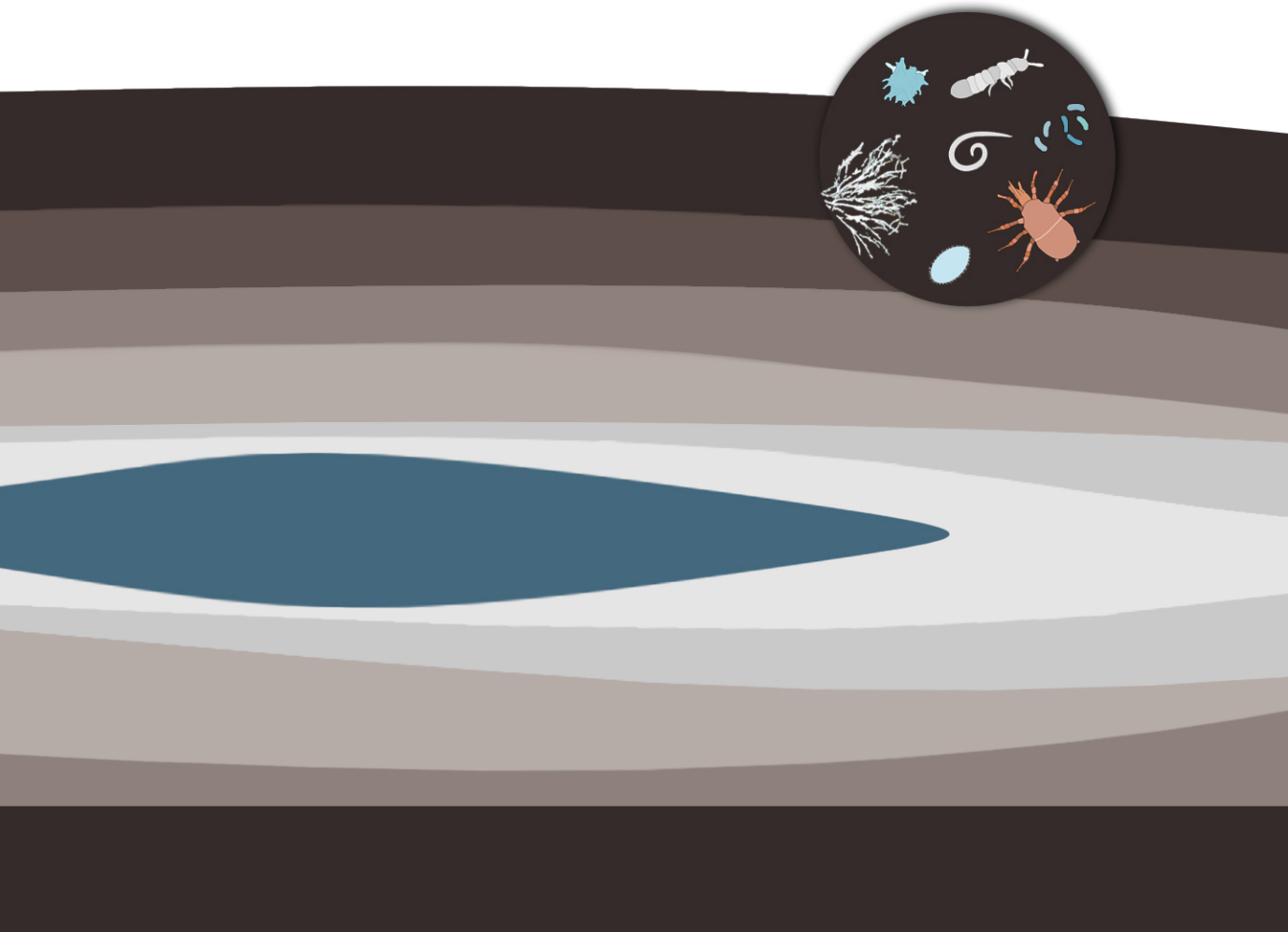
PhD Thesis 2016

SOIL BIOTA UNDER HIGH CO_2 EMISSIONS: FROM AN EXPERIMENTAL APPROACH TO NATURAL ENVIRONMENTS

Assessing environmental impacts of potential CO_2 leakages from a geological storage

Irena Fernández Montiel



universidad de león

Departamento de Diversidad y Gestión Ambiental
Área de Ecología

SOIL BIOTA UNDER HIGH CO_2 EMISSIONS: FROM AN EXPERIMENTAL APPROACH TO NATURAL ENVIRONMENTS

Assessing environmental impacts of potential CO_2 leakages from a geological storage

---

BIOTA EDÁFICA SOMETIDA A EMISIONES ELEVADAS DE CO_2: DE UN SISTEMA EXPERIMENTAL A UN AMBIENTE NATURAL

Evaluando el impacto ambiental de posibles fugas de CO_2 desde un almacén geológico

Irena Fernández Montiel

2016

universidad de león

Departamento de Diversidad y Gestión Ambiental
Área de Ecología

Memoria presentada por la Licenciada en Biotecnología Irena Fernández Montiel para optar al título de Doctor por la Universidad de León.

Directores de tesis:

Dr. **Eloy Bécares Mantecón**

Universidad de León

Dr. **Anna Pedescoll Albacar**

Universidad de León

A mis padres

Duda siempre de ti mismo,
hasta que los datos no dejen lugar a dudas.

Louis Pasteur

Table of Contents

List of Tables

List of Figures

Índice

Resumen

El uso de combustibles fósiles supone la mayor fuente de emisiones de CO_2, por ello, los esfuerzos para el control de dichas emisiones se concentran en un conjunto de medidas que incluyen, entre otras, 1) el incremento en el uso de energías renovables y energía nuclear, 2) la mejora de la medida de eficiencia energética y 3) la reducción de la intensidad de carbono (el ratio de emisiones de CO_2 por combustión fósil dividido entre el PIB). En éste último caso destaca una tecnología nueva: la Captura y Almacenamiento de Carbono (CAC). La CAC consiste en un proceso de separación del CO_2 emitido por la industria y fuentes relacionadas con la energía, seguido de su transporte a un lugar de almacenamiento, donde se confina a largo plazo para aislarlo de la atmósfera. La integridad del depósito, la resistencia de la roca que rodea el almacén y los distintos mecanismos de retención del gas van a determinar la permanencia de CO_2 en el depósito. Por eso, el emplazamiento se selecciona teniendo en cuenta estas características, a fin de evitar cualquier fuga posible del CO_2 almacenado.

No obstante, la implementación de esta tecnología conlleva el estudio de potenciales fugas desde el lugar de almacenamiento, así como las consecuencias derivadas de las mismas, con el objetivo de evitar daños irreparables en el ecosistema, y desarrollar sistemas de monitorización y control. La enorme diversidad de microorganismos en el suelo, así como su relevancia en los procesos edáficos y su extrema sensibilidad a cambios en las condiciones que les rodean, hacen de ellos excelentes indicadores de alteraciones en el ecosistema.

Esta tesis tiene por objetivo principal estudiar las consecuencias de una potencial fuga de CO_2 desde un almacén geológico sobre las comunidades microbianas del suelo. Para ello, se analizó la microbiota de una planta experimental que simula una fuga controlada de CO_2 (experimento $PISCO_2$). Paralelamente se estudiaron las comunidades microbianas de una zona de emisiones naturales de CO_2 (La Sima, Campo de Calatrava) con el fin de comparar el sistema experimental con una emisión natural. Las zonas de muestreo fueron seleccionadas en función de los flujos de CO_2: en el sistema $PISCO_2$ se estudiaron flujos bajos de CO_2 (50 g m^{-2} d^{-1}) y flujos altos (100 g m^{-2} d^{-1}), además de un control no expuesto a CO_2 (10 g m^{-2} d^{-1}). En La Sima los flujos analizados fueron 10 (control), 50 (bajo) y 900 g m^{-2} d^{-1} aproximadamente (alto), incluyéndose además en este caso el estudio de zonas de flujos extremos (más de 10.000 g m^{-2} d^{-1}). Los objetivos específicos del trabajo fueron: 1) caracterizar la funcionalidad (mediante BIOLOG Ecoplates), diversidad genética (mediante DGGE) y abundancia de microorganismos (mediante qPCR) en ambos sitios; 2) evaluar la influencia de la estacionalidad, tiempo de exposición al gas y el sistema de emisiones

(artificial o natural) en la respuesta microbiana y 3) observar el efecto del CO_2 sobre las relaciones entre la microbiota y otros grupos edáficos (protistas, nematodos, mesofauna). Como objetivo final, se discutió la idoneidad de los microorganismos como bioindicadores de fugas de CO_2 desde almacenes geológicos.

Los resultados variaron en función de la zona estudiada. En la planta experimental $PISCO_2$, tras 15 meses de exposición a flujos relativamente bajos CO_2, las comunidades microbianas no se vieron significativamente afectadas en ninguna de las variables estudiadas. Las emisiones naturales de La Sima, sin embargo, causaron un profundo impacto sobre la abundancia y riqueza de la microbiota edáfica. Se observó una reducción significativa del número de copias en bacterias, arqueas y hongos en los flujos altos de CO_2 (260-600 g m^{-2} d^{-1}), pérdidas de diversidad genética bacteriana y una disminución de la actividad metabólica potencial. Aunque la exposición al CO_2 en La Sima no afectó a las relaciones de abundancia entre grupos (ej. Bacterias/protozoos, protozoos/mesofauna), el efecto negativo del incremento del CO_2 sobre bacterias, hongos y arqueas se vio acompañado por una disminución de diversidad y abundancia en el resto de grupos: una disminución en el número de amebas, una casi desaparición de los nematodos y una reducción en la abundancia y riqueza de la mesofauna. Los flujos más extremos de La Sima (más de 10^4 g m^{-2} d^{-1}) provocaron, sin embargo, una recuperación de la actividad bacteriana y de la biomasa de la mayoría de poblaciones estudiadas, a pesar de que la pérdida de diversidad se mantuvo, sugiriendo que las condiciones extremas de CO_2 se asociaban con comunidades adaptadas, de elevada abundancia y baja riqueza de especies.

Un factor que explicaría las diferencias de resultados entre las zonas podría ser el tiempo de exposición al gas: la surgencia de La Sima lleva emitiendo durante varios siglos, probablemente milenios, por lo que las comunidades podrían mostrar una adaptación a las condiciones extremas de la zona. La mayoría de grupos edáficos estudiados presentaron menor diversidad (bacterias, ciliados, mesofauna) y bajas abundancias (bacterias, hongos, amebas, nematodos y mesofauna) con el aumento de CO_2, y en los flujos más extremos (más de 10^4 kg m^{-2} d^{-1}) la riqueza de biota se vio seriamente afectada. Estos resultados podrían deberse, por tanto, a la combinación de flujos elevados de CO_2 con emisiones a escala geológica. Las fluctuaciones estacionales influyeron en la respuesta de las comunidades, tanto en La Sima como en $PISCO_2$. En concreto, la humedad del suelo se relacionó con variaciones en el número de organismos (hongos en $PISCO_2$, bacterias y arqueas en La Sima) y estimuló significativamente la actividad metabólica potencial bacteriana.

En relación con la biomonitorización de proyectos de CAC, la biota edáfica podría ser considerada indicador de fugas de CO_2 desde almacenes geológicos: una menor diversidad de microorganismos, o las bajas densidades de grupos tróficos superiores (protozoos, nematodos o mesofauna) podrían reflejar un incremento de los flujos de CO_2 en el suelo. No obstante, hay que tener en cuenta que la capacidad de respuesta

de la biota edáfica viene determinada por varios factores, como son la intensidad del flujo de CO_2 a la que se expone, el tiempo de exposición o las variaciones estacionales. En consecuencia, la biomonitorización de la CAC mediante biota edáfica estaría indicada para controlar fugas leves que no han sido detectadas durante largos periodos o bien escapes de CO_2 de gran flujo. Por lo tanto, los futuros estudios deberían centrarse en identificar 1) el tiempo de exposición necesario para detectar cambios en la biota edáfica debidos a pequeñas fugas, y 2) la concentración umbral de CO_2 a partir de la cual puedan detectarse grandes fugas producidas en poco tiempo.

1. Introducción

1.1. El cambio climático y las emisiones de CO_2

El calentamiento del sistema climático es inequívoco. El cuarto informe de evaluación del Panel Intergubernamental en Cambio Climático (IPCC en sus siglas en inglés) declara que "la mayor parte del incremento observado en las temperaturas medias globales desde la mitad del siglo XX se debe con toda probabilidad al aumento en la concentración de gases de efecto invernadero (GEI) de carácter antropogénico", siendo el dióxido de carbono (CO_2) el gas con mayor contribución a la concentración de GEIs en la atmósfera.

Desde la era preindustrial, la concentración atmosférica ha aumentado en un 40 % aproximadamente, con un crecimiento promedio anual de 2 ppm/v (IEA, 2014). La última medición global alcanza 400,1 ppm (NOAA, junio 2015). A finales del siglo XXI, dichas concentraciones atmosféricas podrían oscilar entre 535 y 983 ppm. Si no se mitiga, y considerando las emisiones de metano y otros GEIs, estos cambios podrían causar un incremento de entre 1,1 y 6,4 ºC en 2090-2099, respecto al periodo 1980-1999. Teniendo en cuenta la correlación entre la cantidad de CO_2 en la atmósfera y la temperatura superficial terrestre, la comunidad científica sugiere que se establezcan objetivos para limitar las concentraciones a 450-500 ppm, con la esperanza de acotar el incremento de la temperatura global a 2 ºC (máximo calentamiento permisible para evitar interferencias antropogénicas peligrosas sobre el sistema climático) (RISCS, 2014).

El uso de energía es la mayor fuente de emisiones de CO_2, siendo la quema de combustibles fósiles la que contribuye en mayor grado (Figura 1.1) (IEA, 2014). Concretamente, es la responsable de dos terceras partes de las emisiones de CO_2 desde el inicio de la revolución industrial (Global Carbon Atlas, 2015). En 2013, se liberaron 36 Gt de CO_2 debido a la quema de combustibles fósiles.

En España, durante el mismo periodo, se emitieron 240 Mt de CO_2 (OECD, 2015a). La intensidad de carbono de España (el ratio de emisiones de CO_2 por combustión fósil dividido entre el Producto Interior Bruto –PIB–) disminuyó más del 20 % desde 2000, situando España entre los 10 países miembros de la OECD con menor intensidad de CO_2 (OECD, 2015b). Esta reducción se debe, en primer lugar, al incremento en el uso de energías renovables y la mejora de la medida de eficiencia energética y, en segundo lugar, al profundo impacto causado por la crisis global en España. La industria –incluyendo el sector energético– y el transporte son los mayores consumidores de energía y por tanto, las mayores fuentes de emisiones de GEIs, entre las que el CO_2 cuenta con un 80 % (Figura 1.2). De esta manera, España no ha conseguido el objetivo establecido por el Protocolo de Kyoto en 2008-2012 (el 15 % sobre los niveles de 1990), y las emisiones en 2012 han alcanzado 356 Mt, excediendo en 15 % dicho objetivo

(MAGRAMA, 2013). Se prevé que para el 2020 las emisiones de GEIs se sitúen un 9 % por encima del objetivo del Protocolo de Kyoto (EC, 2104).

Considerando estas predicciones, resulta esencial desarrollar diferentes medidas que nos permitan alcanzar dichos objetivos (reducción de las emisiones al 80-95 % del nivel de 1990 para el 2050). El IPCC sugiere un conjunto de acciones destinado a reducir las emisiones de CO_2, que incluye el incremento en el uso de las energías renovables y nuclear, la mejora de la eficiencia energética y la reducción de la intensidad de carbono en los combustibles fósiles (IPCC, 2007). Dentro de esta última opción se propone la implementación de una innovadora tecnología: la Captura y Almacenamiento de Carbono.

1.2. ¿Qué es la Captura y Almacenamiento de Carbono?

La Captura y Almacenamiento de Carbono (CAC) es un proceso que consiste en la separación del CO_2 emitido por la industria y fuentes relacionadas con la energía, su transporte a un lugar de almacenamiento y su aislamiento de la atmósfera a largo plazo (IPCC, 2005). El IPCC destaca la CAC como una tecnología clave en la mitigación de las emisiones debidas a la producción de energía y la industria ya que su contribución a la reducción global de emisiones podría llegar al 20-30 %. Teniendo en cuenta únicamente la mejora de la eficiencia energética y el incremento en el uso de energías renovables no se podría alcanzar la disminución de emisiones requerida para conseguir estabilizar las concentraciones atmosféricas en el periodo establecido, por lo que la CAC podría considerarse un elemento esencial a corto plazo para una reducción sustancial de las emisiones antropogénicas de CO_2 (Damen et al., 2006). Por otra parte, un quinta parte de todas las emisiones de CO_2 provienen de procesos industriales, los cuales pueden ser mejorados o reemplazados para reducir las emisiones y su impacto. Sin embargo, dichas emisiones no podrán ser completamente eliminadas ya que necesitamos los productos que las causan (ZERO, 2015).

El proceso de CAC comienza con la separación del CO_2 de los gases exhaustos que provienen de la fuente emisora, como centrales térmicas, plataformas de gas natural y otras industrias. Actualmente, se están investigando tres métodos para la captura de CO_2: 1) postcombustión, que separa el CO_2 utilizando solventes químicos, 2) pre combustión, que retira químicamente el carbono, dando por resultado hidrógeno y CO_2, y 3) combustión oxígeno-gas, que quema el carbón o el gas en aire desnitrificado para producir sólo CO_2 y agua (Haszeldine, 2009). Tras la separación, el CO_2 se presuriza a 70 bares, dando lugar a un líquido que puede ser transportado por tuberías o enviado por barco al lugar de almacenamiento (Figura 1.3).

El CO_2 puede ser almacenado en diferentes formaciones geológicas, incluyendo yacimientos de petróleo o gas agotados, capas de carbón no explotables y formaciones salinas profundas (formaciones de roca sedimentaria porosa saturada con agua salobre

o salmuera), a una profundidad de 800-1000 m (Figura 1.4). También puede utilizarse este CO_2 en la recuperación mejorada de petróleo (RMP). A una temperatura superior a 31,1 ºC y una presión de 72,8 bar (las condiciones típicas de rocas de almacenamiento a profundidades de 800 m), el CO_2 forma una fase fluida relativamente densa, es decir, un fluido supercrítico. La consecuente pérdida de volumen bajo esas condiciones permite que se pueda inyectar más CO_2 en el mismo espacio (RISCS, 2014). De esta forma, se consigue un uso eficiente del espacio disponible en los poros de las rocas sedimentarias. Otro requisito para llevar a cabo el almacenamiento es que las formaciones tengan una roca de cubierta estanca que actúe como barrera sobre el depósito de almacenamiento a fin de que el CO_2 permanezca retenido. El informe especial sobre CAC del IPCC (IPCC, 2005) concluye que un depósito apropiadamente seleccionado y controlado puede retener "muy probablemente" más del 99 % del CO_2 almacenado durante más de 100 años y un 99 % "probablemente" durante más de 1000 años. En cuanto a la capacidad de almacenamiento se sabe que las formaciones salinas profundas ofrecen el mayor volumen de depósito (10.000 Gt de CO_2), seguidas de los yacimientos agotados de petróleo y gas (675-900 Gt) y las formaciones de carbón no explotables (3-200 Gt).

1.2.1. CAC en España

La CAC está aún en sus primeras fases en España. La "Fundación Ciudad de la Energía" (CIUDEN) es la principal entidad del Gobierno dedicada a desarrollar las tecnologías de captura, transporte y almacenamiento, a través del Proyecto Compostilla-OXY-CFB-300 (www.compostillaproject.eu). Este proyecto, que finalizó en 2013, es el único de su clase –un proyecto piloto a gran escala que incluye la cadena completa del proceso CAC– avalado por el Programa Energético Europeo para la Recuperación (EERP). Está formado por un conjunto de acciones desarrolladas entre la CIUDEN y ENDESA (CIUDEN, 2009) y comprende el Centro de Desarrollo Tecnológico para la captura de CO_2 localizado en Cubillos del Sil, cerca de la central térmica de Compostilla (León, noroeste de España) y la Planta de Desarrollo Tecnológico para el almacenamiento de Hontomín, a 250 km al este (Burgos, norte-centro de España). Hontomín, considerado como una de las mejores opciones para el almacenamiento en el país, es un acuífero salino terrestre (1600 m de profundidad), con una capacidad de 100.000 toneladas de CO_2. Representa un buena oportunidad para desarrollar actividades de inyección y almacenamiento en un acuífero salino profundo carbonatado de baja permeabilidad, lo que supone una nuevo enfoque en el almacenamiento geológico de CO_2 y puede proporcionar nuevos conocimientos para operaciones en sitios "no ideales" (Global CSS Institute, 2015).

1.2.2. Riesgos asociados a la CAC: fugas potenciales y sus consecuencias para el medio ambiente

Existen numerosas evidencias que indican que un emplazamiento de almacenamiento de CO_2 puede ser elegido de manera que se minimice la probabilidad de fuga (RISCS, 2014), entre ellas los depósitos naturales de CO_2 que han permanecido estables durante millones de años, ensayos que demuestran la integridad de la roca de cubierta (Bennion and Bachu, 2007) o la experiencia de proyectos de almacenamiento de CO_2 en desarrollo (Sleipner en Noruega, In Salah en Algeria, Weyburn en Canadá o Ketzin en Alemania). Sin embargo, uno de los factores que va a determinar la puesta en marcha de los proyectos de CAC es el riesgo asociado al almacenamiento de CO_2. La estimación de riesgos es uno de los primeros pasos a tener en cuenta en la estrategia de establecimiento de medidas de control y gestión del almacenamiento de CO_2 para minimizar fallos. Además facilitará la elaboración de estándares y marcos normativos necesarios para llevar a cabo la CAC a gran escala (Damen et al., 2006). En este sentido, la UE ha establecido un marco jurídico para garantizar el almacenamiento geológico de CO_2 en condiciones seguras para el medio ambiente (Directiva 2009/31/EC), así como para minimizar los impactos ambientales y de salud humana, y asegurar la integridad climática. En España, la Directiva se transpuso a la legislación nacional mediante la Ley 40/2010.

El CO_2 inyectado en los yacimientos geológicos podría escaparse del depósito a través del subsuelo, migrar lateralmente en las formaciones de cobertura y finalmente filtrarse a la atmósfera/biosfera. La probabilidad de fuga viene determinada por la integridad del depósito y la roca de cubierta y por los mecanismos de retención. El CO_2 puede ser retenido en los reservorios por diferentes mecanismos, en el caso de acuíferos salinos profundos –como Hontomín– la migración desde la zona de inyección a la superficie podría llevar entre miles y millones de años, ya que estas formaciones presentan tasas de flujo extremadamente bajas (retención hidrodinámica). Además, existen otros mecanismos de retención propios de acuíferos salinos profundos (Figura 1.5). La permeabilidad de la cobertura –las formaciones que se sitúan encima del depósito– es otro factor crítico en la posible fuga, ya que determina el tiempo de retención del CO_2 en el subsuelo (Damen et al., 2006). Las formaciones salinas profundas tienen como ventaja una menor probabilidad de fuga de CO_2, ya que al no ser de interés económico para su explotación no tendrán pozos de inyección como los presentes en yacimientos de petróleo o gas agotados. No obstante, hemos de tener en cuenta que el espacio disponible para el almacenamiento de CO_2 sólo se alcanza tras la compresión de los fluidos (salmuera o agua salobre) en el reservorio, o por desplazamiento de los mismos a formaciones adyacentes o a la superficie (Holloway, 1996). Esto daría lugar a un incremento de presión que podría potenciar zonas de fractura y como consecuencia, el CO_2 migraría hacia zonas superiores (Over et al., 1999). El principal mecanismo de fuga en estas formaciones sería la difusión a través de la roca de cubierta, aunque muy lentamente; la modelización indica que este

proceso podría durar millones de años (Lindeberg y Bergmo, 2003). Asimismo, las simulaciones realizadas en el depósito salino de Sleipner muestran que la precipitación mineral causada por el CO_2 reduciría la porosidad y permeabilidad de la roca de cubierta (Johnson y Nitao, 2003).

No obstante, si se produjera una fuga desde un almacenamiento geológico, podría dar lugar a riesgos potenciales derivados de las concentraciones elevadas de CO_2 (Figura 1.6). Se sabe que a concentraciones superiores al 2 % el CO_2 tiene un fuerte efecto sobre el sistema respiratorio humano, y que a concentraciones superiores al 7-10 % pueden causar desvanecimiento y la muerte de humanos y la mayoría de los animales aerobios (Benson et al., 2002; IPCC, 2005). Por otra parte, los riesgos ecológicos y medioambientales pueden tener consecuencias locales o globales: desde la disolución del CO_2 en acuíferos útiles o aguas subterráneas potables –que darían lugar a una reducción del pH y la movilización de metales– a la fuga de CO_2 al ambiente, causando acidificación, cambios en los nutrientes y con ello una profunda alteración del ecosistema del suelo y el crecimiento vegetal. Aunque las plantas suelen tener una mayor resistencia al CO_2 que los mamíferos, una fuga persistente podría inhibir la respiración en la rizosfera. Otras consecuencias serían los efectos derivados del desplazamiento del fluidos en el reservorio (salmuera, otros gases), la invasión por parte del CO_2 de otros recursos subterráneos o la elevación de la superficie topográfica debido a cambios en las propiedades geomecánicas del subsuelo (PTECO$_2$, 2012).

Se ha de tener en cuenta que la probabilidad de filtración y escape de CO_2 desde un depósito hace del almacenamiento de CO_2 una opción menos efectiva en la mitigación del cambio climático. Así, la cuestión esencial es determinar qué índices de fuga serían aceptables para asegurar la estabilización de las concentraciones atmosféricas de GEIs en el próximo siglo (450-750 ppm). La mayoría de autores coincide en que el índice de fuga medio anual no debería sobrepasar el 0,1 % (Damen et al., 2006). La rigurosa selección del sitio de almacenamiento, la supervisión reglamentaria eficaz, un programa de monitorización apropiado y la aplicación de métodos correctivos para detener o controlar una filtración inicial son distintas maneras de evitar una fuga potencial (IPCC, 2005).

El impacto de una posible fuga de CO_2 va a venir determinado por varios factores como las características del área afectada (geología, tipo de suelo, topografía, clima) o las condiciones climatológicas en el momento de la fuga (por ejemplo la humedad del suelo, la velocidad del viento o la temperatura del aire). Dichos factores pueden influir en el flujo y concentraciones de CO_2 del suelo: la creación de caminos preferenciales para el gas puede causar altas concentraciones focalizadas y con mayor impacto, o una mayor humedad puede incrementar la concentración de CO_2 en el suelo ya que impide el escape del gas. Por consiguiente, el nivel de CO_2 en el suelo puede mostrar importantes variaciones estacionales (RISCS, 2014).

La implementación de la CAC en la última década ha fomentado la investigación sobre posibles escapes de CO_2 y sus consecuencias en ambientes terrestres (y marinos), incluyendo sus pobladores. A pesar de que existen numerosos estudios sobre concentraciones elevadas de CO_2 atmosférico, estas no tienen apenas relevancia para los potenciales efectos de una fuga, ya que la concentración de CO_2 en el suelo es mucho mayor que los incrementos atmosféricos aplicados en estas investigaciones. Estudios recientes (ej. RISCS, 2014) corroboran que los aumentos en la concentración edáfica de CO_2 tienen un efecto mucho más marcado que los ligeros incrementos en los niveles atmosféricos (Jones et al., 2015). Por este motivo, algunos experimentos hacen uso de emisiones naturales de CO_2 como análogos, mientras que otros se centran en sitios de inyección experimentales, para estudiar impactos sobre el suelo, las plantas o los microorganismos.

1.3. ¿Por qué estudiar las comunidades microbianas del suelo en procesos CAC?

Por un lado, es una cuestión de su relevancia ecológica. El suelo presenta la mayor diversidad de microorganismos de la Tierra (Torsvik et al., 2002), y las transformaciones llevadas a cabo por ellos son fundamentales para el funcionamiento de ecosistema terrestre, ya que son responsables del reciclado de nutrientes y del mantenimiento de la estructura del suelo (Prosser, 2007). Además, el suelo presenta una enorme heterogeneidad espacial, lo que da lugar a una gran variabilidad de microbiota en unos pocos metros, que está extremadamente influenciada por propiedades del suelo como el pH, por la vegetación o por impactos antropogénicos (Fierer and Jackson, 2006). Hoy por hoy, contamos con un número relativamente pequeño de estudios sobre las respuestas microbianas a una fuga de CO_2, por lo que serían necesarias más investigaciones para identificar cambios específicos debidos al CO_2 y no otros factores (Jones et al., 2015). Asimismo, los ambientes extremos (como una fumarola de CO_2) representan una fuente de microorganismos únicos que poseen estrategias metabólicas interesantes y pueden ayudar a dilucidar el papel de las especies en el funcionamiento de estos ecosistemas (Maier and Neilson, 2015).

Por otro lado, los microorganismos pueden ser extremadamente sensibles a los cambios en las características del suelo, actuando como buenos indicadores de la calidad del mismo (Winding et al., 2005). Serán el primer punto de contacto –biológico– con los compuestos químicos tóxicos y/o beneficiosos que entren al suelo o al agua subterránea como consecuencia de una fuga de CAC, de manera que pueden manifestar los primeros cambios en la monitorización de impactos (Noble et al., 2012). Cualquier alteración asociada con un escape de CO_2 (modificaciones del pH, Eh, salinidad, temperatura, movilización de metales) puede influir en el número y actividad de los microrganismos, proporcionando indicadores tempranos. Igualmente, y dada su diversidad, es muy probable que existan especies indicadoras muy sensibles específicas de tipos de hábitat determinados.

Dejando aparte las propiedades químicas o físicas del suelo, las comunidades microbianas están también fuertemente influenciadas por sus predadores. Se sabe que las cascadas tróficas pueden ser funcionalmente significativas para dar forma a las comunidades microbianas e influenciar la toma de nutrientes por las plantas. Por ejemplo, es de especial relevancia el papel de los protozoos debido a sus preferencias alimentarias y a su altas tasas de consumo (Clarholm et al., 2007). Respecto a la evaluación de riesgos aplicada a la CAC, es determinante conocer no sólo su impacto en los microrganismos, sino también cómo las relaciones de éstos con otros grupos bióticos del suelo pueden verse afectadas. En este contexto, existe muy poca investigación hasta la fecha sobre los efectos de los incrementos del CO_2 del suelo en niveles tróficos superiores (protistas, micro o mesofauna).

1.3.1. Metodología utilizada en este trabajo

Existen diversas técnicas para estudiar las comunidades microbianas del suelo, teniendo en cuenta su complejidad. En lugar de utilizar métodos dependientes de cultivo, que sólo nos permiten analizar una pequeña porción de la microbiota –entre el 0,1 y el 10 % de los microorganismos totales de un determinado ambiente– decidimos combinar diferentes técnicas moleculares basadas en el ADN para tener una visión más completa de los impactos del CO_2 sobre las comunidades microbianas: la reacción en cadena de la polimerasa cuantitativa (qPCR, por sus siglas en inglés) y la electroforesis en gel con gradiente desnaturalizante (DGGE).

Con el fin de estudiar la presencia de microorganismos en ambientes terrestres, la qPCR ofrece una excelente sensibilidad y es capaz de cuantificar poblaciones específicas usando primers seleccionados. Sin embargo, como en todos los métodos basados en PCR, pueden producirse inconvenientes asociados a la extracción de los ácidos nucleicos, la especificidad de los primers y las condiciones de la PCR (Nybroe et al., 2007). Además, los ácidos húmicos del suelo pueden inhibir la PCR, pero una continua optimización de los protocolos experimentales puede evitar este problema. La DGGE es una técnica basada en el *fingerprinting* de comunidad que se fundamenta en el distinto comportamiento de desnaturalización de los productos de PCR de doble cadena debido a diferencias en la estructura primaria de los fragmentos de ARNr (Oros-Sichler et al., 2007). El resultado es un gel con un perfil de bandas, cada banda corresponde –teóricamente– con una especie microbiana de la comunidad analizada. Este método permite estimar la diversidad de comunidad (número de bandas por cada muestra) y se puede combinar con análisis adicionales, por ejemplo escindiendo y secuenciando una banda seleccionada podemos identificar la afiliación filogenética de los ribotipos que componen dicha banda. Como desventaja, esta técnica sólo amplifica el ADN de organismos dominantes en una comunidad (0,1-1 % de todas las dianas posibles) y tiene también los inconvenientes asociados al uso de ARNr 16S como marcador (un mismo organismo puede contener varias copias de un gen que darán lugar a distintas bandas en un gel, o bandas que corresponden a distintos organismos

pueden ocupar la misma posición en el gel). No obstante, esta técnica es rápida y con un relativo bajo coste por lo que representa una herramienta muy adecuada en la investigación de dinámicas de población de las comunidades microbianas del suelo, lo que la hace más que apropiada para nuestros objetivos.

También incluimos un método poco utilizado en la evaluación de riesgos de la CAC en el ecosistema terrestre: BIOLOG EcoPlates™. También conocido como perfil fisiológico a nivel de comunidad (CLPP en sus siglas en inglés), esta técnica proporciona un indicador del funcionamiento del ecosistema, su potencial metabólico y la diversidad funcional de las comunidades microbianas en el suelo (Timms-Wilson et al., 2007). BIOLOG Ecoplate es una microplaca de 96 pocillos que contiene 31 de las fuentes de carbono más usadas para el análisis de comunidades de suelo, replicadas tres veces. Cada pocillo contiene también tetrazolio como tinte indicador, de manera que cuando los microorganismos utilizan la fuente de carbono el color vira a morado. Así, estas placas pueden medir la magnitud y la variedad de fuentes de carbono que la comunidad ambiental analizada puede usar potencialmente, a través del patrón de desarrollo que aparece en la placa (similitud), la tasa de cambio de color de cada pocillo, o la riqueza de respuestas en los pocillos (diversidad). No se ha de olvidar que esta técnica nos ofrece la actividad "potencial" de la comunidad microbiana más que su actividad *in situ*, y que la mayoría de los microorganismos no son cultivables. A pesar de esto, BIOLOG EcoPlate proporciona un método sencillo y económico para evaluar el perfil metabólico microbiano.

Las características de la aplicación de estas técnicas a este trabajo se detallan en los capítulos 3, 4 y 5.

1.4. Investigación experimental con CO_2 en sistemas construidos

Hasta la fecha, hay pocos experimentos construidos como simulaciones de fugas de CO_2 desde un almacén geológico, entre ellos los proyectos ASGARD (Reino Unido), ZERT (EEUU), Grimsrud farm (Noruega), Resacada Farm (Brasil) y Gininderra (Australia). En algunos de ellos se han evaluado los efectos del CO_2 sobre las comunidades microbianas (Tabla 1.1), mientras que en Grimsrud farm sólo se estudiaron las respuestas de las plantas, y el proyecto de Brasil aunque tiene previsto un estudio microbiológico aún no está publicado. Asimismo, se han llevado a cabo análisis biológicos en varios de los sitios de inyección y almacenamiento de CO_2: análisis de referencia de flora y microbiota en In Salah (Jones et al., 2011) o estudios microbiológicos en profundidad (dentro del pozo de inyección) en Ketzin, comparando el estado previo y posterior a la inyección (Morozova et al., 2011). No obstante, en esta tesis nos centraremos en los impactos del CO_2 sobre las comunidades edáficas subsuperficiales. En este sentido, la mayoría de los estudios que se han llevado a cabo hacen uso de análogos naturales; sin embargo los sistemas experimentales en campo permiten controlar las tasas de inyección y, a diferencia de los experimentos en

laboratorio, introducen la complejidad del clima y otros factores ambientales (Jones et al., 2015). Por contra, dichos factores pueden enmascarar el efecto del CO_2, con lo que los resultados de los estudios en sistemas experimentales construidos en campo son diversos y están altamente influidos por la estacionalidad. Por ejemplo, en ASGARD vieron que tras dos años de emisiones de CO_2 las respuestas de la microbiota no eran claras, ya que podrían asociarse a cambios en las condiciones ambientales (Jones et al., 2015); Morales y Holben (2013) por su parte obtuvieron cambios sujetos a la estacionalidad.

En esta tesis se estudió la planta experimental $PISCO_2$, perteneciente al Proyecto Compostilla-OXY-CFB-300.

1.4.1. Caso de estudio: el proyecto $PISCO_2$

La Planta de Investigación en Suelos con CO_2 ($PISCO_2$) es un sistema experimental para estudiar el impacto de la inyección de CO_2 en suelos, localizado en el Centro de Desarrollo Tecnológico para la Captura de CO_2 de Cubillos del Sil, dentro de las instalaciones de la Fundación CIUDEN (Figura 1.7). El principal objetivo de este proyecto es desarrollar herramientas económicas y ecológicas de biomonitorización para el control seguro del almacenamiento geológico de CO_2. A diferencia de los análogos naturales, donde el CO_2 ha estado emitiéndose durante largos periodos, en el proyecto $PISCO_2$ se analizaran efectos de flora y fauna no expuestos previamente. Por otra parte, se sabe que grandes fugas de CO_2 como las existentes en análogos naturales producen cambios significativos visibles en los focos y entornos pero, ¿qué sucedería si la filtración de CO_2 fuese mucho más leve de manera que la concentración del mismo en superficie aumentase poco a poco pero sin cambios a simple vista? En esta premisa se basa el $PISCO_2$, y con ello se diferencia de otros sistemas experimentales que usan flujos de CO_2 mucho mayores (Tabla 1.1).

La planta experimental $PISCO_2$ (Figura 1.8) presenta un diseño factorial conformado por 24 celdas (8 m^2 cada una) en las que se aplicaron tres niveles de CO_2 sobre dos suelos diferentes; con cuatro réplicas por tratamiento. Las celdas se rellenaron (de abajo a arriba) con una capa de 20 cm de grava, cubierta por 170 cm de arena y finalmente una capa de suelo de 40 cm. En la mitad de las celdas se colocó un suelo franco arenoso proveniente de una pradera de Hontomín (Burgos) –el lugar donde se llevó a cabo el almacenamiento geológico de CO_2– y la otra mitad con un suelo franco procedente de un pasto localizado en el mismo lugar que el $PISCO_2$, Cubillos del Sil. El banco de semillas presente en cada suelo determinó el tipo de vegetación durante el experimento. Se aplicaron dos flujos de CO_2 (20 y 40 l h^{-1}) a través de unas parrillas localizadas a 1 y 2 m bajo el suelo, quedando 8 celdas (4 de cada suelo) sin inyección como controles. Los tratamientos se asignaron de forma aleatoria a cada celda experimental. En comparación con experimentos similares (Smith et al., 2013), en este sistema se suministró un flujo difuso y continuo a través de parrillas, lo que proporcionó

flujos homogéneos en superficie. La emisión de CO_2 fue constante y sin interrupciones durante todo el experimento, comenzó 4 meses antes de que el suelo fuese colocado para evaluar la heterogeneidad espacial de los flujos en cada celda.

Otros investigadores han estudiado el impacto del CO_2 en la planta experimental $PISCO_2$. Gabilondo et al. (2015) analizaron los efectos del CO_2 sobre los protozoos edáficos sin encontrar patrones claros en la abundancia de amebas, ciliados o flagelados; mientras que la composición de la comunidad de ciliados (equitabilidad, índice Margalef o el ratio Colpodea/Polyhymenophorea) mostró diferencias significativas como respuesta a la inyección de CO_2. Por su parte, Sáenz de Miera et al. (en revisión) investigaron la composición y estructura de las comunidades bacterianas mediante pirosecuenciación, observando cambios en la composición pero no efectos consistentes sobre la diversidad o riqueza, y mayoritariamente en sólo uno de los dos suelos estudiados.

1.5. Investigación centrada en análogos naturales: "mofettes"

Los "mofettes", tal y como se conocen las fumarolas, emisiones naturales de CO_2, se producen sobre todo en zonas de antigua actividad volcánica. Además de dar lugar a emisiones secas, el gas fugado también puede atravesar soluciones acuosas (capas freáticas, ríos) dando lugar a fuentes ácidas. Se han identificado varias regiones donde la concentración edáfica de CO_2 o el flujo del mismo desde el suelo es anómalo –la concentración normal no excede el 0,2-1 % mientras que estas zonas puede llegar al 100 %). Estas surgencias se distribuyen a lo largo de todo el mundo, se han registrado en Alemania, Islandia, Eslovenia, República Checa, Hungría, Rumanía, España, Grecia, Portugal, Austria, Francia, Italia, EEUU, Sudáfrica, Nueva Zelanda y Japón (Pfanz et al., 2004; Paoletti et al., 2005). En estas filtraciones el CO_2, originado en el manto profundo, migra a través de capas permeables o fracturas y fallas hacia la atmósfera. Una vez emerge es dispersado por el viento, aunque en algunas zonas donde los alrededores forman una depresión el CO_2 se dispersa menos fácilmente, con lo que se alcanzan concentraciones más elevadas (Holloway et al., 2007).

Las emisiones naturales de CO_2 se han estudiado intensivamente en los últimos años ya que pueden ser análogos adecuados para establecer sistemas de monitoreo y detección de los efectos de una posible fuga desde un almacén de CAC (Tabla 1.1). Presentan ventajas sobre los sistemas experimentales, ya que estas emisiones se han prologando durante siglos y por tanto pueden acoger estudios a largo plazo. Además, generalmente presentan gradientes en superficie desde el centro de emisión, con lo que ofrecen un amplio rango de concentraciones de CO_2. Otro beneficio es que no suponen gastos extra: el CO_2 es gratis y no se necesitan instalaciones sofisticadas (Paoletti et al., 2005). Y lo que es más importante, todas las condiciones ambientales son naturales, lo que hace de estas zonas lo más cercano a una fuga de un depósito de CAC (Schütze et al., 2012). Entre las limitaciones de estos sistemas, se incluye la

variabilidad de las concentraciones de CO_2: las surgencias pueden ser intermitentes y el flujo de CO_2 sufre oscilaciones que dependen de las condiciones atmosféricas, la topografía de la zona y la cobertura vegetal. Asimismo, compuestos sulfurosos como H_2S y SO_2 se encuentran habitualmente asociados a estas emisiones y podrían influir en las respuestas al CO_2.

Las elevadas concentraciones edáficas de CO_2 causan un impacto profundo en la zona alrededor de las surgencias. La consecuencia más evidente es una vegetación reducida o irregular, debido a las condiciones anóxicas creadas por una aireación suprimida. Es común encontrar pájaros, mamíferos, reptiles o insectos muertos en las proximidades de las fumarolas (Figura 1.9). El suelo se acidifica gradualmente debido a la meteorización de los minerales y la reducción en contenido de óxidos minerales (Frerichs, 2013), con lo que también se ve afectada la composición química del suelo. En conjunto, la acidificación del suelo, la pérdida de vegetación, la reducción de la rizosfera y el aporte de carbono al suelo, y la limitación en la disponibilidad de oxigeno transforman las comunidades microbianas de las fumarolas de CO_2.

Todas las investigaciones realizadas en emisiones naturales de CO_2 han documentado cambios significativos en la microbiota, causados por los altos niveles de CO_2 en la composición de gases en el suelo (Tabla 1.1). Varios de dichos estudios observaron una transición hacia microorganismos anaeróbicos y ácido tolerantes así como una adaptación del ecosistema a la biogeoquímica edáfica inducida por el CO_2. También se han percibido incrementos en la actividad sulfato-reductora o una metanogénesis estimulada (Jones et al., 2015). Estas diferencias podrían estar relacionadas con la adaptación a largo plazo en estas zonas naturales, ya que no han sido detectadas en sistemas experimentales. Por ejemplo, esta adaptación no se vio en ASGARD tras 24 meses de exposición intermitente al CO_2; aún no está claro con cuanta rapidez se producen estas transformaciones (RISCS, 2014). En relación con la evaluación de riesgos de la CAC cabe destacar que estos ecosistemas han ido evolucionando durante largos periodos con la presencia del gas, por lo que las respuestas a corto plazo a una fuga de CO_2 podrían no ser las mismas.

1.5.1. Caso de estudio: la surgencia de CO_2 de La Sima en el Campo Volcánico de Calatrava

La región volcánica de Campo de Calatrava está localizada en el sur-centro de España (Figura 1.10), en la provincia de Ciudad Real (Castilla-La Mancha). Es una de las tres áreas más importantes con actividad volcánica reciente en la Península Ibérica, junto a Olot (Girona, Cataluña) y Cabo de Gata (Almería, Andalucía) (Higueras y Gallardo, 2011). La actividad volcánica tuvo lugar hace 8,7-0,7 millones de años, durante el Plioceno y el periodo Cuaternario.

Esta región tiene un área total de cerca de 5.000 km^2 y contiene alrededor de 240 edificios volcánicos (Ancochea, 1997). El Campo Volcánico de Calatrava es, junto a La

Selva-Empordà (noreste de España), la mayor área de descarga de CO_2 de toda la España continental; representa uno de los mejores ejemplos de análogos naturales de fugas de CO_2 en el país (Vaselli et al., 2013). El CO_2 presente en el subsuelo y acuíferos se origina por la desgasificación y enfriamiento del magma y se filtra a la superficie a través de fracturas y grietas en la roca (Calvo et al., 2010; Elío et al., 2015). También se da una actividad hidrotermal remanente, visible a través de los conocidos "hervideros" (fuentes termales de baja temperatura) que burbujean debido al CO_2 (Figura 1.9). La emisión total de CO_2 se ha estimado en 1.678 ± 58 t d^{-1} en un área de 758 km^2 (Calvo et al., 2010).

La zona documentada con mayores emisiones de CO_2 es la surgencia de La Sima con cantidades registradas de hasta 324 kg CO_2 m^{-2} d^{-1} (Calvo et al., 2010). La Sima es una fumarola localizada en la ladera noroccidental de la Sierra de Granátula (38° 49' 17.51" N, 3° 45' 19.80" O, 656 m.s.n.m.). El clima es Mediterráneo continentalizado, con veranos cálidos y secos e inviernos húmedos y fríos. El principal uso de la tierra es la agricultura (cultivos de cereales) y la vegetación natural está poco presente (Sáenz de Miera et al., 2014).

El terremoto que se produjo en agosto de 2007, con epicentro en Pedro Muñoz (Ciudad Real), derivó en un drástico incremento de las emisiones gaseosas en La Sima (Peinado et al., 2009). Los flujos de CO_2 aumentaron de 30.000 a 200.000 ppm, con trazas de H_2S, HCl and CH_4 (Gosálvez et al., 2010). Además, surgieron nuevos puntos de emisión pasando de uno a cinco, con evidente daño de la vegetación y animales de los alrededores (Figura 1.11). Actualmente, la temperatura del gas permanece por debajo de 30 ◦C y no se han registrado anomalías térmicas (Gosálvez et al., 2010).

Trabajos previos desarrollados en La Sima (Tabla 1.1) documentaron una menor diversidad en las comunidades protozoarias, con cambios a poblaciones dominadas por Colpodea (ciliados r-estrategas) y una reducción en el porcentaje de ciliados depredadores (Gabilondo y Bécares, 2014). Saénz de Miera (2014) observó que la diversidad y riqueza de comunidad bacteriana decaía notablemente al incrementarse el flujo de CO_2.

En cuanto a la evaluación de riesgos por fugas en CAC cabe mencionar que las cantidades emitidas en el área del Campo Volcánico de Calatrava puede ser del orden máximo de magnitud considerado aceptable para dichas fugas (0,1 % de la cantidad total almacenada por año), en un proyecto de almacenamiento a escala comercial (Elío et al., 2015).

2. Objetivos

La CAC representa una opción de mitigación adecuada para enfrentarse a los niveles atmosféricos de CO_2 en incremento, sin embargo, en la implementación de esta tecnología es de especial relevancia la evaluación de potenciales riesgos sobre el ecosistema terrestre. El objetivo principal de esta tesis es el estudio del efecto de las emisiones subterráneas de CO_2 sobre las comunidades microbianas del suelo. Para el cumplimiento de este objetivo principal se establecieron varios objetivos específicos:

1. Determinar la respuesta de la microbiota edáfica a un gradiente de flujos de CO_2 mediante la caracterización de su funcionalidad, su diversidad genética y la cuantificación de poblaciones específicas.
2. Evaluar la influencia de la estacionalidad en la respuesta microbiana a flujos elevados de CO_2, a través de muestreos temporales en un sistema experimental y en un análogo natural.
3. Valorar el influjo del tiempo de exposición y el sistema de emisiones: comparación de una fuga simulada en un sistema experimental durante un año y una fumarola cuya emisión se remonta a siglos atrás.
4. Estudiar los cambios en las relaciones entre la microbiota y otros grupos edáficos (protistas, mesofauna) debidos a la exposición a CO_2.
5. Verificar la idoneidad de los microorganismos como bioindicadores de posibles fugas de CO_2 desde almacenes geológicos.

Para desarrollar estos objetivos se aplicaron dos enfoques distintos: por un lado un estudio microbiológico en la planta experimental $PISCO_2$, simulando una fuga de CO_2 y por otro, el análisis de una surgencia natural que ha estado emitiendo durante siglos (La Sima, en el Campo de Calatrava). En esta tesis, la investigación desarrollada se presenta en los siguientes capítulos:

En el Capítulo 3, se evalúa el efecto de dos flujos distintos de CO_2 sobre las comunidades microbianas del sistema experimental $PISCO_2$.

En el Capítulo 4, se presenta el estudio de cómo responde la microbiota al amplio rango de flujos de CO_2 de La Sima en el Campo de Calatrava.

En el Capítulo 5, se incluye la evaluación de los efectos del CO_2 de La Sima sobre la micro y mesofauna así como las relaciones de la microbiota y otras comunidades edáficas.

Finalmente, en el Capítulo 6 se desarrolla una discusión general de los resultados obtenidos y se ofrecen las conclusiones de todos los estudios.

3. Resultados y discusión

La captura y almacenamiento de carbono es una opción factible para conseguir una reducción efectiva en las emisiones de CO_2, requerida para cumplir el objetivo del Protocolo de Kyoto en los próximos años. Su aplicación exige una selección cuidadosa de instalaciones de almacenaje adecuadas para evitar fugas no deseadas. Una potencial filtración disminuiría su capacidad como tecnología de mitigación del cambio climático. En relación a esto, han proliferado los estudios de evaluación de riesgos en la última década, que pretenden encontrar indicadores de fugas o que analizan sus efectos sobre el ecosistema terrestre.

En esta tesis se ha hecho uso de varios enfoques para obtener nuevos conocimientos sobre las consecuencias de las emisiones subterráneas de CO_2 en la microbiota edáfica.

Mediante el estudio de diferentes ecosistemas (un sistema experimental y un análogo natural) se intenta responder a las siguientes cuestiones.

3.1. Comparación de flujos de CO_2 experimentales y de origen natural

Para determinar la respuesta de la microbiota edáfica a un gradiente de flujos de CO_2, se estudiaron dos zonas distintas: 1) la planta experimental $PISCO_2$, cuyo objetivo es investigar la influencia de la inyección de CO_2 en suelos para controlar de forma segura el almacenamiento geológico de CO_2, y 2) la fumarola de La Sima, en Ciudad Real, como análogo natural de un depósito geológico con fugas. Con el fin de poder comparar los resultados entre ambos sistemas se utilizó la misma metodología para caracterizar la funcionalidad microbiana, su diversidad genética y para cuantificar las poblaciones microbianas, y se muestrearon los mismos flujos de CO_2 en superficie.

Encontramos distintas respuestas en función del sistema estudiado: mientras que los resultados del $PISCO_2$ (Figuras 3.2 y 3.3) sugieren que las comunidades microbianas no están significativamente afectadas por las emisiones edáficas de CO_2 tras 15 meses de exposición a las mismas, los elevados flujos en La Sima causan un profundo impacto sobre la microbiota (Figuras 4.2, 4.4 y 4.5). Por consiguiente, podemos concluir que no hay respuesta microbiana a corto plazo a bajas emisiones artificiales de CO_2 (hasta 40 l h^{-1}). En nuestro experimento, esta falta de efecto podría ser parcialmente atribuida a la desestructuración del suelo durante el transporte del mismo desde su localización original al sistema experimental. La reconstrucción del suelo puede estar influenciando la estructura de comunidad y este efecto ser más importante que el atribuido al CO_2 (Rygiewicz et al., 2010). No obstante, el factor que más podría haber influido sobre la

falta de efecto son los bajos flujos aplicados en las celdas (20 o 40 l h^{-1}). Otros sitios de inyección controlada como ASGARD (60-180 l h^{-1}) tampoco encontraron respuestas consistentes aunque algunos autores indicaron cambios en las comunidades microbianas tras dos años de exposición intermitente (Smith et al., 2013). En los proyectos de ZERT y Gininderra (Morales and Holben, 2013; Feitz et al., 2014) se documentaron cambios al aplicar CO_2, incluso durante corto tiempo (3 semanas y media, y más de 6 meses respectivamente). Así, la magnitud del flujo de CO_2 sería uno de los factores limitantes para observar una respuesta de la comunidad microbiana. De hecho, flujos mucho más elevados en La Sima sí dieron lugar a poblaciones reducidas y menos diversas (Figura 4.2), con pérdidas de diversidad funcional y una reducción de la actividad metabólica. Igualmente, la escala temporal también ha de ser considerada ya que 15 meses podrían no ser suficientes para visualizar impactos sobre las comunidades edáficas, si tenemos en cuenta los bajos flujos aplicados en $PISCO_2$. A pesar de encontrar alteraciones en las propiedades químicas del suelo (entre ellas, una acidificación significativa en las celdas expuestas a CO_2), éstas aún no se reflejaron en las comunidades microbianas.

3.2. La importancia del tiempo de exposición y los efectos estacionales

La influencia del tiempo de exposición a CO_2 se hace evidente al comparar los resultados de los sistemas experimentales con los de los análogos naturales. Más allá del impacto negativo de la limitación de O_2 sobre las plantas, que provoca una escasez de vegetación alrededor de los centros de emisión, se da una adaptación de las comunidades microbianas a los altos niveles de CO_2 durante periodos prolongados (décadas, siglos). Todos los estudios llevados a cabos en análogos naturales han documentado cambios profundos desde las zonas no influenciadas por el CO_2 a los alrededores o el centro de las fumarolas, donde se establecen especies anaerobias y acidófilas para adaptarse a dicho ambiente. Estos resultados son debidos a la combinación de flujos elevados de CO_2 con emisiones a escala geológica.

En este estudio, los dos sistemas investigados experimentaron respuestas estacionales. En el caso de $PISCO_2$, la mayoría de las variables analizadas tuvieron variaciones estacionales (Figura 3.2), correlacionadas con la humedad del suelo. La actividad microbiana se vio estimulada en estaciones frías, del mismo modo que se incrementaron las poblaciones fúngicas. En La Sima, se observó una clara influencia estacional en la abundancia y actividad microbiana (Figuras 4.2a y 4.2b), esta última estimulada en la estación húmeda. Varios autores han informado sobre distintas respuestas microbianas a las emisiones de CO_2 en función de factores estacionales (humedad y temperatura) (Castro et al., 2010; Frerichs et al., 2013; Morales and Holben, 2013).

3.3. Efecto cascada sobre grupos tróficos superiores

Considerando la escasez de investigación en los efectos de las emisiones de CO_2 en la micro y mesofauna edáfica (sólo Yeates et al., 1999; Russell et al., 2011 y Gabilondo y Bécares, 2014 se han centrado en otros grupos distintos de los microorganismos), decidimos incluir grupos tróficos superiores y su relación con la microbiota en nuestro estudio de La Sima.

Aunque la exposición al CO_2 no afectó a las relaciones entre grupos, el efecto negativo observado en los microorganismos se propagó a través de los múltiples componentes de la cadena trófica del suelo. A medida que los flujos de CO_2 aumentaban observamos una disminución en el número de amebas, una casi desaparición de los nematodos y una abundancia y riqueza de mesofauna reducidas (Figuras 5.1 y 5.2).

Sin embargo, los flujos extremos de La Sima desencadenaron una respuesta diferente. A la vez que las abundancias de microorganismos presentaban tendencias al alza, se percibían aumentos en los números de protozoos (significativos en el caso de los flagelados) y patrones crecientes en la abundancia de mesofauna (Figura 5.2). El déficit de estudios sobre estos grupos dificulta el extraer conclusiones, pero asumimos que tanto la tendencia a la disminución de la micro y mesofauna como la conocida resistencia de los microorganismos a ambientes extremos favorecieron a estos últimos en los flujos extremos de La Sima.

3.4. Biomonitorización de los impactos de la CAC e investigaciones futuras

La aplicación de tecnologías CAC necesita considerar los resultados obtenidos en los estudios de evaluación de impacto, ya que las consecuencias derivadas de una potencial fuga podrían transformar gravemente el ecosistema terrestre, alterando las propiedades químicas del suelo y dando lugar a comunidades alteradas.

En las futuras investigaciones sobre los riesgos de CAC y biomonitorización se deberían considerar varias cuestiones. En primer lugar, son necesarios más estudios sobre la concentración límite de CO_2 para comenzar a apreciar impactos sobre las comunidades edáficas. Para dicho objetivo, las instalaciones experimentales constituyen una buena opción ya que se puede controlar el flujo de CO_2 emitido. También permiten gestionar el tiempo de exposición, otro factor a tener en cuenta, ya que aún ignoramos cuánto tiempo es necesario para observar cambios en las comunidades del suelo. Igualmente, es importante identificar alteraciones específicamente debidas al CO_2 y no a otros factores. En La Sima encontramos efectos significativos sobre las comunidades edáficas pero es difícil determinar si el CO_2 influenciaba directamente a la biota o, por el contrario, las comunidades respondían indirectamente al efecto del CO_2 a través de la modificación de las propiedades químicas del suelo (Šibanc et al., 2014).

Por otra parte, estudios futuros podrían centrarse en el análisis de grupos específicos de microorganismos que pudieran beneficiarse de incrementos en la concentración de CO_2 en el suelo –metanógenos u otras especies anaerobias–. Además, sería preciso considerar otros grupos tróficos además de la microbiota para ampliar el conocimiento sobre las relaciones tróficas en un ambiente de emisiones de CO_2. En relación a esta cuestión, las fumarolas ofrecen un ecosistema extremo donde mejorar nuestros conocimientos sobre especies resistentes.

3.5. Conclusiones

- Los flujos elevados de CO_2 (más de 200 g m^{-2} d^{-1}) tienen un fuerte impacto negativo en la biota edáfica, provocando pérdidas de diversidad y una reducción en abundancia o actividad. Estos cambios sólo han sido observados en una surgencia natural de CO_2, donde los organismos del suelo han estado expuestos a altas emisiones del gas durante siglos. Los flujos extremos (más de 10^4 g m^{-2} d^{-1}) revierten este efecto negativo, las condiciones del suelo en estos puntos favorecen el incremento en el número de microorganismos y sus predadores. En el sistema experimental sin embargo la exposición a corto plazo a bajos flujos de CO_2 no tuvo efecto sobre las comunidades microbianas.

- Existe un notable efecto estacional sobre las respuestas de los microorganismos, principalmente debido a la humedad del suelo, tanto en el experimento $PISCO_2$ como en la fumarola de La Sima. Las estaciones frías y húmedas estimularon la actividad metabólica microbiana en ambas zonas de estudio. No obstante, el efecto estacional sobre las abundancias varió: en $PISCO_2$ no hubo resultados consistentes, mientras que en la sima se incrementaron considerablemente en las estaciones secas.

- La comparación entre una fuga de CO_2 simulada durante un año y una emisión natural cuyo origen se remonta a siglos pasados reveló la influencia de la escala de tiempo sobre el impacto del CO_2 en la biota edáfica. Mientras que en el sistema experimental $PISCO_2$ no se encontraron efectos significativos, La Sima mostró comunidades adaptadas a sus flujos extremos y abundancias y actividades negativamente afectadas en los flujos altos de CO_2. La falta de respuesta en $PISCO_2$ no debe ser solamente atribuida a una corta exposición al gas sino también a los bajos flujos de CO_2 aplicados.

- El efecto de las emisiones elevadas de CO_2 también se observó en otros grupos edáficos, como los protozoos, nematodos y mesofauna de La Sima. En general, siguieron los mismos patrones que la microbiota, viéndose negativamente afectados por el aumento en los flujos de CO_2, y mostrando una tendencia a recuperarse en relación a las emisiones más extremas.

- Los microorganismos pueden ser usados para monitorizar las fugas potenciales de CO_2 si se consideran flujos elevados de CO_2 y un tiempo mínimo de exposición al gas. A corto plazo, se necesitaría flujos relativamente elevados (más de 160 g m^{-2} d^{-1}) para comenzar a observar alteraciones en las comunidades microbianas, mientras que a largo plazo el efecto ya se identificaría con dichos flujos. En consecuencia, la biomonitorización de la CAC mediante biota edáfica estaría indicada para fugas leves que no han sido detectadas durante largos periodos o bien escapes de CO_2 de gran flujo.

Abstract

Burning of fossil fuels is the major source of CO_2 emissions. Consequently, the greatest efforts are directed towards alleviate the emissions from this sector, with a portfolio of actions including 1) increased energy supply from renewable and nuclear sources, 2) increased energy efficiency and 3) a decrease of the carbon intensity of fossil fuels (the ratio of CO_2 emissions from fuel combustion over Growth Domestic Product –GDP–). The latter option includes a new technology: Carbon Capture and Storage (CCS). CCS consists of a process of separation of CO_2 from industrial and energy-related sources, followed by the transport to a storage location, where CO_2 is long-term isolated from the atmosphere. Reservoir integrity, cap rock resistance and the trapping mechanism will determine the retention time of CO_2. Therefore, the storage location is selected considering these characteristics, to avoid any CO_2 potential leakage.

Nonetheless, the implementation of this technology involves the assessment of potential leakages from storage sites and their consequences, with the aim of preventing irreparable damage to the ecosystem, and setting up monitoring and control measures. The great diversity of soil microorganisms as well as their relevance in soil processes and their extreme sensitivity to changes in the environment, make them excellent quality indicators of the soil ecosystem.

The main objective of this thesis was the study of the consequences of a potential leak from a geological storage on soil microbial communities. The microbiota from an experimental plant (the $PISCO_2$ experiment), which simulates a controlled CO_2 escape, was analysed. Simultaneously we studied the microbial communities from a natural CO_2 emission area (La Sima, Campo de Calatrava) to compare an experimental system with a natural vent. At both sites sampling points were selected according CO_2 fluxes: at $PISCO_2$ we studied low fluxes (50 g m^{-2} d^{-1}) and high fluxes (100 g m^{-2} d^{-1}), and also a control with no CO_2 exposure (10 g m^{-2} d^{-1}). At La Sima, we analysed fluxes of 10 (control), 50 (low) and ca. 900 g m^{-2} d^{-1} (high), also including an extreme flux area (more than 10,000 g m^{-2} d^{-1}). The specific objectives of this work were 1) to characterise microbial functionality (by BIOLOG Ecoplates), genetic diversity (by DGGE) and abundances (by qPCR) at both sites, 2) to assess the influence of seasonality, time exposure and type of emission (artificial or natural) on microbial responses, and 3) to observe the effect of CO_2 on relationships between microbiota and other edaphic groups (protists, nematodes, mesofauna). As final objective, the strength of microorganisms as bioindicators of potential CO_2 leakages from geological storage sites was discussed.

Results varied depending on the emission site. At $PISCO_2$ experimental system, after 15 months of exposure to relatively low CO_2 fluxes, the microbial communities were not significantly affected in any of the variables studied. Natural emissions at La Sima, however, caused a profound impact on soil microbial abundances and richness. We observed a significant reduction in bacteria, archaea and fungi gene copy numbers at high CO_2·fluxes (260-600 g m^{-2} d^{-1}), losses in bacterial genetic diversity and a decrease in potential metabolic activities. Even if CO_2 exposure at La Sima did not affect relationships between groups (i.e. bacteria/protozoa, protozoa/mesofauna), the negative effect of increasing CO_2 on soil bacteria, archaea or fungi was accompanied by a reduction in amoebae numbers, almost disappeared nematodes and diminished mesofauna richness and abundance. However, at extreme fluxes in La Sima (more than 10^4 g m^{-2} d^{-1}) metabolic activity and abundances of most of studied groups recovered, even though diversity losses were maintained, suggesting that the extreme CO_2 conditions are associated with high abundances of well- adapted communities, although with very low diversity.

A factor that might explain the different results between $PISCO_2$ experiment and La Sima vent was the time exposure to CO_2. La Sima have been emitting for several centuries, probably millennia, so that edaphic communities might have adapted to these extreme conditions. Most of soil biota was less diverse (bacteria, ciliates, mesofauna) and less abundant (bacteria, fungi, amoebae, nematodes and mesofauna) when increasing CO_2 flux, and at extreme fluxes (more than 10^4 kg m^{-2} d^{-1}) biota richness was deeply affected. These results may be due to the combination of high CO_2 fluxes with geological timescale emissions. Seasonal variations also influenced community responses to CO_2 exposure, both at La Sima and at $PISCO_2$ site. Soil moisture in particular was related to changes in microbiota numbers (fungi at $PISCO_2$, bacteria and archaea at La Sima) and significantly stimulated bacterial metabolic activity.

With respect to biomonitoring CCS projects, edaphic biota may be considered indicator of CO_2 leakages from geological storages: a reduced microbial diversity or low densities of higher trophic levels (protists, nematodes or mesofauna) might reveal increments in soil CO_2 fluxes. However, we should take into account that several factors will determine their responsiveness, i.e. CO_2 flux intensity, time exposure and seasonal variations. Accordingly, biomonitoring of CCS using soil biota may be used to assess small leaks that have been undetected for a long time or leaks with very high CO_2 fluxes. Therefore, future research should focus on identifying 1) the time exposure required to appreciate the impact of small leaks on edaphic communities, and 2) the CO_2 threshold from which large leaks can be quickly detected.

.

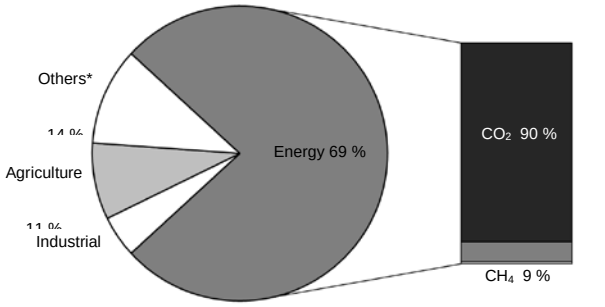
1 Introduction

1.1 Climate change and CO_2 emissions

Warming of the climate system is unequivocal. Intergovernmental Panel on Climate Change's (IPCC) fourth assessment report (IPCC, 2007) stated that "most of the observed increase in global average temperatures since the mid-20th century is very likely due to the observed increase in anthropogenic greenhouse gas (GHG) concentrations", being carbon dioxide (CO_2) the most contributing gas to GHGs concentration in atmosphere.

Since pre-industrial era, atmospheric CO_2 concentrations have growth about 40 %, with an average growth of 2 ppmv/year in the last ten years (IEA, 2014). The latest global measure reaches 400.11 ppm (NOAA, June 2015). By the end of the 21st century, these increasing concentrations could range between 535 to 983 ppm in the atmosphere. Without mitigation, and considering methane and other GHG emissions, these changes may cause an increase of 1.1–6.4 °C in 2090-2099 relative to 1980-1999. Taking into account the correlation between the amount of CO_2 in the atmosphere and earth surface temperature, scientific community have suggested setting goals to limit concentrations to 450-500 ppm, in the hope of limiting global temperature increases to a 2 °C target (maximum allowable warming to avoid dangerous anthropogenic interference in the climate) (RISCS, 2014).

Energy use is the major source of CO_2 emissions, with burning of fossil fuels as the greatest contributor (Figure 1.1) (IEA, 2014). Fossil fuels combustion are responsible for 2/3 of the emissions of CO_2 since the beginning of industrial revolution (Global Carbon Atlas, 2015). In 2013, 36 Gt CO_2 were released from the burning of fossil fuels.



*Others include large-scale biomass burning, post-burn decay, peat decay, indirect N_2O emissions from non-agricultural emissions of NO_x and NH_3, waste and solvent use

Source: IEA estimates for CO_2 from fuel combustion and EGDAR 4.2 FT2010 estimates for all other sources.

Figure 1.1 Shares of global anthropogenic GHG, 2010 (IEA, 2014)

In Spain, during the same period, 240 Mt of CO_2 were emitted from fossil fuel sources. (OECD, 2015a). Spain's carbon intensity (the ratio of CO_2 emissions from fuel combustion over Growth Domestic Product –GDP–) has decreased by more than 20 % since 2000, placing Spain among the 10 OECD member countries with the lowest CO_2 intensities (OECD, 2015b). The reasons for this decline are first, the increasing share of renewable energies and more rigorous measure for energy efficiency and second, the deep impact of global economic crisis in Spain. Industry –including the energy sector– and the transport sector are the largest consumers of energy and major sources of GHG emissions, and CO_2 accounts for more than 80 % of GHG emissions (Figure 1.2). Thereby, Spain did not achieve its target under the Kyoto Protocol for 2008-2012 (15 % above 1990 level), and in 2012 emissions reached 356 Mt, exceeding in 15 % the mentioned target (MAGRAMA, 2013). Expectations for 2020 are that GHG emissions will situate 9 % above the target (EC, 2014).

Considering this disturbing future, it is essential to develop different measures to enable us to meet our EU and global climate change objectives (emissions cuts of 80-95 % below 1990 levels by 2050). The IPCC suggest a portfolio of actions to reduce CO_2 emissions, including increased energy supply from renewable and nuclear sources, increased energy efficiency and a decrease of the carbon intensity of fossil fuels (IPCC, 2007). The latter option includes the implementation of a new and innovative low-carbon technology: Carbon Capture and Storage.

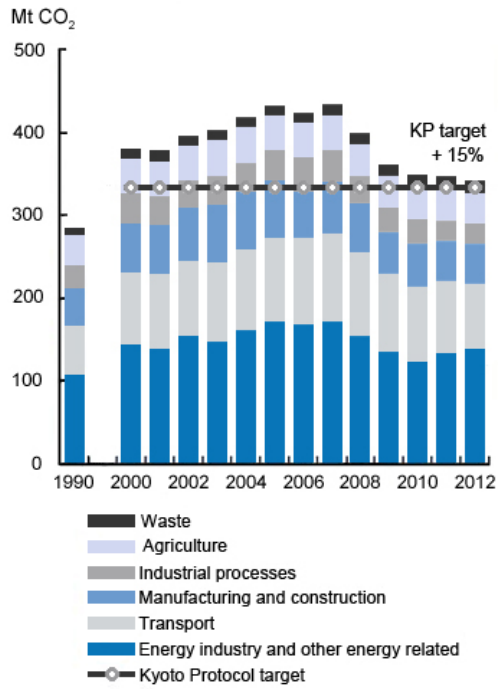


Figure 1.2 Spain's GHG emissions and Kyoto Protocol target for 1990-2012 period, by sector. Source: (OECD, 2015b).

1.2 What is Carbon Capture and Storage?

Carbon dioxide capture and storage (CCS) is a process consisting of the separation of CO_2 from industrial and energy-related sources, transport to a storage location and long-term isolation from the atmosphere (IPCC, 2005). IPCC emphasizes CCS as a key technology to enable cuts in the emissions from energy production and the industry; its contribution to global reduction may be of 20-30 %. It must be borne in mind that energy use efficiency and increasing share of renewables cannot achieve the emission reductions required to reach long-term atmospheric stabilization targets, therefore CCS can be considered a potential element of a strategy to substantially reduce global anthropogenic CO_2 emissions in the short-term (Damen et al., 2006). Furthermore, a fifth of all CO_2 emissions comes from industrial processes and, while some of these can be improved or replaced to reduce their impact, these emissions cannot be completely eliminated as long as we need the products they produce (ZERO, 2015).

CCS process begins with CO_2 capturing from the exhaust gases of large emitters, such as power plants, natural gas production platforms and industry. Three methods of CO_2 capture are currently being investigated: 1) postcombustion that separates the CO_2 with the use of chemical solvents, 2) precombustion, which chemically strips off the

carbon, leaving hydrogen to burn, and 3) oxyfuel combustion that burns coal or gas in denitrified air to yield only CO_2 and water (Haszeldine, 2009). After that, the captured CO_2 is pressurized to 70 bar, forming a liquid that can be transported by pipe or ship to a storage site (Figure 1.3).

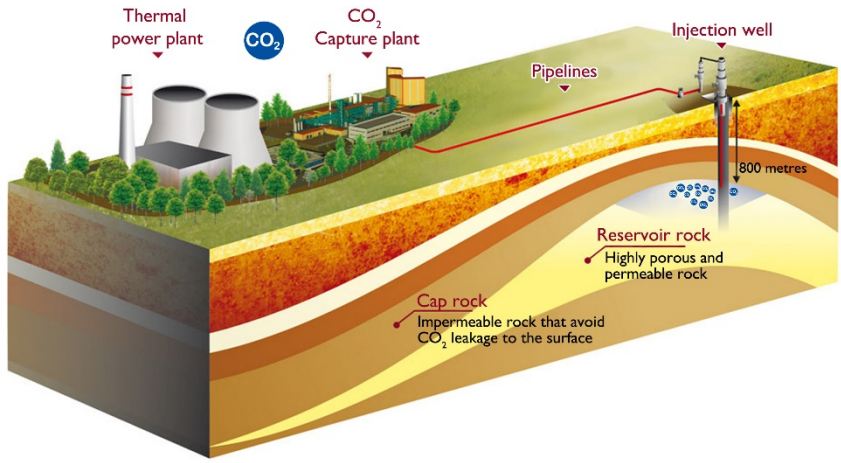


Figure 1.3 Full CCS process. CO_2 is captured from industrial and energy-related sources and transported through pipelines to the selected geological storage site, where is injected more than 800 m deep (modified from CIUDEN.es http://www.ciuden.es/index.php/en/tecnologias/tecnologias-cac)

CO_2 can be stored in different geological formations including depleted oil and gas reservoirs, unmineable coal seams, and deep saline reservoirs (deep underground porous sedimentary rocks saturated with brackish water or brine), at depths below 800-1,000 m (Figure 1.4). It also can be used for Enhanced Oil Recovery (EOR). At temperatures above 31.1 °C and pressures above 72.8 bar (those typical of reservoir rocks at depths of more than about 800 m), CO_2 forms a relatively dense fluid phase, a supercritical fluid. The consequent decrease in volume above those temperature and pressure values allows more CO_2 to be injected into the same pore space (RISCS, 2014). This way we obtain the efficient utilization of storage space in the pores of sedimentary rocks. Another requisite is that these geological formations have an extensive cap rock or barrier at the top of the formation to contain the CO_2 permanently. IPCC Special Report on CCS (IPCC, 2005) concluded that appropriately selected and managed geological reservoirs are 'very likely' to retain over 99 % of the sequestered CO_2 for longer than 100 years and 'likely' to retain 99 % of it for longer than 1,000 years. How much could be stored? Deep saline formations offer the biggest storage capacities (10000 Gt CO_2), depleted oil and gas reservoirs have a capacity of 675-900 Gt CO_2 and unmineable coal formations vary between 3-200 Gt.

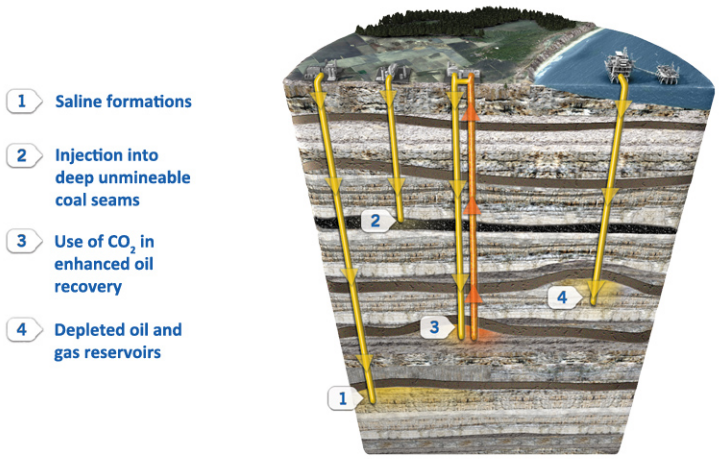


Figure 1.4 Overview of storage options for CCS technology (modified from Global CCS Institute).

1.2.1 CCS in Spain

CCS is still in its early stages in Spain. The "Fundación Ciudad de la Energía" (CIUDEN) is the Spanish Government's principal instrument for developing CO_2 capture, transport and storage technologies, through the Compostilla-OXY-CFB-300 Project (www.compostillaproject.eu/en). The project, which ended in 2013, was the only one of its kind (a full chain CO_2 large-pilot scale including capture by oxy-combustion, transport and geological storage in saline aquifers) endorsed by the European Energy Recovery Programme (EERP). This Spanish project is composed of a set of actions being developed in coordination between the CIUDEN and the Spanish utility ENDESA (CIUDEN, 2009). It involves the Technology Development Centre for CO_2 Capture located in the village of Cubillos del Sil (Leon, north west Spain), close to the existing Compostilla power plant, and the Hontomín CO_2 Storage Technology Development Plant (TDP), located 250 km eastwards, in the province of Burgos (north central Spain). Considered one of the best options for geological storage in the country, Hontomín is an on-shore saline aquifer (1,600 m depth) with a capacity of 100,000 tonnes of CO_2. The Hontomín site represents an opportunity to develop injection and CO_2 storage-related activities in a low permeability, carbonate deep saline aquifer – which is a novel approach for dedicated geological storage, and could provide learnings for potential storage operations in other 'less than ideal' sites (Global CSS Institute, 2015).

1.2.2 Risks associated to CCS: potential leakages and consequences for environment

There is a large body of evidence that a CO_2 storage site can be chosen so that the probability of leakage will be very low (RISCS, 2014), i.e. natural accumulations of CO_2 which remained stable for millions of years, experiments that shown the integrity of caprocks (Bennion and Bachu, 2007) or experience with CO_2 storage projects (Sleipner in Norway, In Salah in Algeria, Weyburn in Canada or Ketzin in Germany). However, a key factor affecting the implementation of CCS are the risks associated with underground CO_2 storage. Risk assessment is a first step in a strategy to set up management and control measures to minimise risks of underground CO_2 storage. In addition, it helps to facilitate the formulation of standards and regulatory frameworks required for large-scale application of CCS (Damen et al., 2006). The EU has therefore established a legal framework for the environmentally safe geological storage of carbon dioxide (Directive 2009/31/EC), to ensure that the environmental and human health impacts are minimised, and the climate integrity of the technology is assured. Spain transposed the EU CCS Directive into its legal system through Law 40/2010.

When CO_2 is injected in geological reservoirs, it might potentially migrate out of the reservoir through the subsurface, migrate laterally in overburden formations and finally leak into the atmosphere/biosphere. The potential for leakage will depend on well and cap rock (seal) integrity and the trapping mechanism. CO_2 can be retained in reservoirs by means of different trapping mechanisms; in the case of deep saline aquifers -as Hontomín- might take thousands to millions of years to migrate from injection point to the surface due to the extremely low flow rates encountered in these formations (hydrodynamic trapping). There are also other different trapping mechanisms in deep saline aquifers (Figure 1.5).The permeability of the overburden – the formations above the target reservoir– is another critical factor for leakage, since it determines the retention time of CO_2 in the subsurface (Damen et al., 2006). As advantage, deep saline aquifers have a lower potential for CO_2 leakage through/along wells, since they are not of economic interest and have a lower number of penetrating wells. Nonetheless, the space to store CO_2 only becomes available as a result of compression of the fluids and rock in the reservoir, or displacement of formation water into adjacent formations or to the surface (Holloway, 1996). This will cause a pressure increase that might trigger fracture zones, which might end up in CO_2 migrating upwards (Over et al., 1999). The main leakage transport mechanism would be diffusion through the cap rock, though very slow; modelling indicates that this process will take millions of years (Lindeberg and Bergmo, 2003). Furthermore, model simulations of Sleipner storage site showed that mineral precipitation caused by CO_2 would have decreased the porosity and permeability of the cap rock base (Johnson and Nitao, 2003).

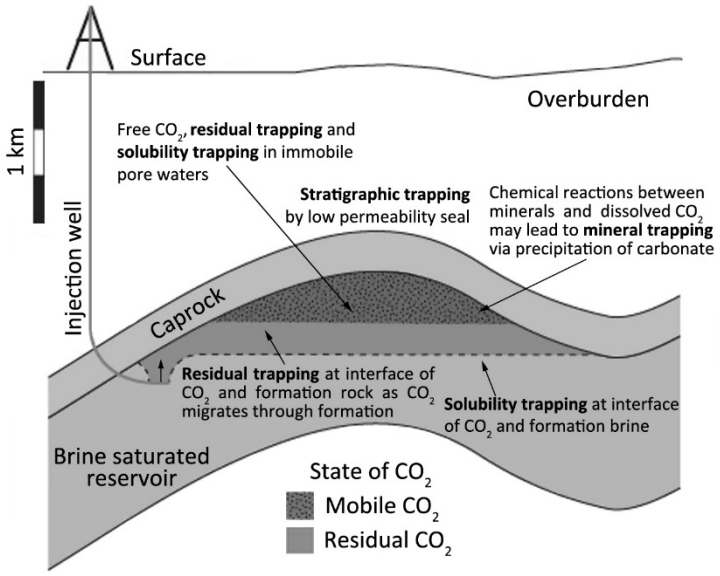


Figure 1.5 Geological trapping mechanisms in a saline reservoir (Burnside and Naylor, 2011).

Nonetheless, if a leakage from a geological storage does occur, potential hazards could derive from high CO_2 concentrations in the environment (Figure 1.6). For humans and the majority of air-breathing animals, it is known that at concentrations above 2 %, CO_2 has a strong effect on respiratory physiology and at concentrations above 7–10 %, it can cause unconsciousness and death (Benson et al., 2002; IPCC, 2005). In addition, environmental and ecological risks can reach local or global consequences: from CO_2 dissolution in usable aquifers or potable groundwater (resulting in pH reduction and metal elements mobilization) to CO_2 escape to environment, causing changes in lower pH, impacted chemistry of nutrients and therefore, an altered soil ecosystem and plant growth. Although plants usually have a higher resistance against CO_2 than mammals, persistent leaks could suppress respiration in the root zone. Other consequences include the effects derived from fluid displacement in the reservoir (brine or other gases), CO_2 invasion of other subsoil resources or elevation of the topographic surface due to changes in subsoil geomechanic properties (PTECO$_2$, 2012).

It is important to consider that a potential leakage of CO_2 from reservoirs would make CO_2 storage less effective as mitigation option. Therefore, the crucial question is what leakage rates are acceptable to ensure the stabilisation of atmospheric greenhouse gases concentrations in the coming century (450–750 ppm). Most of authors seem to agree that the mean annual leakage rate should not exceed 0.1 % (Damen et al., 2006). A careful site selection, an effective regulatory oversight, an appropriate monitoring program and the implementation of remediation methods to stop or control a release are different ways to avoid a potential leakage (IPCC, 2005).

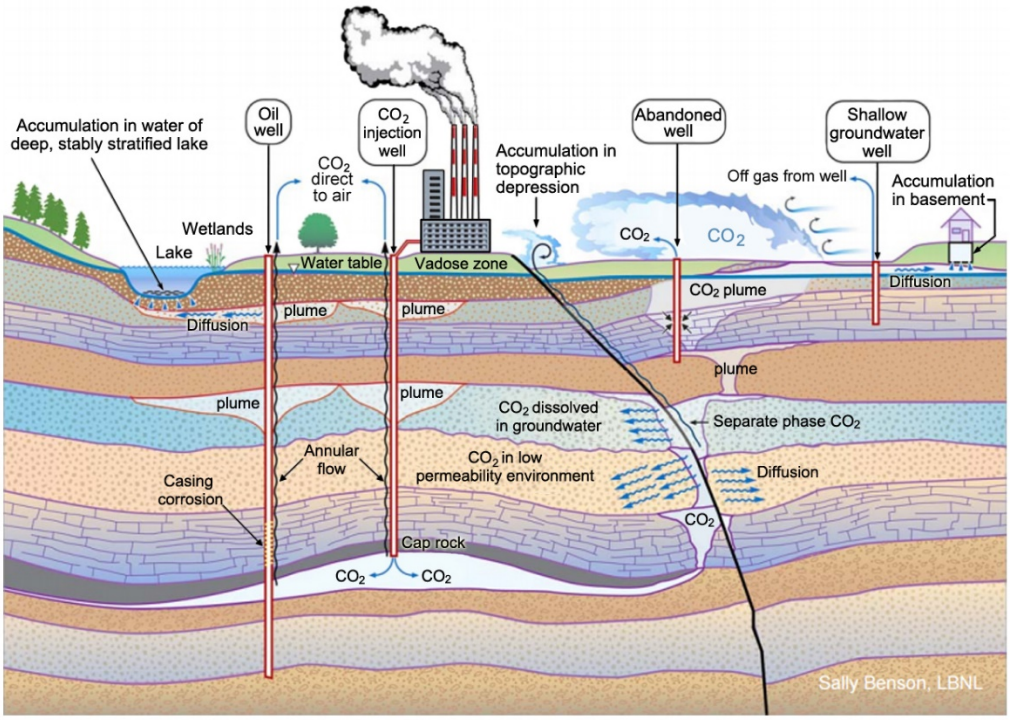


Figure 1.6 Potential mechanisms of leakage from a geological storage site and derived consequences (Sally Benson, LBNL)

Several factors will influence the impacts of a potential CO_2 leakage such the characteristic of the affected area (geology, soil type, topography and climate) or the weather conditions at that moment (e.g. soil moisture, wind speed, air temperature). These factors can affect the flux and concentrations of CO_2 in the soil; e.g., preferential pathways will cause locally higher CO_2 concentrations with greatest impact, or high moisture can increase soil CO_2 due to impeded gas escape. Therefore, soil CO_2 level might show a strong seasonal variability (RISCS, 2014).

CCS implementation in the last decade has triggered the research on CO_2 potential leakages and its derived effects on soil (and marine) environments, including their inhabitants. Previous research on rising atmospheric concentrations of CO_2 appears to have little relevance to the impacts of a potential leakage, as soil CO_2 concentration is much higher than those atmospheric increments. Recent studies (e.g. RISCS, 2014) corroborate that increased CO_2 soil concentration have a much more marked impact than slight rises in atmospheric levels (Jones et al., 2015). For this reason, some studies take advantage from natural occurring CO_2 vents as surrogates, whereas others focus on experimental injections sites to study impacts on soil, plants or microorganisms.

1.3 Why assess soil (micro)biota communities in CCS process?

On one hand, it is about their ecological relevance. Soil has the greatest diversity of microbial communities on Earth (Torsvik et al., 2002), and microbial transformations are fundamental to the operation of the soil ecosystem, as they are responsible of the main nutrient cycling processes and the maintenance of soil structure (Prosser, 2007). Moreover, soil also presents a huge spatial heterogeneity, leading to high variability of soil microorganism in a few meters, which are extremely influenced by edaphic properties such as pH, vegetation or anthropogenic impacts (Fierer and Jackson, 2006). To date, only a relatively small number of studies on microbial responses to CO_2 leakage are available, hence further research is needed to identify changes specific to CO_2 rather than other factors (Jones et al., 2015). Moreover, extreme environments (like a CO_2 emitting vent) represent a proved resource of unique microorganisms that exhibit interesting metabolic strategies and could help to elucidate the role of species in ecosystem function (Maier and Neilson, 2015).

On the other hand, microorganisms can be extremely sensitive to changes in soil characteristics acting as good indicators of soil quality (Winding et al., 2005). They will be the first point of biological contact with toxic and/or beneficial chemicals entering the soil and groundwater as a result of CCS leakage so that may display the first changes that can be detected by monitoring for effects other than CO_2 (Noble et al., 2012). Any change associated with CO_2 leakage (changes in pH, Eh, salinity, temperature, metal mobilisation) can influence the abundances and activities of microorganisms, thus providing early indicators. Furthermore, given the diversity, highly responsive indicator species for specific habitat types are likely to be present.

Leaving out soil physical or chemical properties, microbial communities are strongly influenced by their predators. There is evidence that trophic cascades can be of functional significance for shaping microbial communities and influencing plant nutrient supply. For example, it is of special relevance the role of protozoa as a result of their feeding preferences and high consumption rates (Clarholm et al., 2007). Considering CCS risk assessment, it is crucial to gain knowledge not only in the impact on microorganisms, but also in how the relationships with other soil biota groups will be changed. In this context, little research has been conducted about soil CO_2 increments on higher trophic levels (protists, micro or mesofauna).

1.3.1 Methods used in this work

Several methodologies are available to study soil microbial communities, taking into account their complexity. Instead of using culture-dependent methods, which only allow to study a small part of the microbiota (0.1-10 % of the total microorganisms of a given environment), we decided to combine molecular approaches to have a more

complete view of CO_2 impacts on microbial communities: quantitative polymerase chain reaction (qPCR) and denaturing gradient gel electrophoresis (DGGE).

To assess the occurrence of microorganisms in soil environments, the qPCR offers excellent sensitivity and is able to quantify specific populations by using selected primers. However, as all PCR-based methods, there are biases associated with nucleic acid extraction, primer specificity and general PCR conditions (Nybroe et al., 2007). In addition, soil humic acids can act as PCR inhibitors, but continuous optimisation of assays protocols can avoid this problem. DGGE is a molecular community fingerprinting technique based on different melting behaviour of the double-stranded PCR products due to differences in the primary structures of the target rRNA gene fragments (Oros-Sichler et al., 2007). As result, we obtain a gel with a band profile: each band theoretically representing a different microbial population in the community. This method allows to estimate the community diversity (number of bands in the fingerprint) and to be combined with additional analysis, i.e. excision of selected bands to sequence them can lead to identification of the phylogenetic affiliation of the ribotypes that make up a band. As drawbacks, the method only amplifies DNA of dominant organisms in the population (0.1-1 % of the total potential targets) and also, the known caveats of using 16S rRNA gene as marker (one organism can contain several copies of the gene-several bands on the gel-, bands of different organisms can overlap). However, this rapid and relatively low-cost technique represents a very suitable tool for investigating population dynamics of microbial communities in soil, which makes it perfectly adequate for our purpose.

We also introduce a little explored method in CCS risk assessment of soil ecosystem: BIOLOG EcoPlates™. The so-called community level physiological profiling (CLPP) provides a potential indicator of ecosystem function, metabolic potential and functional diversity of the microbial communities in soil (Timms-Wilson et al., 2007). The BIOLOG EcoPlate is a microtiter plate of 96 wells that contains 31 of the most useful carbon sources for soil community analysis, repeated 3 times as replicates. Each well also contains a tetrazolium dye so that formation of purple colour occurs when the microbes can utilize the carbon source and begin to respire. In this way, these plates can measure the range and scope of carbon sources that the particular environmental community tested can potentially use, through BIOLOG EcoPlate pattern development (similarity), the rate of colour change in each well, or the richness of well response (diversity). We have to keep in mind that this technique mainly reports on the "potential" activity of the microbial community rather that its "in situ" activity, and that many organism are unculturable. Nonetheless, BIOLOG EcoPlate provides an easy, low-cost method to evaluate microbial metabolic profile.

The details of the implementation of these techniques are explained in the following chapters.

1.4 Experimental research on CO_2: engineered systems

To date there are few experiments developed in engineered systems simulating a controlled CO_2 leakage from a geological storage: ASGARD (UK), ZERT (USA), Grimsrud farm (Norway), Resacada Farm (Brazil) and Gininderra (Australia). Some of them have evaluated CO_2 effects on microbial communities (Table 1.1), while Grimsrud farm only evaluated plant response and Brazil's project have not published yet its microbiological monitoring. Additionally, in some CO_2 storage sites biological studies have been developed. Floral and microbiological baseline studies were undertaken at In Salah, Algeria (Jones et al., 2011). At Ketzin, Germany, Morozova et al. (2011) showed changes in deep microbiota (inside the injection well) comparing before and after CO_2 injection samples. Nevertheless, in this work, we will focus on CO_2 impacts on soil subsurface communities. In this context, most of the monitoring and risk assessment studies have taken place in natural analogues. Experimental approaches, however, allow injection rates to be controlled and, unlike laboratory experiments, introduce real world complexity (Jones et al., 2015). Per contra, the impact of CO_2 may be overlain to varying degrees of stress such as weather conditions or other factors introduced by the environment. Therefore, results of CO_2 impact on soil microbiota are diverse depending the experimental site studied and they are highly influenced by seasonality. Hence, in ASGARD found that after 2 years of exposure, results were not clear, as the variations observed may not result from CO_2 injection but from changes in weather conditions (Jones et al., 2015) and Morales and Holben (2013) reported seasonal-dependent changes at ZERT.

In this thesis, the $PISCO_2$ experimental site was studied, which belongs to Compostilla-OXY-CFB-300 Project.

1.4.1 A case study: The $PISCO_2$ project

The $PISCO_2$ project is an experimental system for the research of the influence of CO_2 injection in soils, located at the Technology Development Centre for CO_2 Capture located in the village of Cubillos del Sil (León), within the facilities of the CIUDEN foundation (Figure 1.7). The main objective of this project is to develop economic and ecological biomonitoring tools for safety control of CO_2 geological storage. In contrast to natural analogues where CO_2 has been emitting for long periods, in the $PISCO_2$ project, the effects on non-adapted flora and fauna are tested. Furthermore, it is well known that large leaks like those presented in natural vents cause visible changes at the centre and surroundings but, what if the CO_2 leak was slighter so that surface concentrations rose gradually without visible changes? This is the basic idea of $PISCO_2$ and the main difference with other experimental systems that use much higher CO_2 fluxes (Table 1.1).

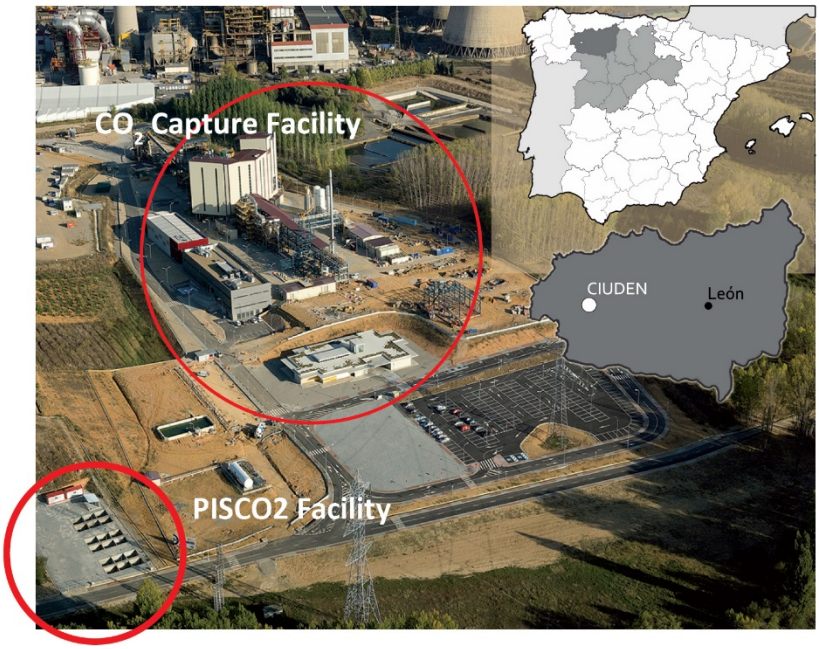


Figure 1.7 CIUDEN's facilities: CO_2 capture technology plant and $PISCO_2$ experimental system locations (modified from CIUDEN, 2015).

Two different CO_2 levels (20 and 40 l h^{-1} CO_2) were applied through two grills at depths of 1 and 2 m below ground level. There were 8 plots that remain without injection as controls. The treatments were assigned randomly to each experimental cell. In comparison with similar experiments (Smith et al., 2013), in this system a diffuse and continuous flow of CO_2 was assessed through underground CO_2 releasing grills, providing homogeneous fluxes on the surface of the cells. The emission of CO_2 was constant and without interruption during the whole experiment. The gassing started four months before the soil was placed in the plots to test the spatial heterogeneity of CO_2 fluxes in each of the plots.

Figure 1.8 PISCO$_2$ experimental plant. Photographs from part of the plots (the beginning of the experiment and the first sampling). Detail of the sampling procedure. After each sample was took, the hole was immediately filled with soil from the same location to avoid undesired CO_2 leaks.

A more detail description of the cells and the soils exposed to CO_2 is given in the chapters 3 and 4.

Other researchers have studied CO_2 impacts at PISCO experimental site. Gabilondo et al. (2015) report on CO_2 effect on edaphic protozoa, finding no clear patterns in the abundance of amoebae, ciliates, or flagellates in response to the injection of CO_2, whereas composition of the ciliate community (Equitability, Margalef index, or the *Colpodea* to *Polyhymenophorea* ratio) showed significant differences with increases in CO_2. Sáenz de Miera et al. (under revision) investigated composition and structure of soil bacterial communities by high-throughput sequencing of 16S RNA genes. They observed shifts in bacterial community composition, but no consistent effects on the richness and diversity in one of the soil types studied, even at low CO_2 flux exposure.

Table 1.1 CO_2 vents and experimental injection sites (*) where biological studies have been developed. MPR: (potential) methane production rates, MOR: methane oxidation rates, SRR: sulphate reduction rates, CPR: CO_2 production rates (in aerobic conditions), AOA: ammonia-oxidizing archaea, SRB: sulphate reduction bacteria. ATP: adenosine triphosphate

Location	Fluxes	Concentrations (depth)	Studied group	Methodology used	Main effects observed	References
Cheb Basin, Czech Republic	> 28,000 l h^{-1}	> 99.83 % (60 cm)	Microbiota	Pyrosequencing, acetogenic/ methanogenic	Lowered diversity, favoured acidotolerant, and anaerobic methanogens and acetogens	Beulig et al., 2015
		20-100 % (average of 10, 20, 40 and 60 cm)	Collembola	Berlese-Tullgren funnels	Decreased richness, moffetophilous species, abundances increased when CO_2 low fluxes	Russell et al., 2011
Florina, Greece	40-10,000 g m^{-2} d^{-1}	8- 90 % (20 cm)	Microbiota	qPCR, MPR	Trends to decrease in bacterial and archaeal numbers (65 cm deep), increased methanogenesis	Ziogou et al., 2013
Hakanoa Springs, New Zealand		0.04-0.39 % (atmospheric, 20 cm in height)	Nematoda	Whitehead tray method	Decrease in abundance and diversity	Yeates et al., 1999
Laacher See, Germany	177-550 g m^{-2} d^{-1}	4-30 % (15 cm) 20-90 % (60 cm)	Microbiota	qPCR, DGGE, MPR, MOR, SRR, CPR	Increased AOA (Thaumarchaeota) and SRR; decreased methanogenic archaea and methane oxidation	Frerichs et al., 2013
		20-90 % (60 cm)	Microbiota	qPCR, MPR, MOR, SRR, CPR	Lower bacterial numbers, Archaea increased. Reduction in methane oxidation, favoured methanogenesis	Krüger et al., 2009; 2011

Latera Caldera, Italy	Up to 2,000-3,000 g m^{-2} d^{-1}	Up to 93 % (20 cm)	Microbiota	qPCR, DGGE, MPR, MOR, SRR, CPR	Decreased Bacteria, Archaea and Eukarya numbers, enhanced methanogens and SRB	Oppermann et al., 2010
	10-2,000 g m^{-2} d^{-1}	10-100 % (20 cm)	Microbiota	ATP biomass, bacterial counts, qPCR, MPR, MOR, SRR	Lowered microbial biomass, bacterial and archaeal abundances, increased SRR	Beaubien et al., 2008
Mammoth Mountain, USA	25 to >6,000 g m^{-2} d^{-1}	< 1 to > 90 %	Microbiota	PLFA, ARISA, qPCR	Lowered microbial biomass, fungi highly affected, low abundance of bacteria and fungi gene copies	McFarland et al., 2013
Muszyna, Poland		> 25 %	Microbiota	Bacterial culture	*Clostridium kluyveri,* Thiobacillus and nitrification as possible bioindicators	Tarkowski, 2008; 2009
Sima de Calatrava, Spain	0.57 g m^{-2} d^{-1}-547 kg m^{-2} d^{-1}		Microbiota	Pyrosequencing	Relative abundance of Chloroflexi increased, Acidobacteria, Verrucomicrobia and Gemmatiomonadetes decreased	Sáenz de Miera et al., 2014
	14.7-1,163 g m^{-2} d^{-1}		Protozoa	Protozoan culture and counting	Shift to Colpodea dominated community, lowered diversity, decreased rapacious ciliates	Gabilondo and Bécares, 2014
Stavešinci, Slovenia		0.2 %-13.8-85.5 % (15 cm)	Microbiota	T-RFLP, clone libraries	Changes to anaerobic; increased Methanomicrobia, lowered Thermoplasmata , Cloroflexi, Firmicutes	Šibanc et al., 2014
	228 - 400 µmol m^{-2} seg^{-1}		AM fungi	T-RFLP, pyrosequencing, clone libraries	Dominance of specific taxa in hypoxic soil, community turnover (beta diversity)	Maček et al., 2011

Table 1.1. (continued)

Location	Fluxes	Concentrations (depth)	Studied group/ exposure time	Methodology used	Main effects observed	References
ASGARD, Nottingham (UK)*	60 l h^{-1}	14.5±4 % (30 cm)	Microbiota/ 10 weeks	Microbial biomass and activity (Biolog GN_2 and respiration)	No significant changes in microbial biomass or carbon utilisation; trend towards reduced microbial respiration	Pierce and Sjörgersten, 2009
	180 l h^{-1}	Up to 75-87 % (20 cm)	Microbiota/ 19 weeks	Total bacterial numbers, activity (ATP)	Bacterial numbers decreased, ATP below detection limits at 87 %-CO_2 plot	West el al., 2009
	180 l h^{-1}	Up to 50 %	Microbiota/ 1 year and 4 months	Total bacterial numbers, qPCR, activity (ATP), CPR, MPR, MOR	Increased methanogenesis and decrea-sed methane oxidation, decreasing bacterial and archaeal DNA copy umbers; seasonal-dependent variations	Smith et al.,2013
PISCO$_2$, León (Spain)*	20/40 l h^{-1}		Protozoa	Protozoan culture and counting	Changes in ciliate community composition, decrease in Equitability and Margalef index, and increased Colpodid/Polyhymenophorean ratio	Gabilondo et al., 2015
	20/40 l h^{-1}		Microbiota	Pyrosequencing	Partial shifts in bacterial community composition, but no consistent effects on the richness and diversity, in one soil type	Sáenz de Miera et al., under revision
ZERT, Montana (USA)*	700-6,500 g m^{-2} d^{-1}	Up to 13-14 % (30 cm)	Microbiota/ 1 + 2.5 weeks	Pyrosequencing, qPCR (specific functional groups mediating N and C transformat.)	Seasonal-dependent variations; altered abundance (DNA) and activity (mRNA) of; decrease in bacterial richness, shifts in bacterial community composition	Morales and Holben, 2013; 2014
Gininderra, Australia*	144/218 kg d^{-1}	80 % (100 cm)	Microbiota/ 2+2+2.5 months	Geochip	Favoured anaerobic and acid/metal tolerant species, increased Nitrospira and Firmicutes	Feitz et al., 2014

1.5 Research focused on natural environments: mofettes

Mofettes, also referred as natural CO_2 vents or seepages, mostly occur at sites of former volcanic action. Besides forming dry mofettes, the escaping CO_2 gas may also pass aqueous solutions (water tables, rivers) leading to acidic springs. Several regions have been identified where soil CO_2 concentration or the CO_2 efflux from the soil is anomalous –normal concentration does not exceed 0.2-1 %, whereas in these areas can reach 100 %–. Mofettes are distributed all around the world, having been reported in Germany, Iceland, Slovenia, Czech Republic, Hungary, Romania, Spain, Greece, Portugal, Austria, France, Italy, USA, South Africa, New Zealand and Japan (Pfanz et al., 2004; Paoletti et al., 2005). In these seepages deep mantle origin CO_2 migrates through permeable layers or along fractures and faults to the atmosphere. The emerging CO_2 is usually dispersed by the wind; however, at some sites where the immediately surrounding area form of a depression the CO_2 is less easily dispersed, reaching higher CO_2 concentrations (Holloway et al., 2007).

Natural CO_2 vents have been extensively studied in the last years as they provide suitable analogues to establish detection and monitoring systems on the effects of possible leakages from a CCS storage reservoir (Table 1.1). They have advantages over short-term experimental manipulations as long-term exposure studies can be developed at these areas –they have been emitting for centuries up to millennia-. In addition, CO_2 fluxes often form a gradient on the surface, so a wide range of CO_2 concentrations are available. Other benefit is that there is no extra cost: CO_2 is free and no sophisticated installation is required (Paoletti et al., 2005). And most important, all environmental conditions are natural, making mofettes the closest approximation of leakage from CO_2 storage (Schütze et al., 2012). Their limitations include the variability in atmospheric CO_2 concentrations: vents may be intermittent and CO_2 flux can suffer oscillations depending on atmospheric conditions, site topography and vegetation cover. Moreover, sulphur compounds, mostly H_2S and SO_2, are often associated with CO_2 emissions.

High CO_2 soil concentrations cause a deep impact in the vent-surrounding environment. The most visible consequence is a patchy or reduced vegetation, derived from anoxic conditions due to suppressed aeration. Dead bodies of birds and mammals, reptiles, and insects in the vicinity of mofettes are also usual (Figure 1.9). Soil is gradually acidified as minerals are weathered and reduced in their content of mineral oxides (Frerichs, 2013). Therefore, soil chemistry is also affected. Together, soil acidification, loss of vegetation and reduced rhizosphere and carbon input, and limited oxygen availability transform the microbial communities in the CO_2 vents.

Figure 1.9 A bubbling spring (or "hervidero") in CVF and detail from death animals.

All the natural CO_2 seepages analyses have reported significant changes in the soil microbial community caused by high CO_2 levels in the soil gas (Table 1.1). Several studies observed a shift toward anaerobic and acid tolerant microorganisms as well as an ecosystem adaptation to the CO_2 induced soil biogeochemistry. Other changes include increases in sulphate reduction activity, or stimulated methanogenesis (Jones et al., 2015). These differences may be related to long-term adaptation at the natural sites because there are not observed at experimental systems. Such community adaptation was not seen at ASGARD over a period of 24 months-intermittent-exposure; it is not clear how rapidly these changes occur (RISCS, 2014). Considering CCS risk assessment should be noticed that these ecosystems have usually adapted over long periods to the presence of the gas so that short-term responses to leaking CO_2 may not be the same.

1.5.1 A case study: La Sima CO_2 vent in Campo de Calatrava Volcanic Field

The volcanic region of Campo de Calatrava is located in South-Central Spain (Figure 1.10), in the province of Ciudad Real (Castilla-La Mancha). It is one of the three most important areas with recent volcanic activity in the Iberian Peninsula, together with those of Olot (Girona, Catalonia) and Cabo de Gata (Almeria, Andalusia) (Higueras and Gallardo, 2011). Volcanic activity took place there between 8.7 and 0.7 million years ago, during the Pliocene epoch and Quaternary Period.

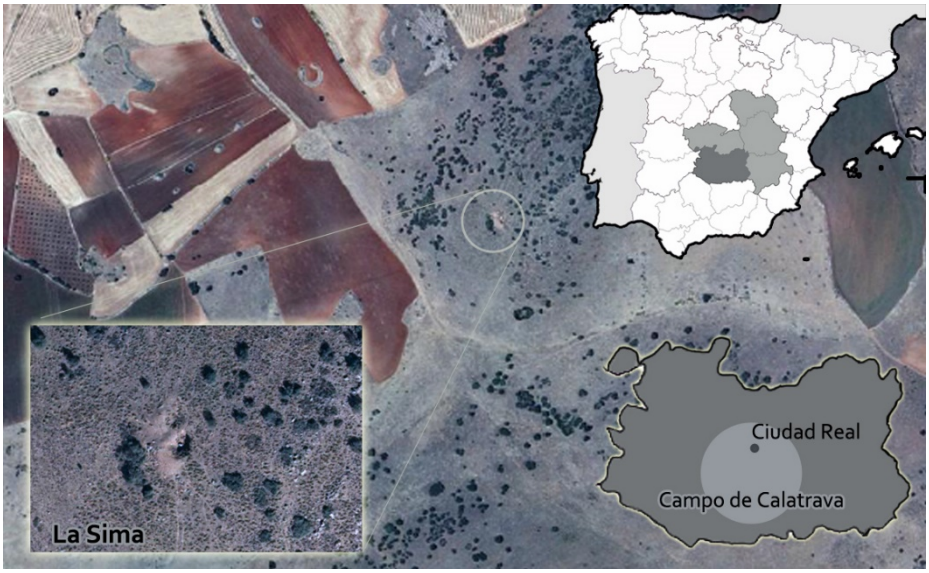


Figure 1.10 Situation of CVF and La Sima mofette. Aerial photograph from la Sima.

The volcanic region has a total area of about 5,000 km², and includes some 240 different volcanic edifices (Ancochea, 1997). The Calatrava Volcanic Field (CVF), along with La Selva-Empordà (NE Spain), likely represents the highest CO_2 discharge area in the whole continental Spain; it can be assumed as one of the best examples of natural analogues of CO_2 leakages in Spain (Vaselli et al., 2013). CO_2 presence in subsurface and aquifers has its origin in magma degassing and cooling processes and it discharges to the surface through fracture and cracking in rocks. (Calvo et al., 2010; Elío et al., 2015). It exists also a remaining hydrothermal activity, visible through the so-called "hervideros" –thermal springs with low temperatures-, which bubble due to CO_2 presence (Figure 1.9). The total CO_2 output is estimated in 1,678 ± 58 t d^{-1} for an area of 758 km² (Calvo et al., 2010).

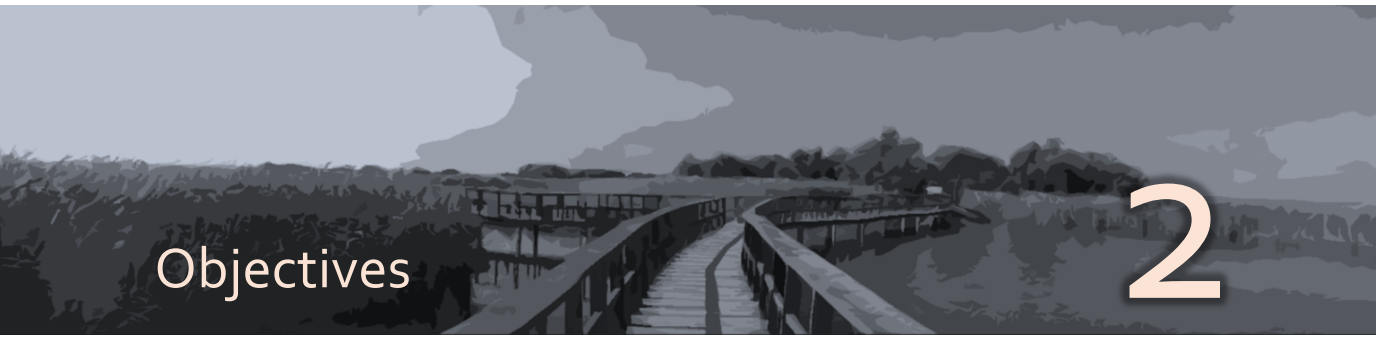
The site with highest CO_2 emissions has been recorded at La Sima vent; Calvo et al. (2010) reported up to 324 kg m^{-2} d^{-1}. La Sima is a mofette located in the north-western slope of Sierra de Granátula (38° 49' 17.51" N, 3° 45' 19.80" W, 656 m.a.s.l.). The local climate is Continentalised Mediterranean, with hot dry summers and cool wet winters. The main land use is farming (cereal crops), and natural vegetation is scarcely represented (Sáenz de Miera et al., 2014).

Figure 1.11 Photographs from La Sima mofette, with emission points and affected area marked. Dead animals and insects.

A seismic crisis in August 2007 (epicentre about 100 km away, in Pedro Muñoz, Ciudad Real) produced a dramatic increase in gas emissions at La Sima (Peinado et al., 2009). CO_2 fluxes increased from 30,000 to 200,000 ppm, showing traces of H_2S, HCl and CH_4 (Gosálvez et al., 2010). After the earthquake, new CO_2 vents opened (from one to five emission points) with apparent damage to the surrounding vegetation and animals (Figure 1.11). Currently, gas temperature remains under 30 ◦C and thermal anomalies have not been registered (Gosálvez et al., 2010).

Previous work has been carried out at La Sima (Table 1.1). Gabilondo and Bécares (2014) found a decreased diversity of edaphic protozoan communities, a shift to Colpodea dominated communities (r-strategist ciliates) and a decrease in the percentage of rapacious ciliates was observed as CO_2 increased. Sáenz de Miera (2014) noticed that bacterial community richness and diversity notably decreased with increasing CO_2 flux.

In the perspective of assessing gas leakages it is worth mentioning that the amount of CO_2 emitted from these points (CVF) can be of the order of magnitude of the maximum leakage rate considered acceptable (0.1 % of the total amount in storage per year) for a commercial storage project (Elío et al., 2015).



Objectives 2

CCS represents a favourable mitigation option to deal with increasing atmospheric CO_2 levels; however, the importance of assessing potential risks on soil environment is a major issue in the implementation of this technology. The main objective of this PhD research is to study how CO_2 belowground emissions affects soil microbial communities. To elucidate this, several specific objectives were established:

1. To determine the response of edaphic microorganisms to a gradient of CO_2 fluxes, by characterizing their functionality, genetic diversity and quantifying the populations.
2. To assess how seasonality affect to the microbial response to high CO_2 fluxes, through seasonal samplings in an experimental system and a natural CO_2 vent.
3. To evaluate the time and site influence on CO_2 effects by comparing between a simulated leakage for a year and a natural emitting vent for centuries.
4. To study the changes in microbial relationships with other edaphic groups (protists and mesofauna) due to elevated CO_2 exposure.
5. To verify the strength of microorganisms as bioindicators of potential CO_2 leakages from geological storage sites.

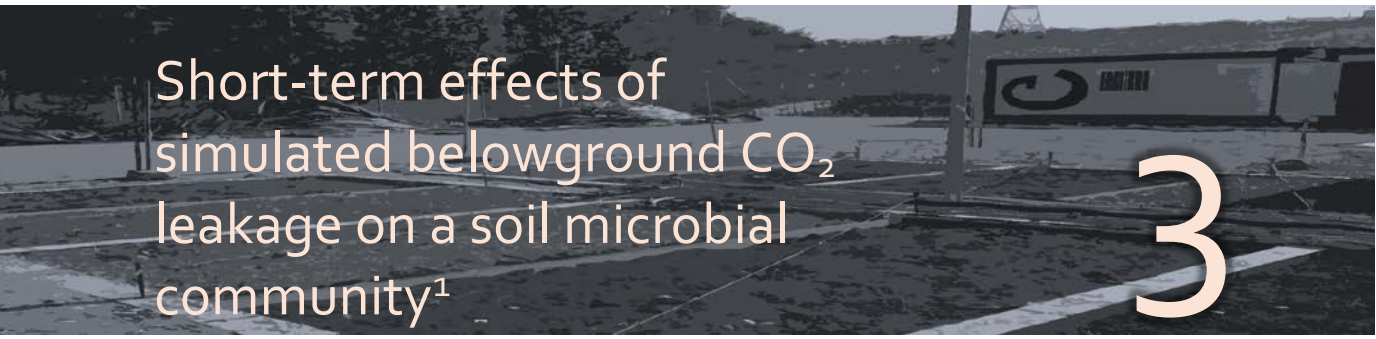
To address these objectives two different approaches were accomplished: a microbiological study by using an experimental plant, which simulated a CO_2 leakage ($PISCO_2$), and the assessment of a natural vent where CO_2 had been emitting for centuries (La Sima of Campo de Calatrava). In this thesis, the research developed is presented in the following chapters:

In chapter 3, the effect of two different CO_2 fluxes on microbial communities is evaluated at $PISCO_2$ experimental plant.

In chapter 4, we present the study of the response of microbiota to a great range of CO_2 fluxes in Campo de Calatrava's La Sima CO_2 vent.

In chapter 5, we include the evaluation of CO_2 effects on soil micro and mesofauna and the relationships of microbial and other edaphic communities, at La Sima mofette.

Finally, a general discussion of these results and the conclusions of the overall study are drawn in Chapter 6.



Short-term effects of simulated belowground CO_2 leakage on a soil microbial community[1]

3

The effects of artificial increments in soil carbon dioxide (CO_2) on microbial communities were studied in an experimental plant in Cubillos del Sil (León, Spain). The impact of two fluxes of CO_2 (20 and 40 l h^{-1}) influencing microbial communities and their relationships in two different soils (Cubillos and Hontomín) was evaluated by using three different approaches: community structure by DGGE, qPCR quantification of Bacteria, Archaea and Fungi domains, and community-level physiological profile (CLPP). Soil type was the most important determinant factor in microbial activity and abundance: Cubillos soil showed a significantly higher qPCR copy numbers (58.68, 275.92 and 375.4 %, for Bacteria, Archaea and Fungi, respectively) with a richer metabolism than Hontomín soil. No significant changes were observed in relation to CO_2 increase with any of the methods employed. Soil microbial communities proved to be resilient to short-term below-ground low CO_2 emissions. Short-term studies are useful for developing methods to detect possible leakages and assessing the likelihood of undesirable consequences on soil ecosystem.

[1] This chapter is based on the article:

Fernández-Montiel, I., Touceda, M., Pedescoll, A., Gabilondo, R., Prieto-Fernández, A., & Bécares, E. (2015). Short-term effects of simulated below-ground carbon dioxide leakage on a soil microbial community. *International Journal of Greenhouse Gas Control*, *36*, 51–59. http://doi.org/10.1016/j.ijggc.2015.02.012

3.1 Introduction

The increasing industry of carbon capture and storage (CCS) represents one of the most promising strategies suggested to mitigate greenhouse gas effects on global warming. Deep geological storage of CO_2 has been proposed as a favourable remedy option (Zhou et al., 2013), but it is important to consider all the potential risks associated with an unlikely leakage of CO_2 from the reservoirs (West et al., 2005; 2006). For this reason, studying the effect of elevated CO_2 concentrations on terrestrial ecosystems and understanding the consequences of early CO_2 leaks and associated changes on soil communities are major issues (Noble et al., 2012). Natural environments where CO_2 is released from geological sources have been used in many studies as surrogates to assess the impact of potential leakages from a CCS site (Krüger et al., 2009; Oppermann et al., 2010; Frerichs et al., 2013; McFarland et al., 2013; Gabilondo and Bécares, 2014; Sáenz de Miera et al., 2014), but the fact that natural seepages have been emitting CO_2 for rather long periods needs to be considered, as the ecosystem could have adapted through species substitution or adaptation (Beaubien et al., 2008; Krüger et al., 2011). Moreover, these sites present specific characteristics (soil type, presence of other gases, soil moisture and temperature) that may not be present in the potential scenario of a CCS leak. Therefore, the results from the studies conducted in natural emission sites cannot be extrapolated to the conditions occurring in other areas after possible escapes from anthropogenic CO_2 storage (Ziogou et al., 2013).

In our study, we focus on microbial communities due to their essential role in soil ecosystems. Microbial communities, through their enormous metabolic diversity and versatility, carry out the vast majority of nutrient cycling processes in soil and they participate in the maintenance of soil structure (Prosser, 2007). Changes in microbial community structure may affect both below- and above-ground processes, thus influencing vegetation and key ecosystem functions (Drigo et al., 2008; Eisenhauer et al., 2012). Microorganisms can be extremely sensitive to changes in soil characteristics acting as good indicators of soil quality (Winding et al., 2005). The response of the microbial community to alterations in soil properties could be easily detected by many techniques widely used in soil studies, such as CLPP or methods based on PCR (genetic fingerprints, qPCR) (Oros-Sichler et al., 2007). An increase in CO_2 concentration could cause changes in soil biochemical conditions that would lead to a shift in the functionality or diversity of inhabiting microorganisms (West et al., 2009; Oppermann et al., 2010; Patil et al., 2010; Krüger et al., 2011; Smith et al., 2013)

Instead of using a natural analogue site, where the plant and microbial communities are well established and have been adapted to elevated CO_2 conditions, we used an experimental approach in which the objective was to analyse the first responses of a non-adapted ecosystem to a simulated CO_2 leakage. Our aim was to study the consequences of a belowground low CO_2 emission via a qualitative and quantitative

evaluation of soil microbial community applying the methods described above. For this purpose, we constructed an experimental plant where a continuous below- ground emission was used to simulate a leakage from a geological storage site. To our knowledge, this is the first time that a diffuse and continuous flow of CO_2 is assessed through an injection method based in belowground releasing grills. This chapter focuses on the immediate responses of edaphic microbiota to simulated CO_2 leaks. These short-term studies could be essential for regulatory bodies, stakeholders and society to accept the implementation of CCS projects (Noble et al., 2012).

3.2 Materials and Methods

3.2.1 Study site and experimental design

The study area was located at the PISCO$_2$ experimental site, within the CIUDEN facilities (42°36'50.04"N 6°34'27.30"O; 578 m.a.s.l.), in Cubillos del Sil (León). The region is characterized by Continentalised Mediterranean climate. Average annual precipitation and temperature are about 668 mm and 12.6 ºC, respectively, with an average maximum of 18.1 ºC and a minimum of 7.2 ºC (AEMET, 2015).

The PISCO$_2$ experimental site consisted of a factorial experiment of 24 plots (8m^2 each) in which three fluxes of CO_2 were applied to two different soils and four replicates for each treatment were assayed. The plots were filled from the bottom to the top with a 20 cm gravel layer covered by 170 cm of sand and a 40 cm top soil layer. The CO_2 was released by two grills at depths of 1 and 2 m below ground level (Figure 3.1). Half of the plots were filled with a sandy loam soil from a meadow in Hontomín (Burgos) (42°34'39.53"N 3°38'6.69"O; 923 m.a.s.l.), where geological storage was installed, and the rest with a loam soil from a pasture in Cubillos del Sil. Seed bank present on each soil determined the vegetation throughout the experiment. Both soils were subjected to two different CO_2 levels: 20 l h^{-1}–low–, 40 l h^{-1}–high–, and a control without CO_2 injection. The treatments were assigned randomly to each experimental cell. These fluxes were chosen as being within the expected range in an early CO_2 storage leakage. In comparison with similar experiments (Smith et al., 2013), in this system a diffuse and continuous flow of CO_2 was assessed through underground CO_2 releasing grills, providing homogeneous fluxes on the surface of the cells.

3.2.2 Soil sampling and storage

Soil samplings took place for the first time in July 2012, four months after start-up. Samplings were repeated following seasonal variations during a year (July and October 2012 and January and June 2013). Five soil cores of 5 cm diameter, 10 cm depth, were sampled from each plot, and pooled. After samples were taken, the holes were backfilled with the same soil. Soil samples were transported on ice to the laboratory, sieved to 2 mm, removing all plant material, and stored at -20 ºC until microbial analysis could be completed (only for DNA extractions). In addition, moisture, pH,

organic matter and conductivity were measured using the standard protocols of the Spanish Ministry of Agriculture, Fisheries and Food (Ministerio de Agricultura, Pesca y Alimentación, 1994). The available fraction of the elemental concentrations of ions and metals were analysed with an ICP-AES Optima 2000 DV from Perkin Elmer, using the standard protocols of the Spanish Ministry of Agriculture, Fisheries and Food. Physico-chemical analysis were performed at the beginning and the end of the experiment (July 2012 and June 2013). A net of 18 equidistant points on the surface of each cell was used to evaluate the CO_2 fluxes and to obtain a monthly surface plot of CO_2 fluxes in each one. Gas was measured at each point using a CO_2 field device (LICOR LI820 CO_2 soil flux meter from West Systems). The sampling points were selected according to the CO_2 fluxes measured in the surface plot. Due to the spatial heterogeneity of the CO_2 emission in each cell, only those points with the target CO_2 levels were sampled.

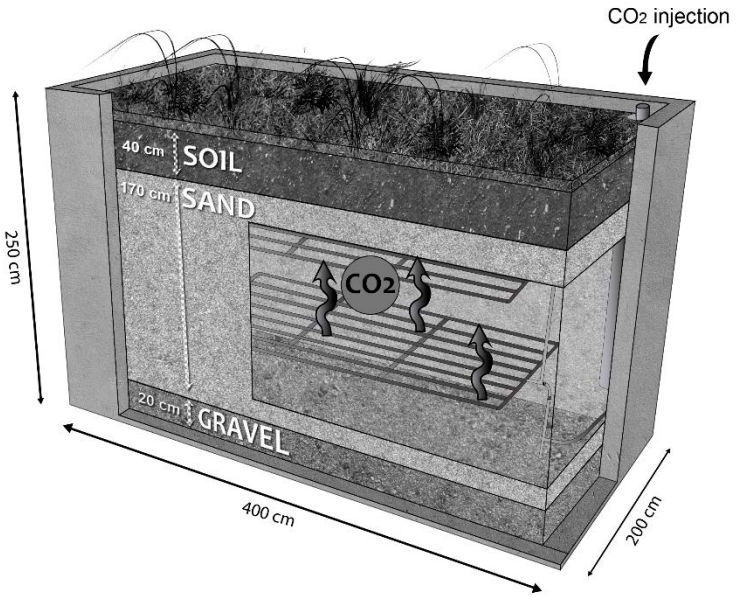


Figure 3.1 Cross sectional scheme of the experimental cells.

3.2.3 DNA fingerprints

Total microbial community DNA was extracted from 0.25 g of soil samples, using the Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., CA, USA) according to the manufacturer's instructions. DNA yield was assessed by electrophoresis in 1.2 % agarose gels stained with RedSafe™ (Intron Biotechnology, Korea) and visualised under UV light.

Table 3.1 PCR primers and conditions used to obtain the products for DGGE analysis and the quantitative PCR.

Target	Primer	PCR conditions			References
		Denaturation	Annealing	Elongation	
PCR-DGGE[1]					
α-Proteobacteria	F203/ R1492	94 °C/1 min	56 °C/1 min	72 °C/2 min	(Gomes et al., 2001) (Weisburg et al., 1991)
β-Proteobacteria	F984/ R1492	94 °C/1 min	64 °C/1 min	72 °C/2 min	(Gomes et al., 2001) (Weisburg et al., 1991)
Bacteria	F984-GC/ R1378	94 °C/1 min	56 °C/1 min	72 °C/2 min	(Heuer and Smalla, 1997) (Nübel et al., 1996)
qPCR[2]					
Bacteria	357F/ 518R	95 °C/15 s	54 °C/10 s	60 °C/30 s	(Turner et al., 1999)
Archaea	771F/ 957R	95 °C/15 s	54 °C/30 s	72 °C/30 s	(Ochsenreiter et al., 2003)
Fungi	FF390/ FR1	95 °C/15 s	50 °C/10 s	60 °C/40 s	(Vainio and Hantula, 2000)

[1] Before each run of cycles (30 for Bacteria, 25 for groups), temperature was held at 94°C for 10 min for Bacteria and Proteobacteria. After the cycles, the temperature was kept at 72°C for 10 min

[2] Before the 40 cycles of amplification an initial denaturation was run for 10 min at 95°C

We analysed total Eubacterial community to provide a screening on total community and Alpha and Betaproteobacteria, as models of one of the most dominant phylum in soils (Janssen, 2006), to evaluate the CO_2 effect on microbial community. PCR was performed to amplify universal and group-specific 16S rRNA gene fragments in a thermal cycler TC-512 (Techne, UK). To analyse total Eubacterial community, the extracted DNA was amplified using the primer set F984GC/R1378 (Table 3.1), targeting variable region V6 of 16S rRNA gene. The reaction mixture (25 µl) was as follows: 1 µl template DNA, 1 X DreamTaq buffer (Fermentas, Lithuania) 0.2 mM dNTPs, 0.2 µM each primer and 0.625 U DreamTaq polymerase (Fermentas, Lithuania). For Alpha, and Betaproteobacteria, (semi)nested-PCR was applied; using group-specific primers in the first round (Table 3.1) followed by primers F984GC/R1378 in the second round. Group-specific 16S rRNA gene fragments were amplified as follows: 1 µl template DNA, 1 X TrueStart buffer (Fermentas, Lithuania), 2.5 mM $MgCl_2$, 2 mM dNTPs, 5 % (v/v) DMSO, 1 mg/ml bovine serum albumin, 0.2 µM primers and 0.625 U TrueStart Hot Start Taq polymerase (Fermentas, Lithuania). For F984GC/R1378 PCR 1 µl of group-specific products was used as a template. PCR conditions are given in Table 3.1. Products were checked by electrophoresis in 1.2 % agarose gels and RedSafe™ staining.

DGGE was performed with a phorU2 apparatus (Ingeny, Goes, The Nederlands), using a double gradient consisting of 46.5-65 % denaturants (100 % denaturant defined as 7 M urea and 40 % v/v formamide) and 6.2–9 % acrylamide (Gomes et al., 2005). For total

community analysis 10 µl of PCR product was loaded on the gel and ca. 5 µl in group-specific DGGEs. Electrophoresis was carried out in 1X TAE buffer at 58 ºC, at a constant voltage of 140V for 17 h. A marker composed of a mixture of F984GC/R1378 PCR products of 6 bacterial isolates was loaded in the extremities and the centre of the gels. After electrophoresis the gels were silver-stained, air dried and scanned (Epson Perfection V750 Pro, Japan). DGGE fingerprints were analysed with the GelComparII v6.0 program (Applied Maths, Ghent, Belgium). Similarities were calculated by Pearson index and cluster analysis was performed applying the unweighted-pair group method using average linkages (UPGMA) to the matrix of similarities. The diversity of the microbial communities was analysed by the Shannon index and band richness was calculated for all conditions studied.

3.2.4 Community-level physiological profiling

The metabolic profile was analysed with Biolog Ecoplate™ (Biolog Inc., Hayward, CA, USA). Briefly, 5 g of each fresh soil sample were homogenised in 45 ml of sterile NaCl solution (0.85 %) by shaking for 30 min at 200 rpm. Slow speed centrifugation was carried out (5 min at 129 g) to decrease soil particles in the supernatant, preserving microbial diversity (Calbrix et al., 2005), and then diluted to 10^{-1}. 150 µl of this suspension were inoculated onto plates. Each Biolog Ecoplate™ consisted of three replicates of 96 wells, each comprising 31 sole carbon sources and one water blank control. Metabolism of the substrate in particular wells resulted in a colour change in the tetrazolium dye. Initial absorbance at 590 nm was recorded using Synergy™ HT microplate reader (BioTek Instruments, Winooski, VT, USA). Plates were incubated at 20 ºC and the DO_{590} nm was read at 24 h intervals over a period of 96 h.

Before statistical analysis within-plate replicates were averaged and raw OD data were corrected by blanking response wells against the control well (containing no carbon source), and negative values were converted to zero. Absorbance values which were lower than initial control well value after the blanking were not considered for the analysis. The average well colour development (AWCD), calculated as the average adjusted absorbance of all wells per plate, was used as an indicator of general microbial activity (Garland & Mills, 1991). AWCD data from 96 h readings were used to compare substrate utilization patterns between different treatments. Differences in microbial functional diversity among soil samples were also compared by Richness (number of positive substrates) and Shannon index. Finally, the absorbance data for the 31 individual carbon substrates at 96 h was used to quantify C-source utilization using principal components analysis (PCA).

3.2.5 qPCR

For the qPCR DNA was extracted following the procedure described above. Quantitative PCR was performed using SYBR Green PCR Master MIX (Applied

Biosystems, Foster City, CA) on the Step One Plus system (Applied Biosystems). Each 20 µl of PCR reaction contained 0.3 µM of each primer (0.9 µM for Archaea), 1X SYBR Green PCR Master MIX, and 2 µl of standard or DNA sample. PCR conditions and primers used are detailed in Table 3.1. Detection of fluorescent product was carried out after each cycle. A melting curve temperature profile was obtained by programming the Step One for one cycle heated to 95 ºC and 15 s at 65 ºC using a slope of 20 ºC s^{-1}, followed by heating to 95 ºC, using a slope of 0.1 ºC s^{-1}. Standards were made from purified PCR products from culture cell extracts, cloned into a pSC-A-amp/kan vector by using the StrataClone PCR Cloning kit (Stratagene, Santa Clara, CA) and confirmed by sequencing. The amplification efficiency of the qPCR reactions was 95 %, 89 % and 82.5 % on average, for Bacteria, Archaea and Fungi, respectively.

3.2.6 Statistical analysis

Data were analysed using SPSS 19.0 software. Statistics were made using all data for assessing each variable response, and averaged data to compare soils and sampling times. Two-way ANOVA was performed to determine the effects of CO_2 level and soil location on each variable studied. Spearman correlations were calculated among physico-chemical parameters and their relation with each variable studied. Each parameter was checked for normality and homoscedasticity; otherwise, data transformation or non-parametric assays (Mann-Whitney tests) were used. LSD and Tukey tests were used in the case of multiple comparisons. Significance for all statistical analyses was accepted at α=0.05.

3.3 Results

3.3.1 Elevated CO_2 and soil chemistry

Soils were subjected to two different below ground CO_2 fluxes: 20 and 40 l h^{-1}. Before the sampling, superficial CO_2 fluxes were measured, with values ranging from 4.5 to 19 (control), 7.3 to 65.6 (low) and 14.2 to 125 (high) g m^{-2} d^{-1} in Cubillos and from 2.9 to 16 (control) 7.4 to 81.3 (low) and 9.3 to 161 (high) g m^{-2} d^{-1} in Hontomín. The main physico-chemical properties of both soils at the beginning of the experiment are shown in Table 3.2. Both soils presented a pH close to neutrality, organic matter around 5 %, low P availability and exchange complex dominated by Ca. Cubillos and Hontomín soils showed significant differences ($p < 0.01$) in most of the properties analysed, except for boron and conductivity. There was no statistical differences between treatments. After one year of CO_2 exposure pH decreased significantly ($p < 0.01$) in gassed plots (from 6.6 to 6.2 for Cubillos and 7.5 to 6.6 in Hontomín), exchangeable Ca decreased slightly ($p < 0.05$) while Mn and Fe levels increased ($p < 0.05$). pH change correlated slightly with the CO_2 applied (Spearman correlation: -0.321, $p < 0.02$), and Ca, Mn and Fe variations were associated with this pH decrease ($p < 0.01$). Soil moisture varied in relation to seasons ranging from 15 % in summer and spring to 25 % in October and up to 40 % in winter.

Table 3.2 Chemical characteristics of the soil in each of the sample conditions at the beginning of the experiment (July 2012). Soils, tested by two-way ANOVA, were significantly different ($p < 0.01$) for all the variables except for those marked with an asterisk.

	Cubillos			**Hontomín**		
	Control	Low	High	Control	Low	High
pH (water)	6,35	6,25	6,25	7,70	7,47	7,38
Organic matter (%)	4,53	4,53	4,65	5,57	5,50	5,59
Conductivity (dS m^{-1})*	0,10	0,10	0,11	0,11	0,10	0,11
Total nitrogen (%)	0,29	0,29	0,30	0,31	0,31	0,31
C:N	9,05	9,02	9,08	10,46	10,34	10,37
Phosporus (Olsen)	< 5,44	< 5,44	< 5,44	< 5,44	< 5,44	< 5,44
Calcium (cmol kg^{-1})	11,40	11,00	11,14	27,69	26,79	26,95
Magnesium (cmol kg^{-1})	3,11	3,12	3,19	0,57	0,59	0,59
Potassium (cmol kg^{-1})	0,24	0,22	0,22	0,71	0,71	0,73
Sodium (cmol kg^{-1})	0,06	0,06	0,07	0,05	0,04	0,04
Ca:Mg	3,66	3,54	3,51	48,45	45,58	45,60
K:Mg	0,08	0,07	0,07	1,25	1,21	1,23
CEC	15,22	16,23	16,74	27,35	26,81	27,51
Manganese (ppm)	5,54	6,36	6,69	8,99	11,34	11,53
Iron (ppm)	83,40	88,33	90,60	13,33	14,40	14,23
Copper (ppm)	1,64	1,70	1,76	0,48	0,54	0,51
Zinc (ppm)	0,85	0,94	0,90	0,38	0,40	0,45
Boron (ppm)*	0,62	0,59	0,57	0,53	0,53	0,57

3.3.2 Quantification of microbial populations

Microbial abundance was quantified using qPCR. Averaged abundances (gene copies g^{-1} dry weight soil) were 4.11×10^9, 4.06×10^9 and 3.48×10^8 in Cubillos, and 2.59×10^9, 1.08×10^9 and 7.32×10^7 in Hontomín, for Bacteria, Archaea and Fungi, respectively. Thus, the number of copies was 59, 276 and 375 % higher in Cubillos than Hontomín, for Bacteria, Archaea and Fungi, respectively (Figure 3.2a). Gene copy numbers of Bacteria and Archaea were similar throughout the first year of operation of the experimental site whereas Fungi followed seasonal variations related to the water content of the soil (Spearman correlation of 0.48, $p < 0.001$). Fungal abundances showed a significant decrease from cold to warm season ($5.45 \pm 2.4 \times 10^8$ to $1.52 \pm 0.5 \times 10^8$ in Cubillos samples, $9.77 \pm 3.9 \times 10^7$ to $4.87 \pm 1.3 \times 10^7$ in Hontomín). In general, no differences were observed between control and gassed soil samples (Figure 3.2a).

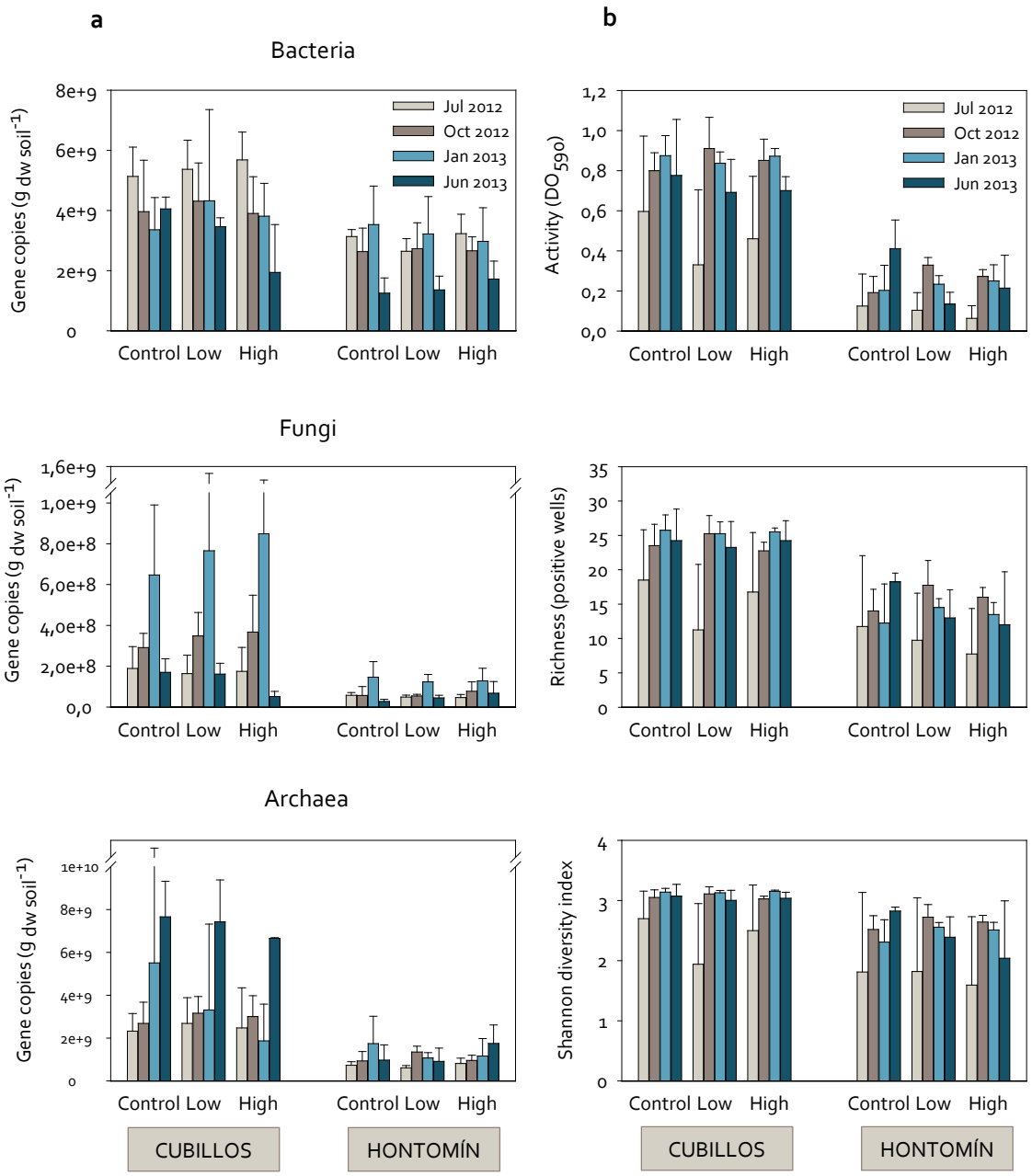


Figure 3.2 a Microbial community quantification (Bacteria, Fungi and Archaea) determined using qPCR in the soil samples of Cubillos and Hontomín, at control, low and high CO_2 fluxes (mean ± sd, n = 4). **b** Average well colour development (AWCD), Richness and Shannon index of Biolog EcoPlates at 590 nm for the tested soils: Cubillos and Hontomín at control, low and high CO_2 fluxes (mean ± sd, n = 4).

3.3.3 *Metabolic activity*

Metabolic profile results confirmed that there was a significant difference between Cubillos and Hontomín soil communities. Bacterial activity (AWCD) was significantly higher in Cubillos (66 %) compared to Hontomín samples, together with the richness (66 %) and Shannon diversity index (25 %) (Figure 3.2b). Principal component analysis applied to 96 h absorbance data of the whole Biolog plate separated the samples into two groups according to the soil location but no CO_2 effect was detected (Figure 3.3). Similarly, no significant changes in AWCD were detected between control and CO_2 influenced plots in either metabolic profile richness (number of positive wells) or Shannon diversity index (Figure 3.2b).

As regards the 31 substrate absorbances, carbon source utilisation profile showed no differences between treatments: L-Asparagine, D-Mannitol and N-acetyl-D-glucosamine were the most utilised substrates in Cubillos, and D-Galacturonic acid, L-Asparagine and Tween 80 in Hontomín. Moisture content significantly influenced microbial activity (AWCD) (Spearman correlation 0.28 $p < 0.001$).

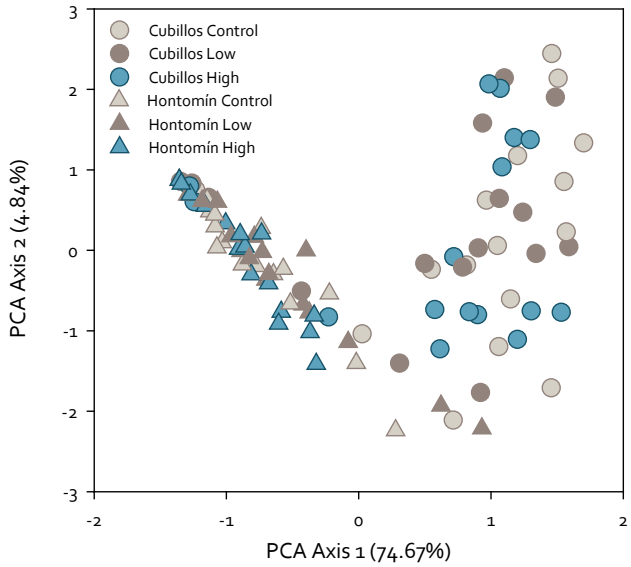


Figure 3.3 Principal components analysis showing how samples distribute, as determined by metabolic profiles using Biolog EcoPlates. Symbols correspond to the different soils studied: circles for Cubillos and triangles for Hontomín.

3.3.4 *Community structure analysis*

DGGE profiles of 16S rDNA fragments (amplified by PCR using universal and group specific primers) were used to evaluate the effect of CO_2 on soil bacterial community.

Cluster analysis of the banding pattern of Alpha- and Betaproteobacterial grouped samples according to CO_2 fluxes applied though their dissimilarity was not significant (Figure 3.4). The Cubillos Betaproteobacterial community at high CO_2 flux differs from control and low fluxes (Figure 3.4b) and at Hontomín, all of the samples taken under high flux in October and January (several months after beginning the experiment) formed a cluster separated from control samples, samples under lower CO_2 flux and samples under high flux taken in July. In the case of Alphaproteobacteria (Figure 3.4a), at Hontomín the "control" and "high" samples were in different clusters, whereas at Cubillos did not show a clear pattern. In terms of total community (Figure 3.5), band richness was higher in Hontomín than in Cubillos ($p < 0.01$), whereas Alphaproteobacteria band richness was higher in Cubillos (showing the most significant decrease in October from 40 –Cubillos– to 30 bands –Hontomín–, $p < 0.01$). In both soils, the number of bands belonging to Betaproteobacteria was about 40. Cluster analysis of total Eubacteria did not show differences between CO_2 fluxes but clearly separated the samples related to soil type (Figure 3.6). The Shannon index was calculated but showed no significant effect of CO_2, following exactly the same trend as richness between soils and sampling times. No significant differences were observed in band patterns between sampling times. Samples taken in June 2013 could not be analysed by DGGE due to technical problems.

3.4 Discussion

The present study proposed an experimental approach to simulate an early leakage from a geological storage site and to evaluate its short-term impact on soil microbial communities. In comparison with natural analogue site studies, where the event duration could have extended for centuries forcing the microorganisms to adapt, we tried to assess the immediate changes caused by a relatively low CO_2 increment in a non-adapted ecosystem.

Moreover, we used two different soils to evaluate the effect of low CO_2 emissions on soil microbial communities, to verify whether the consequences were comparable regardless of soil characteristics. In our study, soil type was the most influential variable on the microbial community, i.e. Cubillos samples showed a significantly higher number of microorganisms and higher and more diverse metabolic activity than those from Hontomín. This result underlines the importance of soil type on microbial communities over and above the effect of low short-term CO_2 emissions. The soils chosen for this study showed different characteristics that could drive the microbial community activity and structure (Standing and Killham, 2007). Besides the differences in soil chemical properties, we also associated this result with the variance in vegetation abundance among the studied soils. Cubillos soil had a vegetal coverage of 80-90 % throughout the experiment whereas Hontomín presented only 5-10 %.

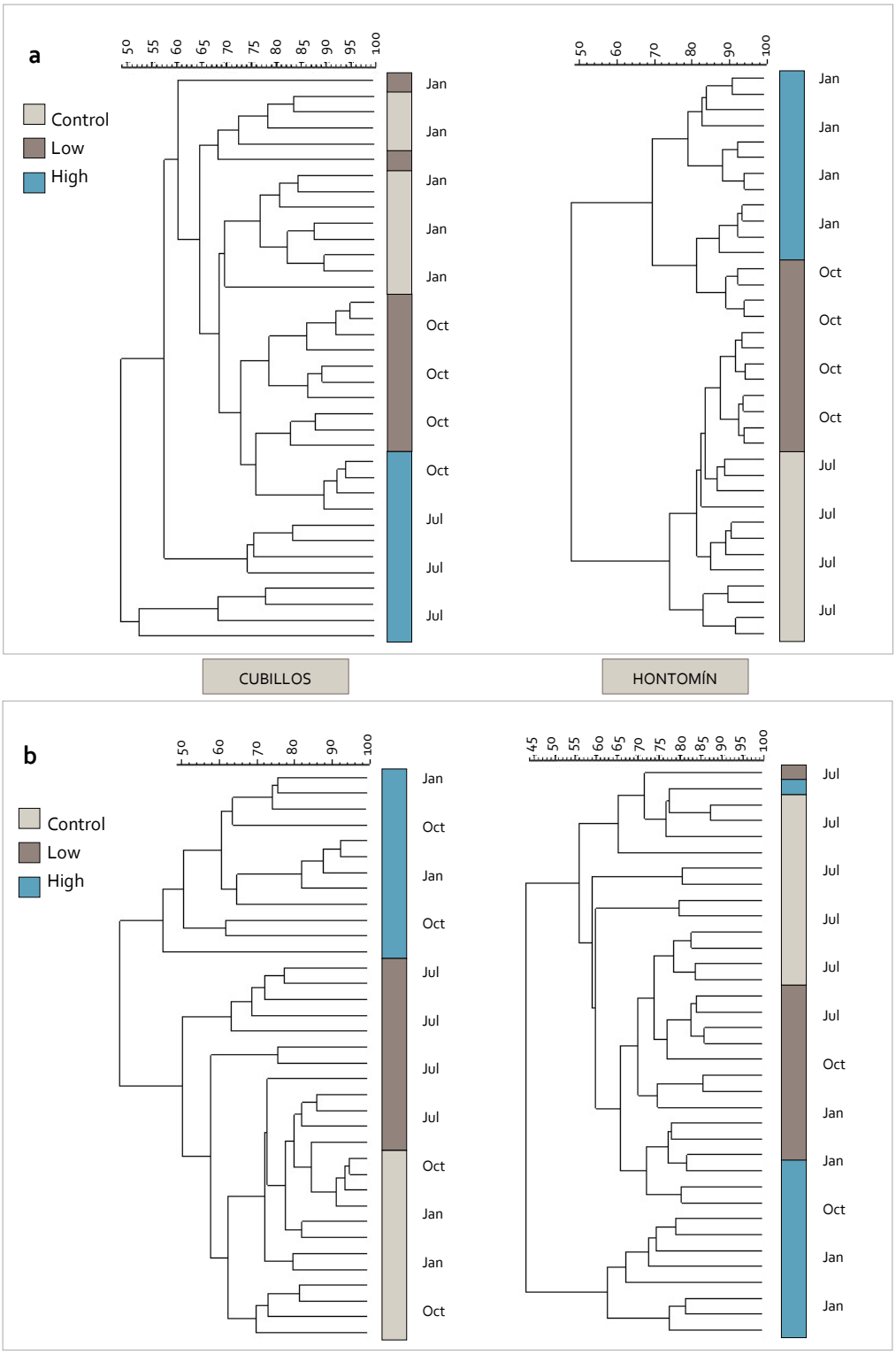


Figure 3.4. Hierarchical cluster analysis results of the (a) Alphaproteobacteria and (b) Betaproteobacteria DGGE profiles demonstrated graphically as an UPGMA dendogram, for July 2012 (Jul), October 2012 (Oct) and January 2013 (Jan) samples.

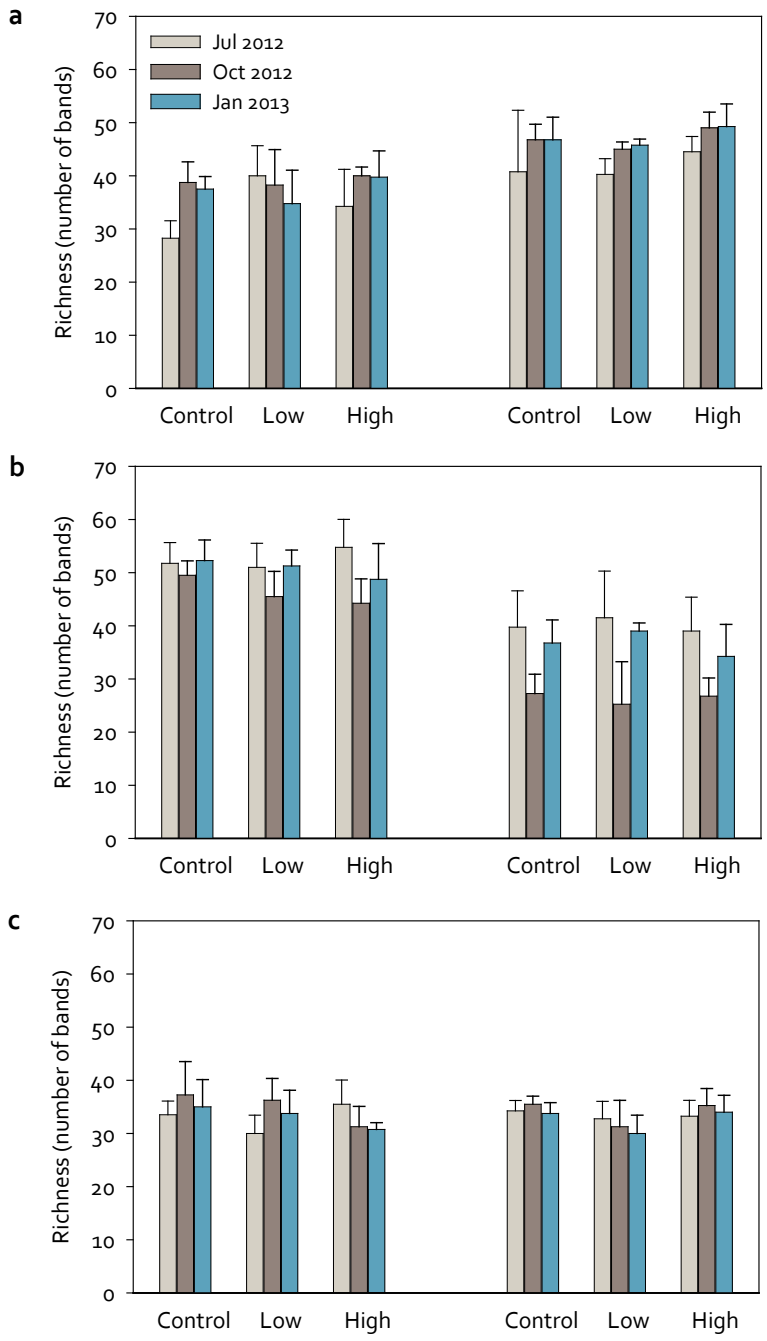


Figure 3.5 Richness (number of bands) of (a) total community, (b) Alpha- and (c) Betaproteobacteria DGGE profiles for Cubillos and Hontomín samples, at control, low and high fluxes (mean ± sd, n = 4).

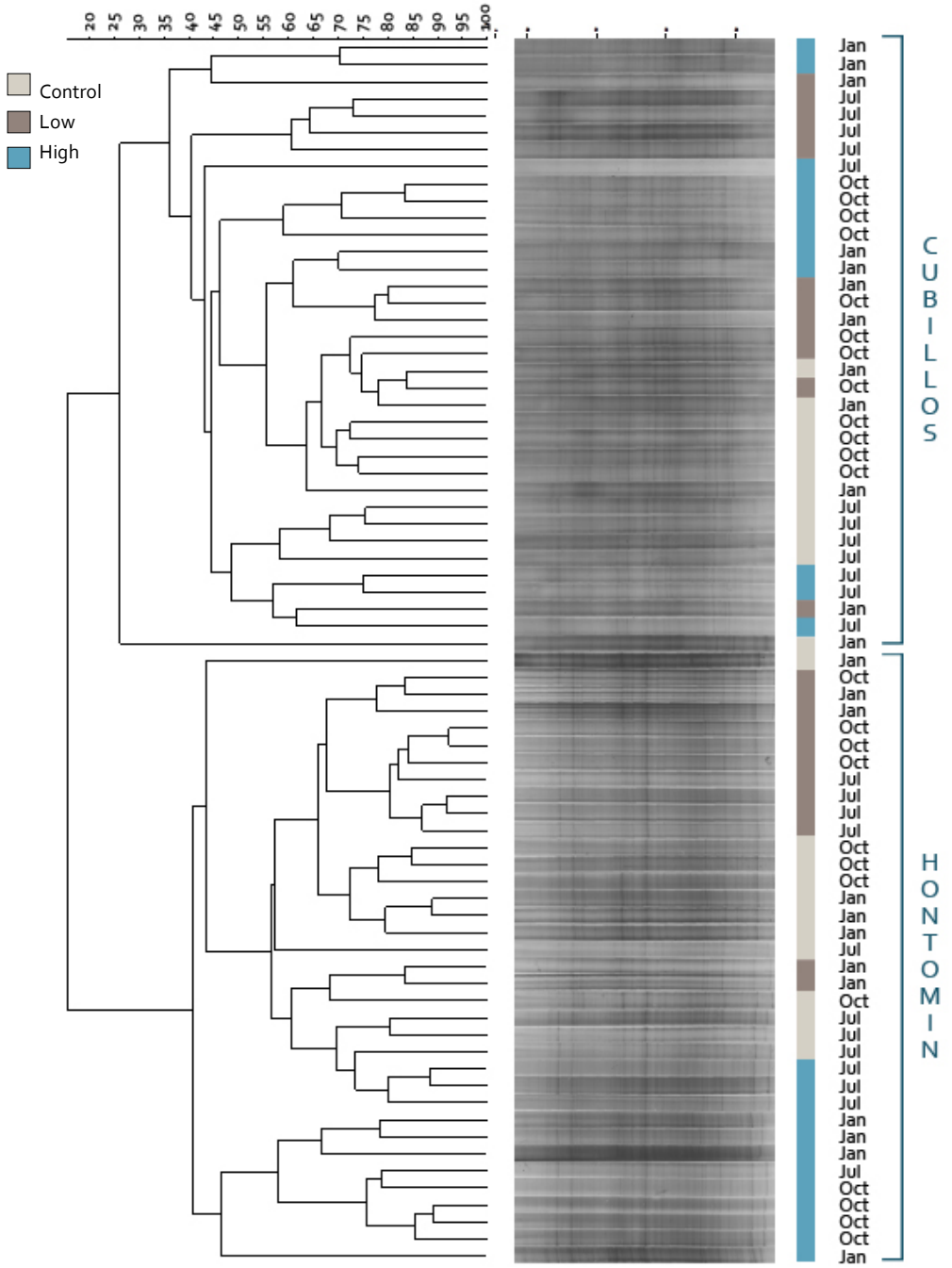


Figure 3.6 Hierarchical cluster analysis results of the Total Bacteria DGGE profiles demonstrated graphically as an UPGMA dendogram, for July 2012 (Jul), October 2012 (Oct) and January 2013 (Jan) samples, comparing Cubillos and Hontomin soils.

Microbial activity is strongly influenced by soil chemistry and vegetation diversity (Gömöryová et al., 2013; McFarland et al., 2013). In that sense, Biolog Ecoplates provides a scheme of potential activity of the microbial community so it was expected that samples from Cubillos soils, the ones with greater vegetation density and thus more carbon availability, showed a higher response in metabolising carbon substrates provided in the Ecoplates. A more elevated and diverse activity in Cubillos soil in comparison to Hontomín's was confirmed by the AWCD results. This fact was also observed in the qPCR as there was lower microbial density in Hontomín soils, with very low vegetation abundance, compared with that from densely vegetated Cubillos soils. Nonetheless, the abundance of Bacteria, Archaea or Fungi was among that expected in this type of soil ecosystems (Oppermann et al., 2010; McFarland et al., 2013). McFarland et al. (2013) also correlated a microbial biomass decrease with the lack of vegetation. DGGE results confirmed the differences between both soils, as they exhibited different microbial community structure (Figure 3.6). In addition, most of the variables analysed followed seasonal variations, in correlation with soil moisture content. Potential carbon-substrates use measured by BIOLOG Ecoplates was stimulated in October 2012 and January 2013 (wet seasons), in the same way as fungal gene copy number rose in January 2013.

After more than a year of exposure we have found that microbial communities were unaffected by a CO_2 increase in their functionality, diversity or number of Bacteria, Archaea or Fungi gene copies. There were many factors that could explain the lack of effect of a CO_2 increase on a microbial community, such as the CO_2 fluxes applied, event duration, the sensitivities of different organisms and external environmental factors (Steven et al., 2010). Pierce and Sjörgersten (2009) studied microbial biomass and carbon utilisation response to CO_2 increments (60 l h^{-1}) at the ASGARD site for 10 weeks, and did not find any differences, although vegetation was significantly reduced. West el al. (2009) found, in the same place, that microbiological changes after 19-weeks exposure (a significant drop in bacterial numbers and ATP concentration) were limited to areas where CO_2 reached high concentrations (more than 75 %). Recent results from the ASGARD site concluded that total microbial numbers increased when CO_2 soil concentrations were between 20-50 % after 2 years´ gassing, but little effect was observed when the emission was below 8 % (West et al., 2013).

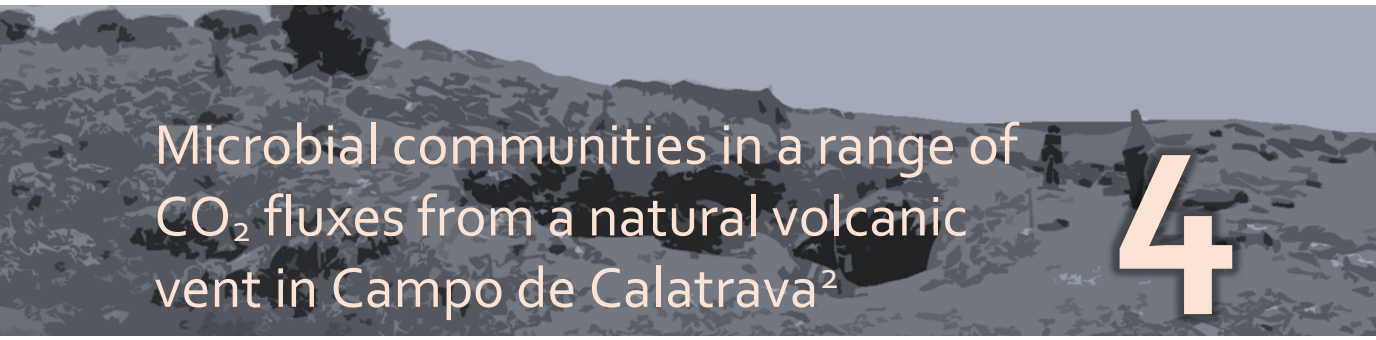
In comparison with experimental sites, studies focused on the effect of high emissions of CO_2 from natural seepages have shown that the microbiota shifted to acidophilic and anaerobic species (Beaubien et al., 2008; Krüger et al., 2009, 2011; Oppermann et al., 2010; Frerichs et al., 2013). Studies which took place in natural environments, such as Latera in Italy (Beaubien et al., 2008; Oppermann et al., 2010), or Laacher See in Germany (Krüger et al., 2009, 2011; Frerichs et al., 2013), found changes in specific groups such as sulphate-reducing Bacteria (SRB), methanogenic Archaea, ammonium-oxidising Archaea (AOA), but the range of CO_2 fluxes present at natural sites are much higher than the ones applied at experimental sites like ASGARD or this one. Frerichs et

al. (2013) did not find any differences in bacterial diversity by DGGE or in the number of Bacteria or Archaea in the Laacher See area, whereas there was an increment in AOA and SRB associated with CO_2 and a decrease in the number of methanogenic Archaea and Geobacteraceae. They also observed an abundance of genetic markers for Crenarchaeota at the points with the highest CO_2 concentration. However, in Latera Caldera Crenarchaeota were the dominant Archaea at the reference –no CO_2 affected site– (Oppermann et al., 2010). These results suggest that microbial communities do not always respond in the same way, and could also depend on uncontrolled factors leading to non-consensus; hence, local conditions could modify the impact of a CO_2 leakage (West et al., 2013). Natural sites have been releasing CO_2 for centuries up to millennia so their ecosystems have evolved after such long periods and hence cannot be considered a reference to infer the consequences expected in short-term CO_2 impacted soils.

Our study demonstrated that short-term low CO_2 emissions had little ecological impact on soil microbial communities. However, we did find changes in soil chemistry, particularly, a significant decrease of pH in gassed plots (West et al., 2009; Patil et al., 2010), although still not reflected in microbial communities. This change highlights the importance of an early detection of CO_2 leaks, to avoid unwanted effects on soil ecosystem. The evaluation of the immediate responses of a terrestrial ecosystem to potential leaks from geological storage sites also has to respond to the public concern on CCS projects. These short-term studies are useful for developing methods to detect possible leakages and assessing the likelihood of undesirable consequences on the ecosystem.

3.5 Conclusions

After 15 months of exposure, our results suggested that microbial communities were not significantly influenced by belowground CO_2 emissions. None of the methods used to analyse the microbial community –BIOLOG Ecoplates, qPCR, DGGE– have shown consistent differences between treatments. In the light of the results presented here, we could state that there would be no microbial response in the short term to a low emission of CO_2 (up to 40 l h^{-1}). Future research could focus on the study of specific groups of microorganisms that may benefit from an increase in soil CO_2 concentration– methanogens, anaerobic species– and research on the CO_2 threshold concentration to appreciate a clear impact on edaphic communities.



Microbial communities in a range of CO_2 fluxes from a natural volcanic vent in Campo de Calatrava[2]

4

A natural CO_2 vent sited in Ciudad Real (Spain) was studied to understand how carbon dioxide emissions affect microbial communities along a gradient of CO_2. We used different molecular methods (quantitative PCR, DGGE and Biolog Ecoplates) to assess changes in abundance, diversity and functionality of main groups of soil microbiota. A general decrease for all studied variables (gene copies and band richness of bacteria, archaea and fungi, and Biolog activities) was observed from control (7-19 g m^{-2} d^{-1}g) to high CO_2 fluxes (260-600 g m^{-2} d^{-1}). On the contrary, at extreme fluxes (more than 10 kg m^{-2} d^{-1}) the microbial community increased their abundance and activity, though remaining less diverse. PCA from carbon use substrate pattern and DGGE dendograms clearly differentiated low fluxes from high and extreme. This chapter proves that increasing CO_2 fluxes cause losses in both structural and functional community diversity, and a decrease in metabolic activities.

[2] This chapter is based on the article:

Fernández-Montiel, I., Pedescoll, A., Bécares, E. Microbial communities in a range of carbon dioxide fluxes from a natural volcanic vent in Campo de Calatrava, Spain (submitted to International Journal of Greenhouse Gas Control)

4.1 Introduction

Natural CO_2 seepages, also known as mofettes, are an example of an extreme ecosystem where we could better understand the mechanisms that influence microbial diversity. In comparison with experimental displays (laboratory scale or mesocosms), natural CO_2 emission sites make it possible to study microbial key players and the factors addressing their activities in a relatively undisturbed and stable long-term scenario (Oppermann et al., 2010; Šibanc et al., 2014). In this context, extreme environments represent a proved resource of unique microorganisms that exhibit interesting metabolic strategies and could help to elucidate the role of species in ecosystem function (Maier and Neilson, 2015).

Moreover, mofettes have recently been used as natural analogues to study the effects of a leakage from a carbon capture and storage (CCS) system (Oppermann et al., 2010; Frerichs et al., 2013; McFarland et al., 2013). Although CO_2 storage sites are selected to avoid any possible leak, this cannot be completely excluded and understanding the consequences of the surrounding environment is of major concern (Krüger et al., 2011). In this sense, several studies have taken place in natural environments, such as Latera in Italy (Beaubien et al., 2008; Oppermann et al., 2010), Laacher See in Germany (Krüger et al., 2009, 2011; Frerichs et al., 2013), Cheb Basin, Czech Republic (Beulig et al., 2015) or Stavensinci, Slovenia (Šibanc et al., 2014), showing a marked shift in the microbial community towards anaerobic and acidophilic microorganisms. Even if they have thrown light on specific functional groups response to upwardly migrating CO_2, the overall effects on microbial community structure are still not fully understood.

To evaluate the changes of increasing CO_2 fluxes on structural and functional properties of microbial communities, we conducted a microbiological study at La Sima, a naturally occurring gas vent located in the Calatrava Volcanic Field (CVF; central-southern Spain). Our aim was to evaluate the consequences of long-term exposure to different fluxes of geological CO_2, including extreme fluxes that have never been measured at other sites (Gabilondo and Bécares, 2014). Although geological characterization is well documented (Peinado et al., 2009; Calvo et al., 2010; Gosálvez et al, 2010; Vaselli et al., 2013; Elío et al., 2015) little is known about the community structure and metabolic profile of microbial populations in La Sima. Our hypothesis is that elevated soil CO_2 caused a profound effect of high CO_2 emissions on edaphic microbiota, through losses of diversity, changes in microbial structure and activities due to adverse conditions in such an extreme ecosystem. In this chapter, we assessed changes in functionality, diversity and abundance of the main groups of soil microbiota through Biolog Ecoplates, DGGE and qPCR analysis.

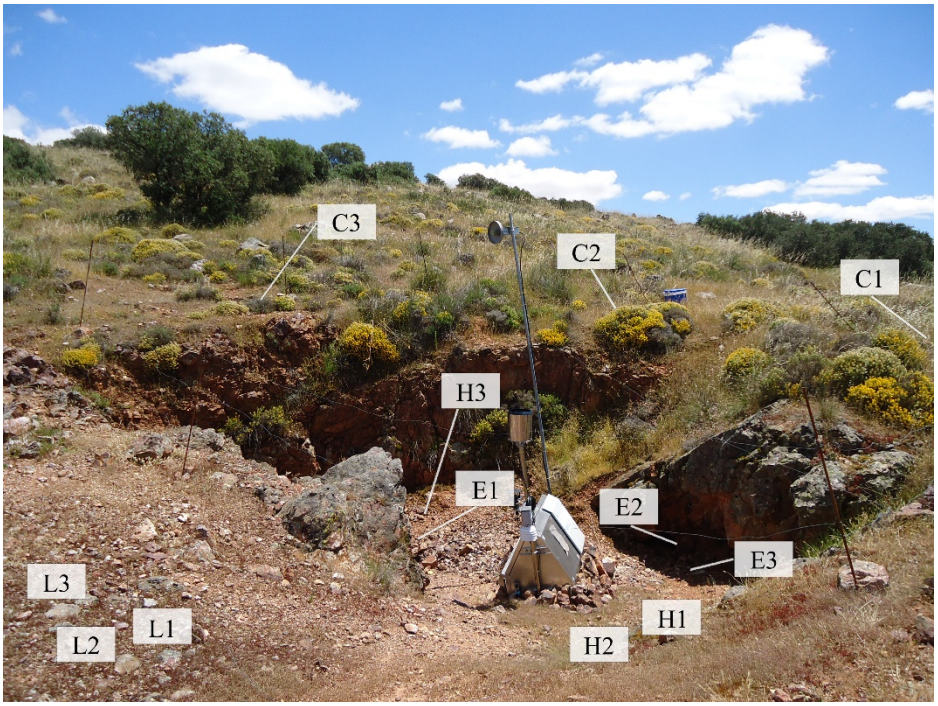


Figure 4.1 A picture of the sampling area, showing the CO_2 vent depression and the non-influenced reference site. Sampling points are indicated by letters: control (C1, 2 and 3), low (L1, 2 and 3), high (H1, 2 and 3) and extreme (E1, 2 and 3).

4.2 Materials and Methods

4.2.1 Site description

The Calatrava Volcanic Field (CVF) is situated in the centre of the province of Ciudad Real (Spain). It occurs in a circular area of about 5,000 km², one of the largest tectonic basins in central-southern Spain (Stoppa et al., 2012) which constitutes one of the more important and recent volcanism zones of Spain. The CVF, with more than 300 emission centres, represents one the highest CO_2 discharge areas in peninsular Spain (Calvo et al., 2010; Vaselli et al., 2013). Volcanic activity took place there between 8.7 and 0.7 million years ago, during the Pliocene epoch and the Quaternary Period (Higueras and Gallardo, 2011). CO_2 presence in subsurface and aquifers has its origin in magma degassing and cooling processes and it discharges to the surface through fracture and cracking in rocks. (Calvo et al., 2010; Elío et al., 2015). Samples were collected at the location called La Sima, near Granátula de Calatrava, Spain (38° 49' 17.51" N, 3° 45' 19.802" W, 656 m.a.s.l.), in the eastern sector of CVF (Figure 4.1). The region is characterized by a Continentalised Mediterranean climate. Average annual precipitation and temperature are about 396 mm and 14.7 °C, respectively, with an average maximum of 21.2 °C and a minimum of 8.2 °C (AEMET, 2015).

La Sima is a CO_2-rich discharge (up to 2 t d^{-1}) consisting of a 5 m diameter depression (Elío et al., 2015). Since a seismic crisis in the area in 2007, CO_2 fluxes increased from 30,000 to 200,000 ppm. Gases also contains traces of H_2S, HCl, CH_4 and Ra (Peinado et al., 2009; Gosálvez et al., 2010) and O_2 measurements remain under 7 % in the gas vent leading to aerobic to microaerophilic conditions. CO_2 values ranges from 15 to 20 % in the spring centre (Gosálvez et al., 2010). CO_2 dominance may be due to the carbonitic nature of the CVF magma (Stoppa et al., 2012). Vegetation presence was reduced to the surroundings, as persistent CO_2 leak has led to vegetation mortality.

4.2.2 Soil sampling and storage

Soil sampling took place in November 2012 and May 2013. Before sampling, distribution of CO_2 fluxes in La Sima vent was measured using a CO_2 field device (LICOR LI820 CO_2 soil flux meter from West Systems). Sampling points were chosen according to fluxes measured at PISCO$_2$ experimental site (Gabilondo and Bécares, 2014; Fernández-Montiel et al., 2015), establishing a gas gradient from the centre of the seepage. CO_2 values ranged from more than 10 kg m^{-2} d^{-1} in extreme (E) fluxes to 260-1,600 g m^{-2} d^{-1} in high fluxes (H) and 40-55 g m^{-2} d^{-1} in low fluxes (L). Finally, control (C) samples (7 to 19 g m^{-2} d^{-1}) were taken from a zone without a CO_2 leak effect (Figure 4.1). Soil cores of 5 cm diameter, 10 cm depth, were sampled from each point and pooled. Extreme samples were only enough for DNA based analysis (qPCR and DGGE), for November 2012 sampling. Soil samples were transported on ice to the laboratory, sieved to 2 mm, removing all plant material, and stored at -20 ºC until microbial analysis could be completed (only for DNA extractions). In addition, moisture, pH, organic matter and conductivity were measured using the standard protocols of the Spanish Ministry of Agriculture, Fisheries and Food (Ministerio de Agricultura, Pesca y Alimentación, 1994). The available fraction of the elemental concentrations of ions and metals were analysed with an ICP-AES Optima 2000 DV from Perkin Elmer, using the standard protocols of the Spanish Ministry of Agriculture, Fisheries and Food.

4.2.3 DNA fingerprints

Microbial community richness was assessed by Denaturing Gel Gradient Electrophoresis (DGGE). We analysed total Eubacterial community, Alpha and Betaproteobacteria to provide a screening on specific groups as models to evaluate the CO_2 effect on microbial community. Extended description of methods applied can be found in Chapter 3. In brief, total microbial community DNA was extracted from 0.25 g of soil samples, using the Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., CA, USA), according to the manufacturer's instructions. DNA yield was assessed by electrophoresis in 1.2 % agarose gels stained with RedSafe™ (Intron Biotechnology, Korea) and visualised under UV light. For DGGE, PCR was performed to amplify universal and group-specific 16S rRNA gene fragments in a thermal cycler TC-512 (Techne, UK). DGGE was performed with phorU_2 apparatus (Ingeny, Goes, The

Nederlands), using a double gradient consisting of 46.5-65 % denaturants (100 % denaturant defined as 7 M urea and 40 % v/v formamide) and 6.2–9 % acrylamide (Gomes et al., 2005). PCR and DGGE conditions and primers used are detailed in Chapter 3 (Table 3.1). DGGE fingerprints were analysed with the GelComparII v6.0 programme (Applied Maths, Ghent, Belgium). Similarities were calculated by Pearson index and cluster analysis was performed applying the unweighted-pair group method using average linkages (UPGMA) to the matrix of similarities. The diversity of the microbial communities was analysed by Shannon index and band richness was calculated for all conditions studied.

Some bands were selected and excised according to their presence/absence in higher flux samples. Sequencing of reamplified DGGE bands was conducted at GATC Biotech AG (Köln, Germany) using forward primer (F984). These bands were checked for their next relatives using the BLAST search in Genbank Data Library (http://www.ncbi.nlm.nih.gov/BLAST/) and RDP (http://rdp.cme.msu.edu/index.jsp) tools.

4.2.4 Community-level physiological profiling

Metabolic profile was analysed with Biolog Ecoplate™ (Biolog Inc., Hayward, CA, USA), following Calbrix et al. (2005). Briefly, 5 g of each fresh soil sample were homogeneized in 45 ml of sterile NaCl solution (0.85 %) by shaking for 30 min at 200 rpm. Slow speed centrifugation was carried out (5 min at 129 g) and then diluted to 10^{-1}. 150 µl of this suspension were inoculated into plates, which were incubated at 20 ºC and the DO_{590} nm was read at 24 h intervals over a period of 144 h. The average well colour development (AWCD), calculated as the average adjusted absorbance of all wells per plate, was used as an indicator of general microbial activity (Garland and Mills, 1991). AWCD data from 120 h readings were used to compare substrate utilization patterns between different treatments. Differences in microbial functional diversity among soil samples were also compared by Richness (number of positive substrates) and Shannon index. Finally, the absorbance data for the 31 individual carbon substrates at 120 h was used to quantify C-source utilization with principal components analysis (PCA). A detailed description of data treatment before statistical analysis is found in Chapter 3.

4.2.5 qPCR

Bacterial, archaeal and fungal abundance was quantified through qPCR. For the qPCR DNA was extracted following the same procedure as that described above. The quantitative PCR was performed using SYBR Green PCR Master MIX (Applied Biosystems, Foster City, CA) on the Step One Plus system (Applied Biosystems). Each 20 µl of PCR reaction contained 0.3 µM of each primer (0.9 µM for Archaea), 1 X SYBR Green PCR Master MIX, and 2 µl of standard or DNA sample. PCR conditions and

primers used are detailed in Chapter 3. The amplification efficiency of the qPCR reactions was 98 %, 94 % and 80 % on average, for Bacteria, Archaea and Fungi, respectively.

4.2.6 Statistical analysis

Data were analysed using SPSS 19.0 software. Statistics were done using all data for assessing each variable response, and averaged data to compare sampling times. Two-way ANOVA was performed to determine the effects of CO_2 level and sampling time on each variable studied. Spearman correlations were calculated among physico-chemical parameters and their relation with each variable studied. Each parameter was checked for normality and homoscedasticity; otherwise, data transformation or non-parametric assays (Mann-Whitney and Kruskal-Wallis tests) were used. LSD and Tukey *post hoc* tests were used in the case of multiple comparisons. Significance for all statistical analyses was accepted at $\alpha = 0.05$. The GelComparII v6.0 programme was used to analyse the DGGE fingerprints and build the UPGMA tree. Principal component analyses were run using CANOCO 5.0.

4.3 Results

4.3.1 Elevated CO_2 and soil chemistry

Soil CO_2 fluxes in La Sima natural upwelling reach values of 300 kg m^{-2} d^{-1} in its central zone, one of the highest ever measured under natural conditions (Beaubien et al., 2008; Krüger et al., 2011; McFarland et al., 2013; Šibanc et al., 2014, Beulig et al., 2015). Soil in the study area was an Alfisol Xeralf, with sandy loam-loam textures (United States Department of Agriculture, 1999). The main physico-chemical properties of soil sampled are shown in Table 4.1. Most of them show statistical differences among CO_2 fluxes: soil pH varied from slightly acid in control sampling points to strongly acid in higher fluxes sites (Table 4.1). Manganese also decreased in relation to CO_2 increments ($p < 0.01$), together with Magnesium ($p < 0.05$). In contrast, phosphorus ($p < 0.05$), iron and copper ($p < 0.01$) significantly increased as CO_2 flux did.

Moisture varied in relation to seasons (20.5 ± 5.5 % in November to 8.2 ± 4.5 % in May) and was strongly correlated with CO_2 fluxes in May (0.87, $p < 0.01$), but not in November.

4.3.2 Quantification of microbial populations

There was no clear pattern in the number of bacterial gene copies in response to the increasing exposure to natural upwelling of CO_2 although a significant decrease ($p < 0.01$) of 87 % was observed in highest fluxes in November, compared with the

Table 4.1 Chemical characteristics of the soil in each sampling time (November 2012 and May 2013). Significant differences ($p < 0.05$) for CO_2 exposure are highlighted in bold letters when both sampling times are considered.

	November 2012				May 2013				
	Control	Low	High	*p*	Control	Low	High	Extreme	*p*
CO_2 flux (g m^{-2} d^{-1})	15.18 ± 3.20	50.14 ± 4.65	1237.90 ± 327.20	< 0.001	8.37 ± 2.38	45.35 ± 4.98	445.64 ± 163.81	39407.50 ± 22020.69	< 0.001
Moisture (%)	17.1 ± 3.5	27.1 ± 1.2	17.4 ± 2.9	< 0.001	5.3 ± 1.2	7.1 ± 2.9	11.9 ± 5.9	41.9 ± 21.3	0.041
pH (water)	5.38 ± 0.43	5.00 ± 0.16	3.89 ± 0.15	< 0.001	5.87 ± 0.03	5.15 ± 0.04	4.53 ± 0.11	4.74 ± 0.28	0.021
Organic matter (%)	3.04 ± 1.12	3.54 ± 0.85	3.56 ± 0.26	0.648	3.37 ± 0.40	3.95 ± 1.35	5.48 ± 1.82	11.10 ± 7.50	0.502
Conductivity (dS m^{-1})	0.02 ± 0.01	0.03 ± 0.01	0.18 ± 0.27	0.178	0.03 ± 0.01	0.02 ± 0.01	0.07 ± 0.04	0.09 ± 0.08	0.290
Total nitrogen (%	0.22 ± 0.08	0.24 ± 0.04	0.26 ± 0.01	0.737	0.23 ± 0.02	0.29 ± 0.02	0.43 ± 0.04	0.84 ± 0.63	0.182
C:N	7.86 ± 0.51	8.41 ± 0.69	8.08 ± 0.51	0.541	8.59 ± 0.63	7.80 ± 2.04	7.27 ± 2.04	8.13 ± 2.22	0.843
Phosphorus (Olsen)	< 5.44	< 5.44	21.62 ± 8.60	0.022	< 5.44	18.22 ± 9.64	19.07 ± 11.94	80.44 ± 13.11	0.023
Calcium (cmol kg^{-1})	3.28 ± 0.67	5.92 ± 0.58	2.62 ± 2.71	0.113	3.68 ± 0.24	2.54 ± 0.85	2.92 ± 1.42	2.15 ± 1.09	0.342
Magnesium (cmol kg^{-1})	0.65 ± 0.10	1.16 ± 0.15	0.09 ± 0.03	< 0.001	0.66 ± 0.07	0.60 ± 0.23	0.20 ± 0.14	0.38 ± 0.14	0.024
Potassium (cmol kg^{-1})	0.22 ± 0.11	0.19 ± 0.02	0.13 ± 0.03	0.113	0.21 ± 0.05	0.20 ± 0.08	0.17 ± 0.02	0.26 ± 0.09	0.416
Sodium (cmol kg^{-1})	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.046	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.02	0.605
Ca:Mg	5.03 ± 0.36	5.13 ± 0.20	24.33 ± 19.18	0.060	5.59 ± 0.43	4.31 ± 0.23	19.68 ± 12.97	5.46 ± 1.19	0.070
K:Mg	0.35 ± 0.17	0.17 ± 0.04	1.33 ± 0.3	< 0.001	0.32 ± 0.08	0.34 ± 0.01	1.09 ± 0.67	0.74 ± 0.25	0.036
CEC	4.82 ± 1.07	7.62 ± 0.48	3.91 ± 1.2	0.008	5.08 ± 0.27	4.11 ± 1.29	4.79 ± 1.33	4.09 ± 1.38	0.644
Manganese (ppm)	13.82 ± 2.73	5.65 ± 3.25	2.20 ± 1.14	0.003	26.39 ± 6.63	9.26 ± 1.23	2.18 ± 0.79	6.00 ± 5.89	< 0.001
Iron (ppm)	102 ± 51.51	128.71 ± 28.65	400.93 ± 73.19	< 0.001	30.02 ± 4.50	134.72 ± 24.99	212.59 ± 53.24	337.22 ± 127.13	0.004
Copper (ppm)	0.20 ± 0.01	0.29 ± 0.03	0.48 ± 0.10	0.002	0.35 ± 0.08	0.58 ± 0.10	0.72 ± 0.30	2.16 ± 0.53	< 0.001
Zinc (ppm)	0.41 ± 0.18	0.35 ± 0.12	0.81 ± 0.1	0.013	0.53 ± 0.21	1.27 ± 0.75	0.89 ± 0.67	3.85 ± 3.71	0.286

control (Figure 4.2a). This drop at high fluxes was reverted at extreme fluxes as bacterial gene copies increased ($p < 0.001$) from 10^8 to 10^9 gene copies in May samples.

Archaeal populations followed a trend to decrease in gene copy numbers related to CO_2 increments (Figure 4.2a), with a significant reduction in high fluxes in the November sampling ($p < 0.01$). As observed with bacteria, this reduction was not maintained in extreme fluxes, as there was an increment of 50 % in November and 190 % in May with respect to the control. The fungal community also showed a lowering trend as CO_2 fluxes increased; in this case, extreme fluxes also diminished fungal gene copy numbers, with a significant decrease of three orders of magnitude in gene copy numbers ($p < 0.01$) in the high CO_2 flux sampling point of November (Figure 4.2a).

Comparing November 2012 and May 2013 sampling times, May samples showed a significantly higher bacterial and archaeal number of gene copies (ca. one order of magnitude, $p < 0.05$) than November. Patterns were different between sampling times, as high fluxes in November caused a pronounced decrease in gene copy numbers, whereas in May there were no differences among control, low or high fluxes. (Figure 4.2a).

4.3.3 Metabolic activity

Bacterial activity (measured as AWCD) showed a clear decreasing trend from control to high CO_2 flux samples (73.6 and 80 % in November and May, respectively), being significant in November ($p < 0.01$, Fig. 4.2b). In the same way as the qPCR results, this trend reverted in extreme CO_2 fluxes ($p < 0.01$), this being the pattern observed both in Richness (number of positive Biolog wells) and Shannon diversity index (Figure 4.2b). Principal component analysis applied to 120 h absorbance data of the whole Biolog plates separated the samples in groups according to CO_2 fluxes (Figure 4.3).

As regards the carbon source utilization profile, no differences were observed among CO_2 levels: the most utilized substrates were carbohydrates and amino acids, specifically D-Mannitol and L-Asparragine, in all cases. There were no changes in consumption patterns of substrates when increasing CO_2, but a drop in AWCD, from control to high CO_2 fluxes, followed by a strong increase of activity in extreme fluxes. Moisture content significantly influenced microbial activity (AWCD) (Spearman correlation 0.62, $p < 0.01$). In fact, control and low fluxes in wet November showed a higher carbon substrate use than their relatives in dry May.

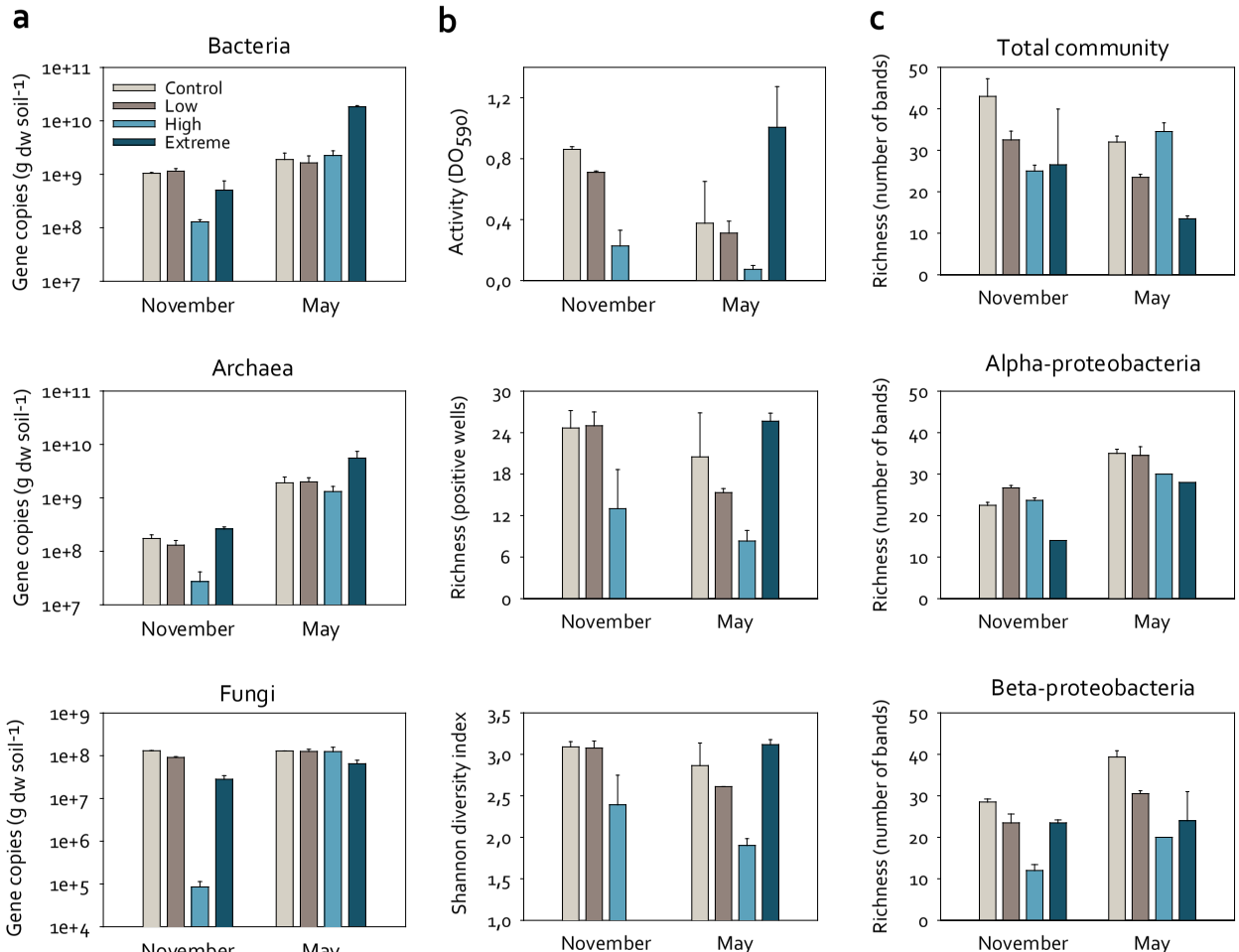


Figure 4.2 a Microbial community quantification (Bacteria, Fungi and Archaea) determined using qPCR for November 2012 and May 2013 at control, low, high and extreme CO_2 fluxes (mean ± sd, n = 3). **b** Average well colour development (AWCD), Richness and Shannon index of Biolog EcoPlates at 590 nm (mean ± sd, n = 3). **c** Richness (number of bands) of total community, Alpha- and Betaproteobacteria DGGE profiles (mean ± sd, n = 3).

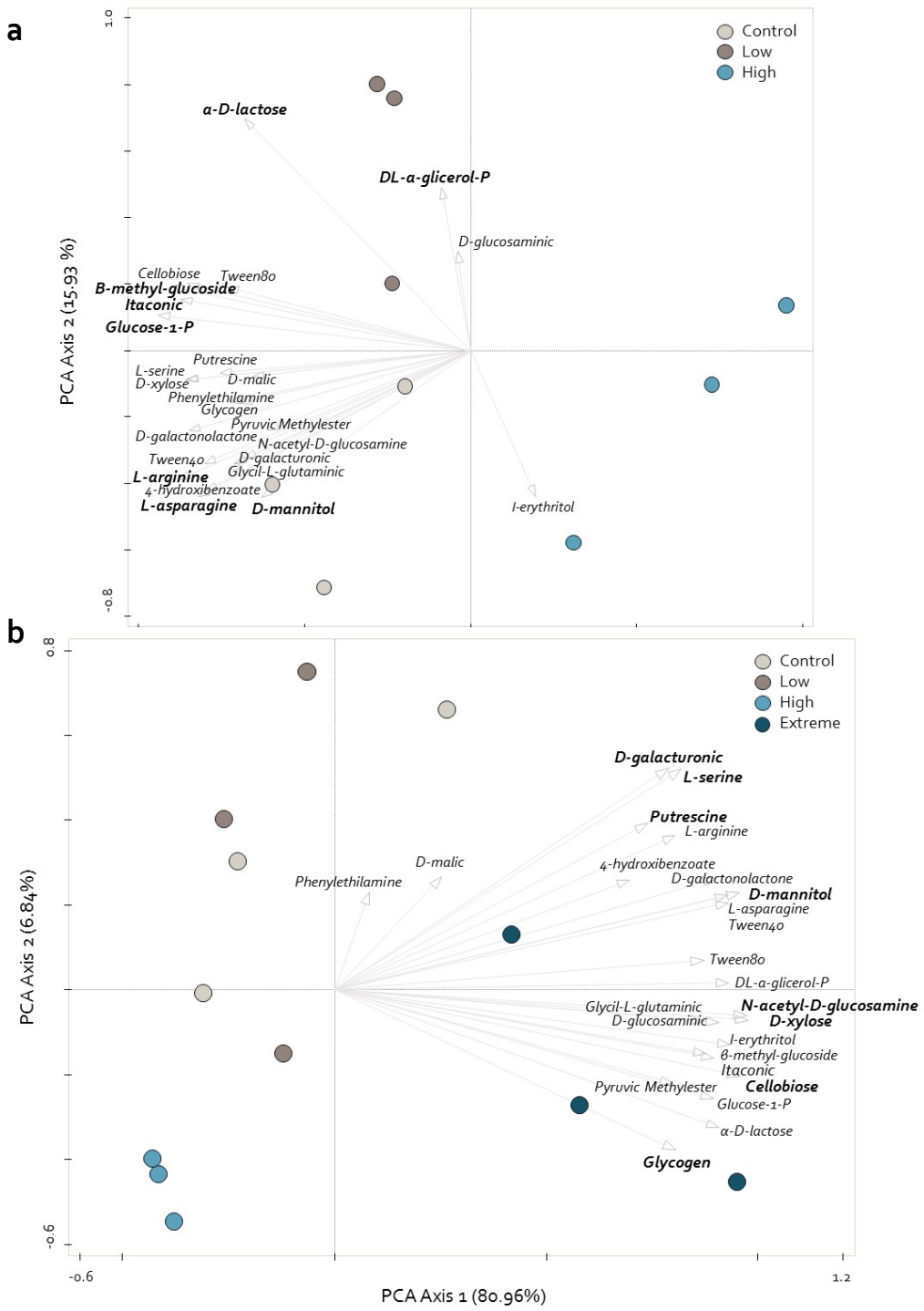


Figure 4.3 Principal components analysis showing how samples distribute, as determined by metabolic profiles using Biolog EcoPlates, for (a) November 2012 and (b) May 2013 samplings. Bold letters indicate which substrates contribute greatest to PC ordination.

4.3.4 Community structure analysis

DGGE profiles of 16S rDNA fragments (amplified by PCR using universal and group specific primers) were used to evaluate the effect of CO_2 on the soil bacterial community. The UPGMA tree (Figure 4.4) showed that samples were grouped according to CO_2 fluxes for each of the bacterial group studied: total community, alpha and betaproteobacterial populations changed from control-low to high-extreme fluxes. Band richness decreased as CO_2 flux increased, from 43 and 29 bands in control to 25 and 13 bands in high fluxes, for total and beta-proteobacterial communities, respectively (Figure 4.2c). Observing the DGGE profiles (Figure 4.5), we saw that as the CO_2 flux increased, the band pattern changed, leading to a new community composition.

Functional richness (number of Biolog positive wells) showed a strong correlation with species richness when all fluxes, except the extreme ones, were considered. In November there was a positive correlation between Biolog and both total community richness ($\rho = 0.87$; $p < 0.00$) and Beta-proteobacterial richness ($\rho = 0.86$, $p < 0.00$), whereas in May functional richness was correlated with Alpha- and Beta-proteobacteria ($\rho = 0.90$ and 0.79, respectively, $p < 0.01$).

As shown in Figure 4.5 the fingerprinting profile of bacterial communities was really complex, with a high number of bands preventing the reamplification and sequencing. In fact, only 7 out of 16 bands were of good enough quality to be sequenced (Table 4.2), most of them from alpha- and beta-proteobacterial profiles. Total community (universal bacterial primers) diversity was so high that only one of the excised bands could be successfully isolated. Interestingly, one of the bands from high-extreme fluxes matched *Methylocella palustris* with 98 % similarity. *Methylocella palustris* is an already described acidophilic methanotroph (Dedysh et al., 2004)

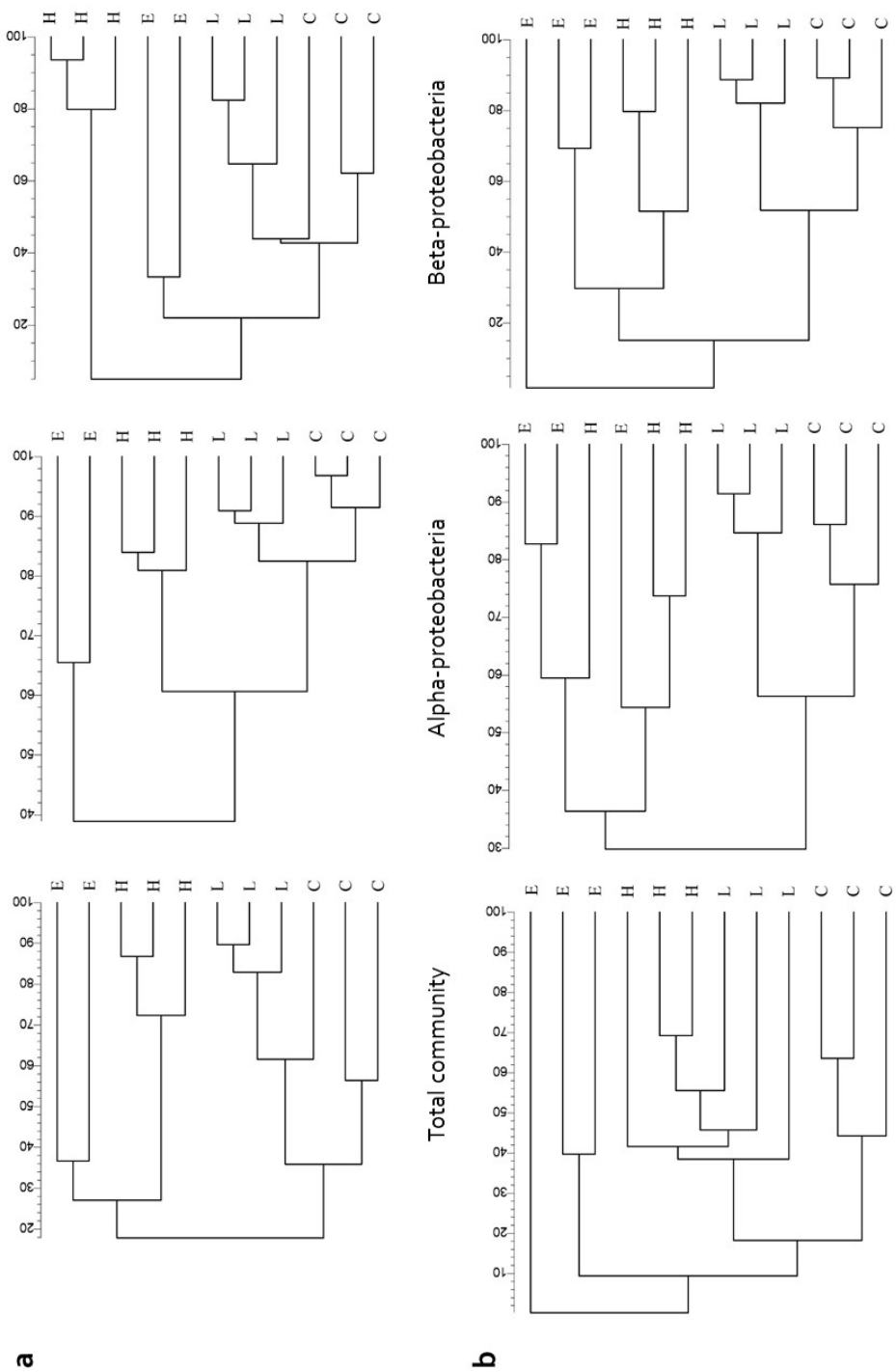


Figure 4.4 Hierarchical cluster analysis results of the Total community, Alphaproteobacteria and Betaproteobacteria DGGE profiles demonstrated graphically as a UPGMA dendrogram, for November 2012 (a) and May 2013 (b) times. Letters show CO_2 treatments: control (C), low (L), high (H) or extreme (E).

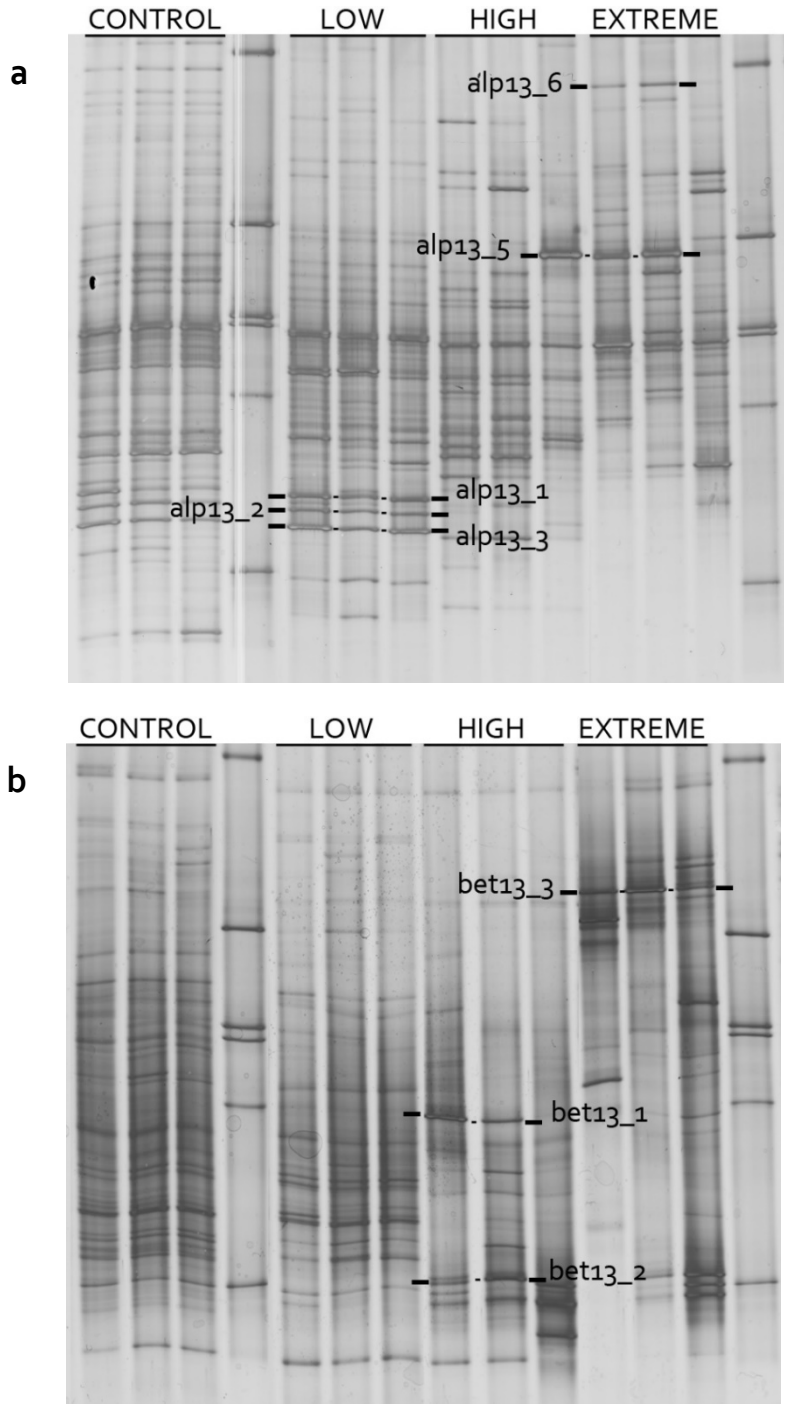


Figure 4.5 DGGE gels from (a) Alphaproteobacteria and (b) Betaproteobacteria in May 2013 sampling, showing excised bands. Note the different profile from control-low to high-extreme flux samples.

Table 4.2 Sequence identity of DGGE excised bands from Alpha-proteobacterial, Total community and Beta-proteobacterial profiles.

DGGE analysis	CO_2 flux	DGGE band	Sequence identity (%)	Organisms or uncultured representative (Accession number)	Isolation source
α-proteobacteria	Low	Alp13_2	99	Bacterial isolate (HQ119568.1)	Loamy sand of *Eucalyptus* forest [a]
	Low	Alp13_3	98	Bacterial isolate (HQ119568.1)	Loamy sand of *Eucalyptus* forest [a]
	High-Extreme	Alp13_5	98	*Methylocella palustris* (AJ563927.1)	*Sphagnum* peatland [b]
Total Community	High	Tot13_6	98	Bacterial clone (EF120704.1)	Conifer decaying wood [c]
β-proteobacteria	High	Bet13_2	99	*Burkholderia* clone (KJ1921955.1)	*Pinus massoniana* soil [d]
	Extreme	Bet13_3	97	*Diaphorobacter* sp (KC352658.1)	Outdoor air [e]

[a] Williamson et al. (2011); [b] Dedysh et al. (2004); [c] Zhang et al. (2008); [d] Shi and Huang (Unpublished); [e] Kim et al. (2014).

4.4 Discussion

Mofettes present extreme environments where soil biota has evolved for long periods, to adapt to elevated CO_2 conditions, leading to specialized communities, which still remain sparsely studied (Maček, 2013). At La Sima mofette, we assessed the impact of belowground high CO_2 emission on soil microbial communities along the greatest gradient ever studied (Beaubien et al., 2008; Krüger et al., 2009; Vodnik et al., 2009; Russell et al., 2011; McFarland et al., 2013). We used different molecular methods to gain a broader understanding of how long-term belowground CO_2 emissions have driven microbial community structure and composition of the edaphic microbiota in this volcanic CO_2 vent. In comparison with experimental approaches (Pierce and Sjögersten, 2009; Morales and Holben, 2013, Fernández-Montiel et al., 2015) where soil microorganisms are still stabilizing and effects are unclear, these extreme environments have been emitting for centuries, and thus the community could be adapted to CO_2 impact (Oppermann et al., 2010; Frerichs et al., 2013; Šibanc et al., 2014). Previous studies on artificial below-ground CO_2 emissions (Fernández-Montiel et al., 2015) found that after more than one year of exposure microbial communities were unaffected by CO_2 fluxes similar to some of those included in this study, although there was a clear significant decrease of pH in gassed plots.

Increasing CO_2 fluxes at La Sima vent caused profound changes in soil characteristics with respect to control zone (not CO_2 affected). We found strong variations in soil physico-chemical characteristics, closely correlated with CO_2 flux, which possibly influenced edaphic microbiota. Most of the studies in mofettes have reported soil acidification (Videmšek et al., 2009; Oppermann et al., 2010; Krüger et al., 2011; Beulig et al., 2015). We also found pH decreased from control to CO_2 high-extreme sites,

whereas P, Fe and Cu increased. In relation to this, the mobilization of metal compounds has been reported as one consequence of elevated CO_2 concentrations (Beaubien et al., 2008; Frerichs et al., 2013; Mehlhorn et al., 2014). Beulig et al. (2015) found an increase in dissolved Fe (II), together with a significant drop in pH and redox potential.

In our study, there was an evident seasonal effect on microbial responses, as already observed by other researchers. Morales and Holben (2013) found seasonal patterns affecting soil microbial communities (moisture, temperature, pressure) even when exposed to elevated subsurface CO_2 concentrations. Other studies (Castro et al., 2010) have reported an interactive effect of CO_2 and temperature suggesting potential seasonally dependent responses. Frerichs et al. (2013) also described an interaction between the CO_2 concentrations and seasonal factors affecting the potential sulphate reductions rates.

In this study, the impact of CO_2 caused marked alterations on edaphic microbiota, as much in abundance as in richness and activity. We found a negative response of microbial communities from control to high fluxes (260 to 1600 g m^{-2} d^{-1}). Bacterial, archaeal and fungal gene copies were reduced because of CO_2 exposure. Many studies have mentioned the negative effect of high CO_2 emissions on microbial abundances, measured as bacterial cell counts (Beaubien et al., 2008; Krüger et al., 2009; 2011), microbial biomass (Beaubien et al., 2008; McFarland et al., 2013), archaea or bacteria gene copy numbers (Beaubien et al., 2008), or bacterial MOTUs (molecular operational taxonomic units) (Šibanc et al., 2014). Additionally, DGGE results showed that there was a negative effect of increasing fluxes of CO_2 on microbial genetic diversity at La Sima mofette. Oppermann et al. (2010) also found a decreased number of bands with CO_2, but Frerichs et al (2013) did not report any changes in bacterial diversity by DGGE. Nonetheless, they reported a distinct shift in the archaeal community composition under elevated CO_2, finding that the majority of the vent centre– associated sequences clustered into the group of the Thaumarchaeota. These results suggest that microbial communities do not always respond in the same way, and could depend on uncontrolled factors leading to non-consensus; hence, local conditions could modify the impact of a CO_2 leakage (West et al., 2013). A previous study by Sáenz de Miera et al. (2014), which reported changes in composition and structure through DNA pyrosequecing in La Sima site, found a high presence of OTUs related to Chloroflexi phylum in the vent centre. In accordance with this study, Sáenz de Miera et al. (2014) also found that bacterial community richness and diversity using pyrosequencing notably decreased with increasing CO_2 fluxes.

An original aspect of this study was to consider the effect of CO_2 vents on functional richness by using a microbial carbon utilization profile through Biolog Ecoplates. The decrease in metabolic activity diversity was in parallel with the drop in band richness, as CO_2 fluxes increased. Hence, increased CO_2 emissions may cause not only a loss of

species diversity (Beulig et al., 2015) but also a reduced functional richness. We did not find changes in the substrate utilization pattern between control and CO_2 exposed samples, so it is possible the metabolic fingerprint was not affected by CO_2 at La Sima mofette. Note that Biolog Ecoplates provide a scheme of potential activity of the microbial community in specific conditions (aerobic, fast-growing, aquatic environment). Other research has focused on specific bacterial activities, i.e. methane and sulphate production or oxidation (e.g. Beaubien et al., 2008; Oppermann et al., 2010; Krüger et al., 2011) so there is no other information available on general metabolic patterns in CO_2 mofettes.

The extremely high fluxes of CO_2 included in this study could be considered as an outlier in comparison with the other fluxes. Nevertheless, it was interesting from the scientific point of view to know which were the characteristics of the microbial communities in such extreme, non-previously reported conditions in soils. This study found that the clear decreasing patterns observed from control to high fluxes suddenly changed when considering extreme fluxes. These extreme fluxes caused an increase in bacteria and archaea abundances, in relation to a greater and more diverse activity in Biolog Ecoplates. However, DGGE community diversity strongly dropped at these extremely high CO_2 fluxes. Presumably local soil conditions (e.g. richer organic matter, higher moisture content) fostered a shift to more active populations, although less diverse and adapted to an acidophilic environment at La Sima extreme sampling points. This aspect has been reported in other mofettes (Oppermann et al., 2010; Frerichs et al., 2013; Beulig et al., 2015). Indeed, we found the presence of an acidophilic methanotroph (*Methylocella palustris*) in extreme fluxes, which produces CO_2 from CH_4 in oxic environments. This evidences that even in extreme CO_2 fluxes, wind effects might be dispersing the CO_2 rapidly (Beaubien et al., 2008), permitting an O_2 concentration high enough to allow this kind of metabolisms. Since O_2 gas measurements remain under 7 % in the gas vent (Gosálvez et al., 2010), bacterial environment varies between aerobic and microaerophilic conditions.

Our results indicated that the effects of the gas upwelling are spatially limited, as observed in Laacher See, Germany (Krüger et al., 2011), between the centre of the vent (high and extreme fluxes) to the surrounding area (low and control). In fact, as shown in the dendograms and PCA results, there was a clear change in microbial community from low (40 to 55 g m^{-2} d^{-1}) to high (260 to 1,600 g m^{-2} d^{-1}) fluxes; which leads us to consider a minimum of 200 g m^{-2} d^{-1} as a limit to begin to appreciate the effect of CO_2 emissions on microbial communities at La Sima site. Frerichs et al. (2013) proposed a potential environmental threshold for soil environments at 10-20 %, as they found changes in microbial potential activities and qPCR at Laacher See vent medium site (with around 20 % CO_2 in the gas phase, and a flux of 177 g m^{-2} d^{-1}) and other investigations found altered vegetation and microbial activities at concentrations of 5-20 % CO_2 in the upper layers of the soil column (Pfanz et al., 2007; Pierce and Sjögersten, 2009).

An applied aspect of these studies is that mofettes offer a monitoring tool to control and evaluate the ecological consequences of the expanding implementation of CCS technologies and the potential environmental effect of accidental gas leakages (Schütze et al., 2012).

4.5 Conclusions

The results confirmed that La Sima CO_2 emissions severely affect soil microbial communities, leading to reduced and less diverse populations. This work supports previous research, confirming that increasing CO_2 fluxes cause losses in functional and community diversity, and a decrease in metabolic activities. Nevertheless, at extremely high CO_2 fluxes, like the ones considered in this research, patterns in abundances and microbial activity shifted, proving that these extreme conditions can harbour very high microbial abundances of archaea and also of well-adapted fungi and bacteria.

Edaphic communities in a wide range of CO_2 belowground emissions at La Sima vent[3]

5

Natural CO_2 vents have received growing interest in the last years due to their relation to CO_2 capture and storage (CCS) risk assessment studies. Despite this increasing body of knowledge, mostly focused on microbial communities, scarce information is available on how geological CO_2 affect mesofauna and microfauna, and their interactions. We studied microorganisms, microfauna i.e. protists and nematodes, and mesofauna communities, i.e. collembola and mites and their relationships in a natural CO_2 vent at La Sima (Spain). Four CO_2 flux intensities from control (7 to 19 g m^{-2} d^{-1}) to high fluxes (260 to 1,600 g m^{-2} d^{-1}), including extreme emissions (more than 10^4 g m^{-2} d^{-1}) were studied. We found that increasing CO_2 emissions from control to high fluxes strongly affected biota abundances and richness, cascading from microorganisms to mesofauna, and resulting in reduced and less diverse populations in each of the groups levels assayed. Nevertheless, at extreme fluxes edaphic biota biomass recovered in most of the communities, suggesting that the extreme CO_2 conditions are associated with high abundances of well- adapted communities, although with very low diversity. Increases in abundance of bacteria, fungi and amoebae, but not ciliates, were related to increases in mesofauna richness and nematode and mesofauna abundances. Results from natural CO_2 vents could be used as models for studying the effects of accidental loses of CO_2 from CCS operations, one of the most promising strategies suggested to mitigate greenhouse gas effects on global warming.

[3] This chapter is based on the article:

Fernández-Montiel, I., Sidrach-Cardona, R., Gabilondo, R., Pedescoll, A., Scheu, S., Bécares E. Edaphic communities in a wide range of CO_2 belowground emissions at La Sima vent (Spain). (Submitted to Soil Biology and Biochemistry)

5.1 Introduction

Rising atmospheric CO_2 concentrations and the relative interest in studying the consequences on soil ecosystems has stimulated research at CO_2 vents (mofettes) (Paoletti et al., 2005). Mofettes are natural ecosystems where cold, geogenic CO_2 migrates upward, through surface water or soil fractures to the atmosphere (Russell et al., 2011). These ecosystems, where CO_2 has been venting for centuries or millenia, represent a stable long-term scenario of basic scientific interest that may serve as model system to assess the effects of anomalous CO_2 concentrations (Šibanc et al., 2014).

The high CO_2 concentrations presented in these areas, sometimes reaching 100 % in the centre, lead to changes in soil gases composition, soil hypoxia or even anoxic conditions (Vodnik et al., 2009) resulting in adverse conditions for microbial and animal life in mofette fields (Paoletti et al., 2005; Gosálvez et al., 2010). Nevertheless, the impossibility of survive for above the ground fauna may not apply to belowground life (Russell et al., 2011). In fact, it is known that soil CO_2 concentrations are 10–100 times higher than the atmospheric level (Drigo et al., 2008; Russell et al., 2011) therefore by at least some edaphic biota may be resistant to mofettes' environment. Moreover, several studies have found microbial communities adapted to high CO_2 concentrations in soils (Tarkowski et al., 2009; Oppermann et al., 2010; Beulig et al., 2015).

Natural CO_2 vents have received growing interest over past years related to emerging CO_2 capture and storage (CCS) applications, as they can be used as a simulation of a potential leakage from a CCS site (e.g. Beaubien et al., 2008; Krüger et al., 2009; McFarland et al., 2013). Although these sites are evaluated to avoid possible leaks, this cannot be completely excluded and understanding the consequences on the environment is a major concern (Krüger et al., 2011).

Mofettes have been studied in several countries (Czech Republic, Poland, Germany, Greece, Italy, Spain, New Zealand, USA), and used in CCS risk assessment (Paoletti et al., 2005; Noble et al., 2012). Biological studies in natural CO_2 vents have focused on microbial communities (mainly Bacteria) (Frerichs et al., 2013; McFarland et al., 2013; Šibanc et al., 2014), but scarce information is available on higher trophic levels. As an exception, Russell et al. (2011) found mofettophilous Collembola in Cheb Basin (Czech Republic) and Yeates et al., (1999) observed negative effects of high CO_2 emissions on nematode in Northland natural vent (New Zealand). However, no studies have focused on other groups of animals or on the relationship among them.

Our investigation takes advantage from natural CO_2 emissions at La Sima vent, located in Calatrava Volcanic Field (CVF), central-eastern Spain. La Sima covers a wide range of CO_2 fluxes (from 40 g m^{-2} d^{-1} to 300 kg m^{-2} d^{-1}) being one of the sites with most extreme fluxes ever studied (Beaubien et al., 2008; Krüger et al., 2011; McFarland et

al., 2013; Šibanc et al., 2014, Beulig et al., 2015). To date there are few biological studies in La Sima vent (Gabilondo and Bécares, 2014; Sáenz de Miera et al., 2014), and to our knowledge, no studies have addressed the effects of CO_2 belowground emissions on organisms of different trophic levels. Hence, the aim of this research is to analyse microbial, protozoal and mesofauna populations from La Sima mofette, to better understand the effect of high CO_2 belowground fluxes on edaphic communities and their relationships.

5.2 Materials and Methods

5.2.1 Site description

The Calatrava Volcanic Field (CVF) is one of the highest CO_2 discharge area of Spain, consisting in more the 300 emission centres throughout an area of about 5,000 km^2. A better description of the area can be found in Elío et al. (2015). La Sima mofette is a CO_2-rich discharge, located at Granátula de Calatrava (Ciudad Real, Spain). Since a seismic crisis in the area in 2007, CO_2 fluxes increased from 30,000 to 200,000 ppm (Peinado et al., 2009; Gosálvez et al., 2010). Details on La Sima site are described in Chapter 4.

Sampling took place in November 2012 and May 2013. Before sampling, distribution of CO_2 fluxes in La Sima vent was measured using a CO_2 analyser (LICOR LI820 CO_2 soil flux meter from West Systems). CO_2 fluxes reached rates of 300 kg m^{-2} d^{-1} at its centre zone, one of the highest ever measured in natural conditions (Beaubien et al., 2008; Krüger et al., 2011; McFarland et al., 2013). Samples were collected along a CO_2 fluxes gradient from the centre of the vent to surrounding area –non CO_2-affected–, with values ranging from 7 to 19 (control), 40 to 55 (low) and 260 to 1600 g m^{-2} d^{-1} (high). An extreme locality with more than 10^4 g m-2 d-1 was also studied, but not included in the statistical analysis. Sampling consisted of five soil cores of 5 cm diameter to a depth of 10 cm, which were pooled and then used for microbial (100 g), protozoan (200 g) and micro / mesofauna (ca. 800 g) analysis. Microbial samples were thoroughly homogenized and transported on ice to the laboratory, sieved to 2 mm and stored at -20 ºC until further analysis. In addition, moisture, pH, organic matter and conductivity were measured with the standard protocols of the Spanish Ministry of Agriculture, Fishery and Food (Ministerio de Agricultura, Pesca y Alimentación, 1994). The available fraction of the elemental concentrations of ions and metals were analysed with an ICP-AES Optima 2000 DV from Perkin Elmer, using the standard protocols of the Spanish Ministry of Agriculture, Fishery and Food. At November 2012 sampling, extreme samples were only enough for microbial and protozoal analysis.

5.2.2 Microbial community

Microbial community richness was assessed by Denaturing Gel Gradient Electrophoresis (DGGE) and bacterial, archaeal and fungal abundance was quantified

through qPCR. Total microbial community DNA was extracted from 0.25 g of soil samples, using the Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., CA, USA), according to manufacturer's instructions. DNA yield was assessed by electrophoresis in 1.2 % agarose gels stained with RedSafe™ (Intron Biotechnology, Korea) and visualised under UV light. For DGGE, PCR was performed to amplify universal and group-specific 16S rRNA gene fragments in a thermal cycler TC-512 (Techne, UK).The quantitative PCR was performed using SYBR Green PCR Master MIX (Applied Biosystems, Foster City, CA) on the Step One Plus system (Applied Biosystems). PCR conditions and primers used as well as a more detailed description of the methods used can be found in Chapter 3.

5.2.3 Protozoan community

Most soil protozoa are bacterial feeders and they have high consumption rates so that they can strongly influence the bacterial species composition and shape the structure of microbial communities (Clarholm et al., 2010). Protozoa were determined as described in Gabilondo et al., 2015. The abundance of amoebae, flagellates and ciliates was measured using the protocol described by Adl et al. (2007). Briefly, 1 g of soil was weighed, mixed with water and the mixture pipetted onto agar in 5 cm Petri dishes and aluminium foil. Amoebae abundance was counted 24 h later. Flagellates were counted in a haemocytometer chamber. To determine the number of ciliates 4 replicates of 100 ml were measured and counted with a light microscope (Madoni, 1984) and. For the analysis of diversity of ciliates, 2 ml of the soil supernatant were fixed with Bouin for posterior Edaphic Quantitative Protargol Staining (EQPS) method (Acosta-Mercado and Lynn, 2003) and 5 ml were stained with a modified silver carbonate method as described in Gabilondo and Bécares (2014). Protozoa were only analysed in November.

5.2.4 Micro- and mesofauna

Soil mesofauna was extracted using modified Berlese funnels, counted (abundance kg^{-1}) and classified at the order level for all animals, and at the family level for the mites orders Prostigmata, Mesostigmata and Oribatida, following Andres et al. (2011). Holometabolous insect larvae and adult were pooled to a single group. For the calculation of microarthropod taxa richness, only morpho-species were distinguished. Nematodes were extracted from 100 g soil (fresh weight) in sterile distilled water using the Baermann funnel technique. After an extraction time of 10 days, nematodes were preserved in ethanol 70 %, counted, and related to g soil dry weight.

5.2.5 Statistical analysis

Data were log-transformed when necessary to meet the assumptions of parametric statistical tests (normality and homoscedasticity of errors). Boxplots and means ± standard error presented in Figures 5.1 and 5.2, and Table 5.1 were calculated using

non-transformed data. Analysis of variance (ANOVA) was performed to test the effects of CO_2.

All variables (abundance and richness values, and edaphic properties) were tested for significant differences ($p < 0.05$) based on the factors "CO_2" and "time" by ANOVA, using the Vegan package (Oksanen et al., 2010) in R (http://www.r-project.org). Spearman correlations were calculated among physico-chemical parameters and their relation with CO_2 effect, and to test relationship between soil biota groups. Regression analysis was also used to examine the correlation between those groups. To analyse whether the structure of edaphic communities varied with the soil characteristics and CO_2 exposure redundancy analysis (RDA) was performed. Analyses were implemented in CANOCO 5.0 (Wageningen, The Netherlands). Extreme CO_2 values were considered outliers and not included in the RDA analysis, nor in the correlations between biota groups.

5.3 Results

5.3.1 Soil chemistry

Soil pH varied from slightly acid in Control sampling points (5.63±0.38 -mean from two sampling times-) to strongly acid in High fluxes sites (4.20±0.37) (Table 4.1). Strong positive correlations were observed between CO_2 flux and P, Fe and Cu (Spearman ρ = 0.83, 0.91 and 0.70 respectively; $p < 0.001$). By contrast, strong negative correlation was found between CO_2 flux and pH (-0.83, $p < 0.001$) or Mn (-0.74, $p < 0.001$). Total nitrogen, organic matter, carbon to nitrogen ratio and the rest of cations and metals were not significantly correlated to CO_2 flux. Moisture varied significantly ($p < 0.01$) in relation to seasons (20.5 ± 5.5 % in November to 8.2 ± 4.5 % in May) and also had a moderate correlation with CO_2 fluxes (0.55, $p < 0.01$). There was also a slightly decrease in Na from November to May ($p < 0.05$) and an increase in Cu ($p < 0.05$).

5.3.2 Effect on soil biota abundance

Bacterial and fungal abundance (measured as gene copy numbers) showed a significant decrease in November sampling (Table 5.1) related to high CO_2 emissions. However, in May we observed a positive trend when increasing CO_2 (Figure 5.1). Archaeal numbers were not affected by CO_2 fluxes. Comparing sampling times, microbial gene copy numbers were higher in May than in November (Figure 5.1).

Protozoal community presented different patterns depending on the group. Abundance of amoebae significantly decreased to zero in high CO_2 flux samples (Table 5.1). However, flagellates and ciliates did not show differences between CO_2 levels, although we observed a trend to decrease with CO_2 (Figure 5.1). Nematodes were severely affected by CO_2 increments, whose abundance declined to 81 % in low fluxes and almost disappeared in high fluxes ($p < 0.001$). Similarly, total mesofauna

abundance showed a negative response to high CO_2 fluxes (p= 0.02) in November sampling, but not in May (Table 5.1). Mesofauna showed a unimodal pattern from Control to high fluxes (Figure 5.1). Acari was the most abundant order, with a significantly increase at low fluxes comparing to Control and High (Table 5.1).

Table 5.1 One-way ANOVA results for CO_2 exposure effects on the different soil groups analyses. Bold letters indicate significant results ($p < 0.05$). Missed values correspond to not available samples.

	November 2012		May 2013	
Soil biota	**F**	**p**	**F**	**p**
Microorganisms				
Bacteria	**8.69**	**0.017**	1.82	0.241
Archaea	0.96	0.432	0.36	0.711
Fungi	**10.81**	**0.010**	1.38	0.321
Protozoa				
Amoebae	**191.00**	**<0.001**	-	-
Flagellates	0.25	0.783	-	-
Ciliates	4.26	0.070	-	-
Nematoda	**622.90**	**<0.001**	-	-
Mesofauna	**7.02**	**0.027**	4.62	0.061
Acari	**7.14**	**0.026**	**5.24**	**0.048**
Collembola	4.07	0.076	1.08	0.397
Soil diversity (sum of all taxa)				
Bacteria richness	2.45	0.166	**6.57**	**0.031**
Total community	0.47	0.643	0.39	0.690
Alpha-proteobacteria	2.03	0.212	**6.56**	**0.031**
Beta-proteobacteria	**18.37**	**0.003**	**27.95**	**<0.001**
Ciliates richness	**10.71**	**0.010**	-	-
Mesofauna richness	**10.79**	**0.010**	2.82	0.137

5.3.3 Effect of CO_2 vent on biodiversity.

Soil biodiversity generally decreased when CO_2 increased, although statistical differences varied depending on the group and data of sampling (Table 5.1). Considering the sum of all, bacteria, ciliates and mesofauna richness, we found a significant decrease of soil biodiversity ($F: 7.63$, $p < 0.01$). Figure 5.2 shows that bacteria richness (sum of total, alpha, and betaproteobacterial number of bands) was negatively affected at high CO_2 flux, although this was only significant in May. Ciliate richness also significantly declined at high CO_2 flux samples. Mesofauna richness responded in the same way as densities: it decreased significantly at high CO_2 fluxes but tended to increase in lower CO_2 fluxes.

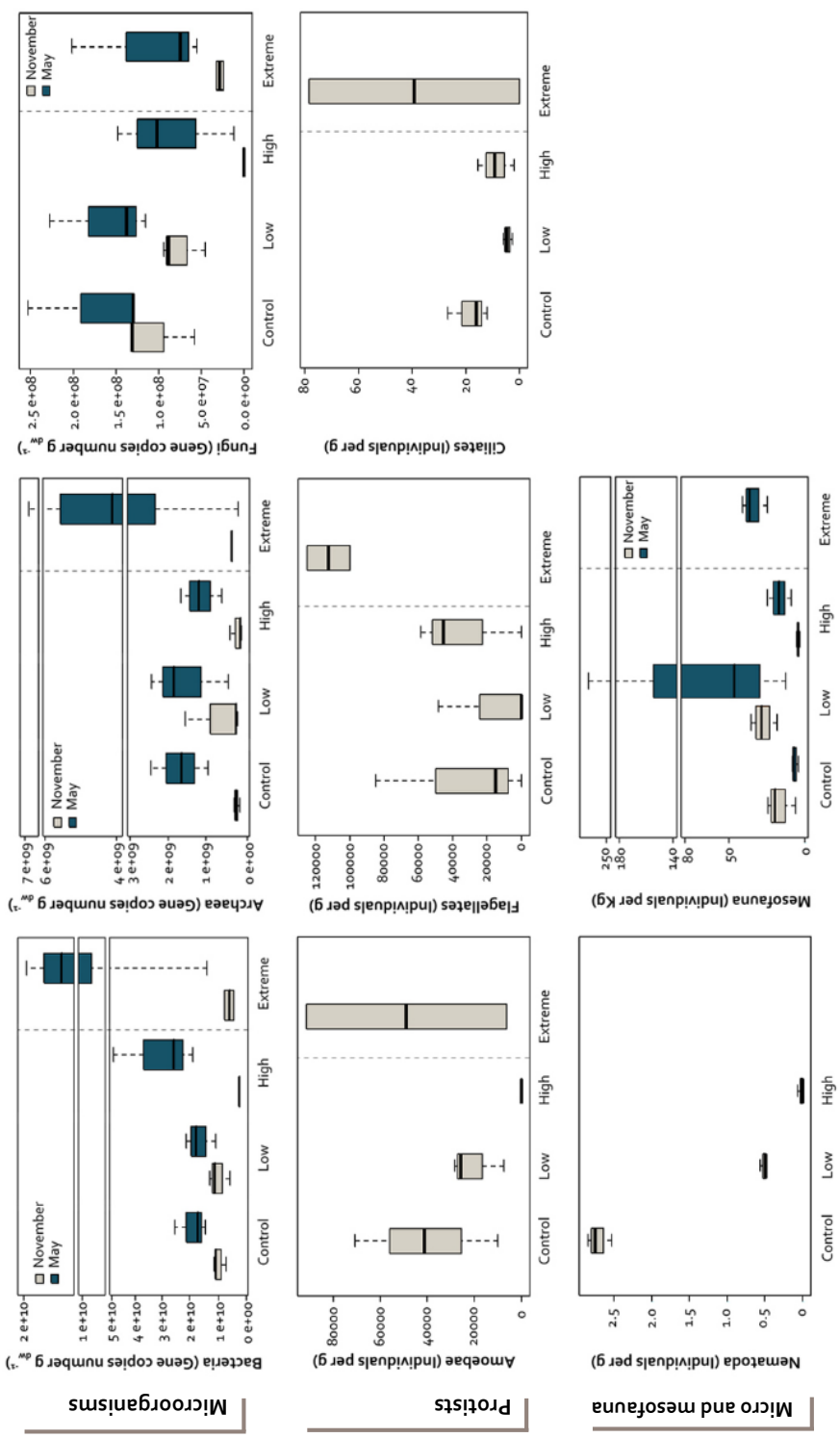


Figure 5.1 Microorganisms: Microbial community quantification (Bacteria, Fungi and Archaea) determined using qPCR at control, low, high and extreme CO_2 fluxes (n = 3). Protists: number of amoebae, flagellates and ciliates (individuals per g) at control, low, high and extreme CO_2 fluxes (n = 3). Micro and mesofauna: number of nematode (individuals per g) and mesofauna (individuals per kg) (n=3). The line across the boxplots represents the median.

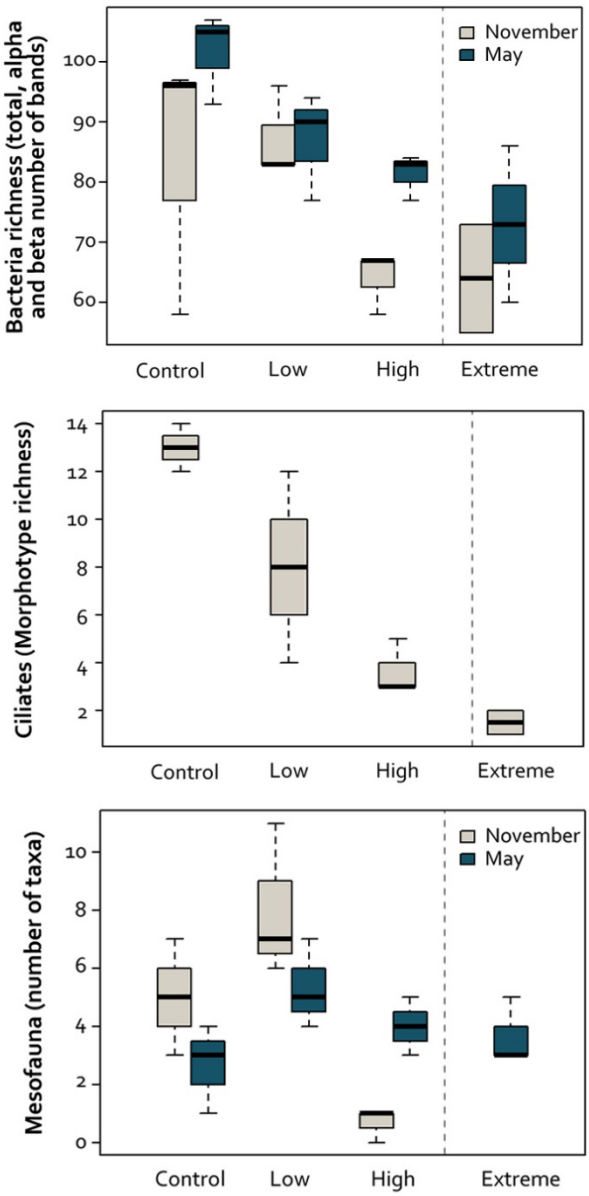


Figure 5.2 Soil biota richness at control, low, high and extreme CO_2 fluxes. Bacterial richness is expressed as the sum of total community, alpha- and beta-proteobacterial number of bands. Ciliates richness represents the morphotype richness whereas mesofauna richness refers to number of different taxa.

Figure 5.3 Redundance analysis biplot for all biological variables studied in each sampling time: a) November and b) May, plotted by soil chemistry variables. Extreme flux samples were excluded from the analysis. Variance explained in November plot is 52.45 and 40.40 (first and second axis, respectively) and 69 and 20.72 for May plot.

As it was expected, increasing mesofauna and ciliates density were associated by an increasing richness. Nonetheless, there was an opposite trend in bacteria: while bacteria gene copies increased in May at CO_2 high fluxes, the richness showed a significant decrease, leading to a specific bacterial community adapted to acidic and anoxic environment.

5.3.4 Relationships among communities

Fungi-to-bacteria ratio was negatively affected by high CO_2 fluxes (linear regression, $R^2 = 0.95$, $p < 0.001$ for November samples, $R^2 = 0.47$, $p = 0.04$ for May). Further, there was a positive relationship between this ratio and mesofauna and nematode abundance ($R^2 = 0.62$, $p = 0.01$ and $R^2 = 0.75$, $p < 0.01$, respectively) in November.

Also, as indicated by linear regression, mesofauna and bacteria ($R2 = 0.83$, $p < 0.001$) as well as mesofauna and fungi ($R^2 = 0.70$, $p < 0.01$) were closely related. Further, positive correlations were observed between ciliates richness and bacterial and fungal numbers (Spearman $\rho = 0.71$ and 0.81 respectively, $p < 0.05$), as well as between mesofauna richness and bacterial and fungal numbers ($\rho = 0.87$, 0.73 respectively, $p < 0.05$). However, most of the correlations were only significant in November and not in May.

To visualise the overall effect of high CO_2 emissions and its derived consequences on soil chemistry, redundancy analysis (RDA) was conducted (Figure 5.3). Samples of high CO_2 flux sites were separated from low flux and control sites, evidencing the negative effect of CO_2 on soil biota abundance and richness, also associated to pH effect. Once again, differences between sampling times were explained by higher soil moisture in November.

5.3.5 Biological communities in Extreme fluxes at La Sima vent

La Sima vent presented the highest flux ever registered in a biological study, ranging from 10 to 300 kg m^{-2} d^{-1}, which contributes to the knowledge about the adaptability of edaphic communities. Soil characteristics from these sampling points were very different from control, low and high fluxes, leading to diverse responses in soil biota. Extreme fluxes tended to reverse negative effects observed in microbiota densities at high CO_2 emissions, with a significant increase in flagellate abundance ($p < 0.05$). Nevertheless, other protozoan groups, i.e. ciliates and amoebae, showed no differences when compared with high fluxes (Figure 5.1). With regard to richness, extreme CO_2 emissions maintained the negative impact observed from control to high fluxes for most groups studied, although the decrease was only significant for alpha and beta-proteobacterial richness ($p < 0.01$).

5.4 Discussion

In the last decade, natural CO_2 vents or mofettes have received increasing attention as they represent extreme environments where not only CCS risk assessment can be developed but also because soil biota responses to long-term environmental changes can be studied. To date, most of biological research on mofettes has focused on microorganisms (e.g.; Oppermann et al., 2010; Frerichs et al., 2013; McFarland et al., 2013; Šibanc et al., 2014). We present the first study about microbial, protozoan, nematode and mesofauna populations in a natural CO_2 vent.

High CO_2 fluxes had profound impacts on soil chemical properties, resulting in an acidified and microaerobic environment (Gosálvez et al., 2010; Sáenz de Miera, et al., 2014). Reduced pH resulted in mobilization of metal compounds (P, Fe and Cu increased), as it has been reported in several studies (Beaubien et al., 2008; Frerichs et al., 2013; Mehlhorn et al., 2014). Despite the seasonal effect observed, and reported in previous studies (Castro et al., 2011; Morales and Holben, 2013), there was a negative effect of increasing CO_2 flux, especially at high CO_2 flux (260 to 1,600 g m^{-2} d^{-1}), on soil biota abundance and diversity from La Sima vent.

Our results suggest that high CO_2 emissions caused a reduced and less diverse bacterial community. Previous research on mofettes has confirmed strong impacts on community structure and composition (Sáenz de Miera et al., 2014; Šibanc et al., 2014; Beulig et al., 2015) and decreasing bacterial numbers or biomass (Beaubien et al., 2008; Krüger et al., 2009; 2011; Oppermann et al., 2010; McFarland et al., 2013). Likewise, we found reduced numbers of fungi at high CO_2 fluxes, consistently with the results of McFarland et al. (2013) in Mammoth Mountain (USA) or the decline in Eukarya copy numbers observed by Oppermann et al. (2010) at Latera Caldera (Italy). These changes led to reduced fungi-to-bacteria ratio, also reported by McFarland et al. (2013) in the area most influenced by CO_2. The well-accepted pattern that fungi-to-bacteria ratio increases due to acidification (Högberg et al., 2007; Joergensen and Wichern, 2008; Rousk et al., 2009) is not valid when considering very high CO_2 conditions. In fact, at fluxes above 260 to 1,600 g m^{-2} d^{-1} and due to the difference in oxygen requirements, bacteria appear to be better adapted than fungi resulting in the ratio to decrease.

Several studies reported changes in archaeal community composition in mofettes, although results varied between sites, and therefore general conclusions are difficult to draw. Most of them observed increments in methanogenic archaea associated to vent centre (Beaubien et al., 2008; Oppermann et al., 2010; Šibanc et al., 2014; Beulig et al., 2015). On the contrary, Frerichs et al (2013) found a reduction in methanogens, whereas Crenarchaeota and Thaumarchaeota phylum were beneficially affected. Oppermann et al. (2010) found reduced Archaea numbers related to higher CO_2 fluxes whereas Krüger et al. (2011) even using the same primers observed them to increase. In our study, archaeal numbers (Crenarchaeota), in contrast, were not significantly

affected by increased CO_2. However, extreme fluxes showed an increasing trend in archaeal abundance suggesting that these extreme conditions can harbour very high microbial abundances of archaea as well as adapted fungi and bacteria.

There is scarce information about CO_2 effects on protist communities and previous papers have focused on atmospheric CO_2 experiments (Treonis and Lussenhop, 1997; Rillig et al., 1999; Hungate et al., 2000; Rønn et al., 2003). Gabilondo and Bécares (2014) studied protozoan communities at La Sima reporting a change in ciliates community composition and a decrease in their diversity. Furthermore, we found a significant reduction in amoebae individuals. There is no consensus in previous research studies regarding the effects of increased atmospheric CO_2 on protozoan abundances, but Treonis and Lussenhop (1997) also described a decrease in amoebic density when they applied twice-ambient CO_2 levels within open-top chambers for 4 weeks. Nevertheless, even if many studies have evaluated potential consequences of elevated atmospheric CO_2 (< 10 %) on soil fauna (Eisenhauer et al., 2012) these findings had little relevance to environments with extreme CO_2 (> 90 %) concentrations (Beulig et al., 2015). In our case, some protists exposed to extreme fluxes were favoured by increasing CO_2 conditions; flagellates significantly increased probably due to elevated humidity and higher bacterial abundance in these extreme points, which might trigger the increased abundances in the bacterivore mesofauna.

Nematodes were strongly affected by CO_2 emissions, almost disappearing at high CO_2 fluxes. Yeates et al., (1999) also found a significant decrease in total abundance and diversity of nematodes in moffete fields. Mesofauna abundance and richness also responded negatively to high CO_2 fluxes, but there was a unimodal, non-significant trend along the CO_2 fluxes. Coincidentally, at Cheb Basin (Czech Republic) abundance of mesofauna (collembola) also increased at intermediate fluxes (Russell et al., 2011), but mesofauna richness was reduced when exposed to higher CO_2 fluxes. Although not statistically significant, extreme fluxes also presented an increased mesofauna abundance compared to high and control fluxes, following the pattern observed in protist and microorganisms, and supporting the idea of an increasing effect of CO_2 on the bottom-up cascade.

Our results showed that high CO_2 emissions affected most groups of soil biota. Relationship between these groups followed a decreasing pattern –except when considering extreme fluxes-, CO_2 did not affect relations between them but its effect propagated through multiple components of soil food web. However, whether CO_2 influenced directly edaphic biota or, on the contrary, soil community indirectly responded to CO_2 effects on soil chemistry (e.g. pH, mobilized mineral compounds, microaerobic environment) is difficult to elucidate (Šibanc et al., 2014). There are too few experiments about mofettes' soil fauna to draw any generalizations. Nevertheless, we speculate that the decreasing trend on the number of mesofauna and microfauna,

and the known resilience of bacteria to such an extreme environment, favoured the latter.

Mofettes could offer a monitoring tool to evaluate and validate methods for the study of the environmental impact of potential accidents from CCS technologies (Schütze et al., 2012). Understanding the consequences of a potential leakage of CO_2 on soil ecosystem is a major issue nowadays; our results evidence the deep ecological impact of high CO_2 emissions, resulting in a reduced, less diverse edaphic biota. Mofettes offer natural CO_2 releasing areas where many research opportunities still not exploited could be conducted (Pfanz et al., 2004). Future studies on mofette's micro and mesofauna are urgently necessary to improve our understanding in these extreme ecosystems and reveal resilient species, which could result a source of potentially useful organisms.

5.5 Conclusions

There was a clear negative response of edaphic community to high CO_2 fluxes at La Sima vent. All biota groups analysed (microbiota, microfauna and mesofauna) were affected by CO_2, which result in a reduced and less diverse soil community, compared with the reference soil. Nevertheless, high microbial and protozoa abundances at extreme CO_2 fluxes proved that there was resilient biota in such an extreme environment. These results need to be considered when applying CCS technologies, as derived consequences from a potential leakage could deeply transform soil ecosystem, changing chemical features and leading to altered communities.

General discussion and conclusions 6

CCS can be a feasible mitigation option to achieve an effective reduction in CO_2 emissions, required to accomplish Kyoto Protocol target in the next years. Its implementation demands a careful selection of favourable storage facilities to avoid undesirable leakages. A possible seepage could diminish its potential as remediation technology in facing climate change. In this context, risk assessment studies have proliferated in the last decade, attempting to find indicators of a potential leakage, or evaluating its impacts on soil ecosystem.

In this thesis, several approaches were applied to gain new insights in the consequences of belowground CO_2 emissions on edaphic microbiota. By studying different ecosystems (experimental systems and natural analogues), we tried to responds to the following questions.

6.1 Comparing natural occurring with experimental CO_2 fluxes

To determine the response of edaphic microorganisms to a gradient of CO_2 fluxes, we studied two different locations: 1) the $PISCO_2$ experimental plant, whose objective is the research of the influence of CO_2 injection on soils for safety control of CO_2 geological storage, and 2) La Sima vent, in Ciudad Real, as natural surrogate of a leaking geological storage site. To compare the results in both systems the same methods have been applied to characterize microbial functionality, genetic diversity and quantifying the populations; and we looked for the same CO_2 surface fluxes.

We found distinct responses depending on the system analysed: while our results at $PISCO_2$ suggested that microbial communities were not significantly influenced by belowground CO_2 emissions after 15 months of exposure, high CO_2 emissions at La Sima mofette caused a profound impact on microorganisms. Therefore, we state that there would be no microbial response in the short term to a low (and artificial) emission of CO_2 (up to 40 l h^{-1}). In our experiment, the lack of response could be partially attributed to physical disruption of soil while transferring from original locations to the

$PISCO_2$ site. Soil reconstruction may be influencing the community structure, this effect being more important than CO_2 effect in the short-term (Rygiewicz et al., 2010). Nevertheless, the most important feature could have been the low fluxes applied to the plots (20 or 40 l h^{-1}). Other injection field test like ASGARD (60-180 l h^{-1}) have failed in finding consistent responses to CO_2 emissions although authors have reported several changes in microbial communities through 2 years of intermittent exposure (Smith et al., 2013). ZERT and Ginninderra sites (Morales and Holben, 2013; Feitz et al., 2014) did report changes even when applying short CO_2 exposure time (3.5 weeks and more than 6 months, respectively). Hence, CO_2 flux would be one of the main limiting factors to observe a response of the microbial community. Indeed, much higher fluxes at La Sima did lead to reduced and less diverse populations, losses in functional diversity and a decrease in metabolic activities. Notwithstanding, time scale needs also to be considered, as 15 months could be not enough to observe any impacts on edaphic communities considering low fluxes of CO_2 such those applied at $PISCO_2$. Despite we did find changes in soil chemistry (a significant decrease of pH in gassed plots), they still were not reflected in microbial communities.

6.2 The importance of time exposure and seasonal effects

The effect of time exposure to CO_2 becomes evident when we compare the results from experimental fields with those from natural analogues. Beyond the negative impact of O_2 limitation on plants, resulting in scarce vegetation in vent areas, there exist an adaptation of microbial communities to continued CO_2 high level for long periods (centuries, millenia). All the studies taking place in natural analogues have reported deep changes from non-CO_2-influenced areas to surrounding or the centre of the vents, where acidophilic and anaerobic species have established to adapt to CO_2 dominated environment. These results are the combination not only of high CO_2 fluxes but also of geological timescale emissions.

In our work, both systems experimented seasonal dependent responses. In the case of $PISCO_2$, most of the variables analysed followed seasonal variations, in correlation with soil moisture content. Microbial activity was stimulated in cold seasons, in the same way as fungal gene copy number rose in winter. At La Sima vent, there was an evident influence of seasonality on abundances and microbial activity, this latter being stimulated in wet season. Many authors have reported different responses of microbial communities to CO_2 emissions depending on seasonal features (moisture and temperature) (Castro et al., 2010; Frerichs et al., 2013; Morales and Holben, 2013). Considering that both sites presented seasonal variations, which influenced microbial response, long-term high CO_2 fluxes at La Sima were determinant to trigger a negative impact whereas short-term lower CO_2 fluxes at $PISCO_2$ did not affect the communities.

6.3 The cascade effect on higher trophic groups

Considering the dearth of research on belowground CO_2 effects on edaphic micro and mesofauna (only Yeates et al., 1999; Russell et al., 2011 and Gabilondo and Bécares, 2014 focused on distinct groups from microbiota), we tried to include higher trophic groups, and their relationship with microorganisms, in our study at La Sima vent.

Although CO_2 emissions did not significantly affect relations between them, their negative effect on soil microbiota propagated through multiple components of soil food web. While CO_2 fluxes increased, we observed a reduction in amoebae, almost disappeared nematode and diminished mesofauna richness and abundance.

Extreme fluxes at La Sima, however, produced a different response. Together with increasing trends in microbial abundance, we perceived a rise in protozoal abundances (significant in the case of flagellates) and growing patterns in mesofauna numbers. The lack of studies in these groups make difficult to draw conclusions but we speculate that the decreasing trend on the number of mesofauna and microfauna, and the known resilience of bacteria to such an extreme environment, favoured the latter.

6.4 Biomonitoring CCS impact and future research

These results need to be considered when applying CCS technologies, as derived consequences from a potential leakage could deeply transform soil ecosystem, changing chemical features and leading to altered communities. Some questions need to be addressed in the future research on CCS risk assessment and biomonitoring.

Firstly, they are necessary more studies on the CO_2 threshold concentration to appreciate a clear impact on edaphic communities. For that objective, experimental systems are a good approach as they allow controlling CO_2 flux release. They also permit to manage time exposure, as we still ignore how much time is necessary to observe changes in edaphic communities (Jones et al., 2015). Moreover, it is important to identify changes specific to CO_2 rather than other factors. At La Sima vent we reported significant effects on soil communities, however, whether CO_2 influenced directly edaphic biota or, by the contrary, soil community indirectly responded to CO_2 effects on soil chemistry is difficult to elucidate (Šibanc et al., 2014).

Furthermore, future research could focus on the study of specific groups of microorganisms that may benefit from an increase in soil CO_2 concentration– methanogens, anaerobic species–. In addition, there is an urgent need to consider other edaphic biota to gain knowledge on trophic relationships under belowground CO_2 exposure. Regarding this question, mofettes offer extreme ecosystems where improve our understanding in resilient species.

6.5 Conclusions

- High CO_2 fluxes (more than 200 g m^{-2} d^{-1}) have a strong negative impact on edaphic biota, causing losses of diversity and a decrease in abundance and activity. These changes were only observed at a natural CO_2 vent, where soil inhabitants have been expose to high belowground CO_2 emissions for centuries. Extreme fluxes (more than 10^4 g m^{-2} d^{-1}) reverted this negative effect; the soil conditions at these points favoured a rise in microbial numbers and in their predators. In the experimental system, short-term exposure to low CO_2 fluxes did not have effect on microbial communities.

- There was an evident seasonality effect on microbial responses, mainly due to soil moisture, both in the $PISCO_2$ experiment and La Sima mofette. Cold and wet seasons stimulated microbial activity at both sites. However seasonal effect on abundances varied from $PISCO_2$ where there was not consistent results, to La Sima where greatly rose in dry season.

- The comparison between a simulated leakage for a year and a natural emitting vent for centuries revealed the time-scale influence on CO_2 impact over soil biota. While non-significant effects were found at $PISCO_2$ experimental system, La Sima showed adapted communities in its extreme points, and negatively affected abundances and activities in relation to high CO_2 fluxes. Lack of response at $PISCO_2$ cannot only be attributed to short-term exposure but also to low CO_2 fluxes applied.

- The effect of high CO_2 emissions was also observed in other edaphic groups, as protists, nematodes and mesofauna, at La Sima vent. In general, they followed the same pattern that microorganisms, being negatively affected by increasing CO_2 fluxes, and showing a trend to recover when exposed to extreme CO_2 emissions.

- Microorganisms can be used to monitor potential CO_2 leakages if we consider high CO_2 flux and a minimum time of exposure. In the short-term relatively high fluxes (more than 160 g m^{-2} d^{-1}) will be need to begin to observe an impact on microbial communities, while in longer periods, the effect can be detected from these fluxes upwards. Hence, biomonitoring of CCS using soil biota is indicated for little leakages that have been undetected for a long time or immediate but huge CO_2 seepages. Considering the results obtained on this thesis and compared with previous research, we could suggest the

biomonitoring of potential CCS leaks through measures on microbial potential activities and genetic diversity and soil biota numbers. However, the effectiveness of this approach will depend on CO_2 flux and the time since the leakage occurs.

7 References

- Acosta-Mercado, D., Lynn, D.H., 2003. The edaphic quantitative protargol stain: a sampling protocol for assessing soil ciliate abundance and diversity. Journal of Microbiological Methods 53, 365-375.
- Adl, S.M., Leander, B.S., Simpson, A.G.B., Archibald, J.M., Anderson, O.R., Bass, D., Bowser, S.S., Brugerolle, G., Farmer, M.A., Karpov, S., Kolisko, M., Lane, C.E., Lodge, D.J., Mann, D.G., Meisterfeld, R., Mendoza, L., Moestrup, Ø., Mozley- Standridge, S.E., Smirnov, A.V., Spiegel, F., 2007. Diversity, nomenclature, and taxonomy of protists. Systematic Biology 56, 684-689.
- Agencia Estatal de Meteorología (AEMET). Guía resumida del clima en España (1981-2010). http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos/valoresclimatologicos. (consulted 27.01.2015).
- Ancochea, E. (1997): La región volcánica central española in: Higueras, P., Sánchez, C. (eds). XVII Reunión Científica de la SEM. Itinerarios Geológicos. Ed. UCLM, pp.27-33.
- Andrés, P., Mateos, E., Tarrasón, D., Cabrera, C., Figuerola, B., 2011. Effects of digested, composted, and thermally dried sewage sludge on soil microbiota and mesofauna. Applied Soil Ecology 48(2), 236–242.
- Beaubien, S.E., Ciotoli, G., Coombs, P., Dictor, M.C., Krüger, M., Lombardi, S., Pearce, J.M., West, J.M., 2008. The impact of a naturally occurring CO_2 gas vent on the shallow ecosystem and soil chemistry of a Mediterranean pasture (Latera, Italy). International Journal of Greenhouse Gas Control 2, 373-387.
- Bennion, D.B. and Bachu, S., 2007. Permeability and relative permeability measurements at reservoir conditions for CO_2-water systems in ultra low

permeability confining caprocks. Society of Petroleum Engineers (SPE) Paper 106995. Proceedings of SPE Europec/EAGE Annual Conference and Exhibition, London, United Kingdom, 11–14 June 2007.

- Benson, S. M., Hepple, R., Apps, J., Tsang, C. F., and Lippmann, M.: 2002, Lessons Learned from Natural and Industrial Analogues for Storage of Carbon Dioxide in Deep Geological Formations, Earth Sciences Division, E.O. Lawrence Berkeley National Laboratory, Berkeley, p. 135.

- Beulig, F., Heuer, V. B., Akob, D. M., Viehweger, B., Elvert, M., Herrmann, M., Küsel, K., 2015. Carbon flow from volcanic CO_2 into soil microbial communities of a wetland mofette. The ISME Journal 9(3), 746–759.

- Burnside, N. and Naylor, M., 2011. Evaluation of CO_2 storage actuarial risk: defining an evidence base. DEVEX 2011, 12th May, Aberdeen. (http://www.devex-conference.org/) (Accessed on 17.09.15)

- Calbrix, R., Laval, K., Barray, S., 2005. Analysis of the potential functional diversity of the bacterial community in soil: a reproducible procedure using sole-carbon- source utilization profiles. European Journal of Soil Biology 41, 11–20.

- Calvo, D., Barrancos, J., Padilla, G., Brito, M., Becerra Ramírez, R., González, E., Gosálvez, R., Escobar, E., Melián, G., Nolasco, D., Padrón, E., Marrero, R., Hernández, P.A., Pérez, N., 2010. Emisión difusa de CO_2 en el Campo de Calatrava, Ciudad Real. Aportaciones recientes en volcanología, 2005–2008. Centro de Estudios Calatravos, UCLM. Ministerio de Ciencia y Tecnología, Madrid.

- Castro H.F., Classen A.T., Austin E.E., Norby R.J., Schadt C.W., 2010. Soil microbial community responses to multiple experimental climate change drivers. Applied Environmental Microbiology 76, 999-1007.

- Clarholm, M., Bonkowski, M., Griffiths, B., 2007. Protozoa and other protista in soil in: Van Elsas J.D., Jansson J.K., Trevors, J.T. (Eds), Modern Soil Biology. Second Edition. CRC Press, Boca Raton, USA. pp. 148-175.

- CIUDEN, 2009. CIUDEN's test facilities for advanced technologies on CO_2 capture and storage in coal power generation. El Bierzo, Spain. May 2009.

- CIUDEN, 2015. $PISCO_2$ project brochure. (Available at http://www.ciuden.es/images/documentos/pisco2_brochure.pdf)

- Damen, K., Faaij, A., Turkenburg, W., 2006. Underground CO_2 storage-Overview of mechanisms and current knowledge. Climatic Change 74, 289-318.

- Dedysh, S.D., Ricke, P., Liesack, W., 2004. NifH and NifD phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. Microbiology 150, 1301–1313.

- Drigo, B., Kowalchuk, G. a. & Van Veen, J. A., 2008. Climate change goes underground: Effects of elevated atmospheric CO_2 on microbial community structure and activities in the rhizosphere. Biology and Fertility of Soils 44, 667–679.

- Eisenhauer, N., Cesarz, S., Koller, R., Worm, K., & Reich, P. B., 2012. Global change belowground: Impacts of elevated CO 2, nitrogen, and summer drought on soil food webs and biodiversity. Global Change Biology 18(2), 435–447.

- Elío, J., Ortega, M.F., Nisi, B., Mazadiego L.F., Vaselli, O., Caballero, J., Grandia, F., 2015. CO_2 and Rn degassing from the natural analogue of Campo de Calatrava (Spain): Implications for monitoring of CO_2 storage sites. International Journal of Greenhouse Gas Control 32, 1–14.

- European Council, 2014. Report from the Commission to the European Parliament and the Council-Progress towards achieving the Kyoto and EU 2020 objectives, COM. 689 Final, European Commission, Brussels.

- Feitz, A., Jenkins, C., Schacht, U., McGrath, A., Berko, H., Schroder, I., Noble, R., Kuske, T., George, S., Heath, C., Zegelin, S., Curnow, S., Zhang, H., Sirault, X., Jimenez-Berni, J., Hortle, A., 2014. An assessment of near surface CO_2 leakage detection techniques under Australian conditions. Energy Procedia, 63, 3891–3906.

- Fernández-Montiel, I., Touceda, M., Pedescoll, A., Gabilondo, R., Prieto-Fernández, A., Bécares, E., 2015. Short-term effects of simulated below-ground carbon dioxide leakage on a soil microbial community. International Journal of Greenhouse Gas Control 36, 51–59.

- Fierer, N. and Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences, 103(3), 626–631.

- Frerichs, J., 2013. The influence of high CO_2 concentrations on microorganisms in different ecosystems. (Doctoral thesis, Leibniz Universität Hannover, Germany) Retrieved from www.dart-europe.eu/full.php?id=818277.

- Frerichs, J., Oppermann, B.I., Gwosdz, S., Möller, I., Herrmann, M., Krüger, M., 2013. Microbial community changes at a terrestrial volcanic CO_2 vent induced

by soil acidification and anaerobic microhabitats within the soil column. FEMS Microbiology Ecology 84, 60-74.

- Gabilondo, R., Fernández-Montiel, I., García-Barón, I., Bécares, E., 2015. The effects of experimental increases in underground carbon dioxide on edaphic protozoan communities. International Journal of Greenhouse Gas Control 41, 11-19.

- Gabilondo, R., Bécares, E., 2014. The effects of natural carbon dioxide seepage on edaphic protozoan communities in Campo de Calatrava, Ciudad Real, Spain. Soil Biology and Biochemistry 68, 133-139.

- Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon- source utilization. Applied Environmental Microbiology 57 (8), 2351–2359.

- Global Carbon Atlas, 2015. http://www.globalcarbonatlas.org/?q=en/outreach (Accessed on 20.09.15)

- Global Carbon Capture and Storage Institute, 2015. Ortiz, G., Kovacs, T., Poulussen, D.F., de Dios, C. (Eds). Hontomín reservoir characterisation tests. Final technical report. June 2015. Global CSS Institute Ltd.

- Gomes, N.C.M., Kosheleva, I., Abraham, W.R., Smalla, K., 2005. Effects of the inoculant strain Pseudomonas putida KT2442 (pNF 142) and of naphthalene contaminations on the soil bacterial community. FEMS Microbial Ecology 54, 21-33.

- Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Mendonça-Hagler, L., Smalla, K., 2001. Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant and Soil 232, 167–180.

- Gömöryová, E., Ujházy, K., Martinák, M., Gömöry, D., 2013. Soil microbial community response to variation in vegetation and abiotic environment in a temperate old-growth forest. Applied Soil Ecology 68, 10-19.

- Gosálvez, R., Becerra, R., González, E., Escobar, E., 2010. Evolución de la emisión de CO_2 en La Sima, Campo de Calatrava (Ciudad Real, España). Aportaciones recientes en volcanología, 2005–2008. Centro de Estudios Calatravos, UCLM. Ministerio de Ciencia y Tecnología, Madrid.

- Haszeldine, R.S., 2009. Carbon Capture and Storage: How Green Can Black Be? Science 325 (5948), 1647-1652.

- Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities, in: Van Elsas, J.D., Trevors, J.T., Wellington E.M.H. (Eds.), Modern soil microbiology. Marcel Dekker Inc., New York, pp. 353–373.

- Higueras Higueras, P.L., Gallardo Millán, J.L., 2011. The Campo de Calatrava volcanic field : geology and resources, in: Ortiz, J.E., Puche, O, Rábano I., Mazadiego L.F. (Eds.), History of Research in Mineral Resources. Cuadernos del Museo Geominero, 13. Instituto Geológico y Minero de España, Madrid, pp 395–404.

- Högberg, M.N., Hogberg, P., Myrold, D.D., 2007. Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? Oecologia 150, 590-601.

- Holloway, S., Pearce, J.M., Hards, V.L., Ohsumi, T., Gale, J., 2007. Natural emissions of CO_2 from the geosphere and their bearing on the geological storage of carbon dioxide. Energy 32, 1194-1201.

- Holloway, S., 1996. The underground Disposal of Carbon Dioxide. Final Report JOULE II Project no. CT92-0031, British Geological Survey, Keyworth, Nottingham, p. 355.

- Hungate, B.A., Jaeger, C.H., Gamara, G., Chapin, F.S., Field, C.B., 2000. Soil microbiota in two annual grasslands: responses to elevated atmospheric CO_2. Oecologia 124, 589-598.

- IEA, 2014. CO_2 Emissions From Fuel Combustion. Highlights. International Energy Agency. Paris Cedex 15, France.

- IPCC, 2007. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor M., Miller H.L. (Eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

- IPCC, 2005. IPCC Special Report on Carbon Dioxide Capture and Storage. Prepared by Working Group III of the Intergovernmental Panel on Climate Change. Metz, B., Davidson, O., de Coninck, H.C., Loos, M., and Meyer L.A. (Eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 442 pp.

- Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Applied Environmental Microbiology 72, 1719–1728.

- Joergensen, R.G., Wichern, F., 2008. Quantitative assessment of the fungal contribution to microbial tissue in soil. Soil Biology and Biochemistry 40, 2977-2991.

- Jones, D. G., Beaubien, S. E., Blackford, J. C., Foekema, E. M., Lions, J., De Vittor, C., ... Queirós, a. M., 2015. Developments since 2005 in understanding potential environmental impacts of CO_2 leakage from geological storage. International Journal of Greenhouse Gas Control 40, 350-377.

- Jones, D.G., Lister, T.R., Smith, D.J., West, J.M., Coombs, P., Gadalia, A., Brach, M., Annunziatellis, A., Lombardi, S., 2011. In Salah Gas CO_2 Storage JIP: Surface gas and biological monitoring. Energy Procedia 4, 3566-3573.

- Johnson, J.W. and Nitao, J.J., 2003. Reactive transport modelling of geologic CO_2 sequestration at Sleipner. In: Gale, J. and Kaya, Y. (Eds.), Sixth International Conference on Greenhouse Gas Control Technologies, Kyoto, vol. I, Pergamon, Amsterdam, pp. 327–323.

- Kim, S.J., Moon, J.Y., Ahn, J.H., Weon, H.Y., Hong, S.B., Seok, S.J. and Kwon, S.W., 2014. Diaphorobacter aerolatus sp. nov., isolated from air, and emended description of the genus Diaphorobacter. International Journal of Systematic and Evolutionary Microbiology 64 (2), 513-517.

- Krüger, M., Jones, D., Frerichs, J., Oppermann, B.I., West, J., Coombs, P., Green, K., Barlow, T., Lister, R., Shaw, R., Strutt, M., Moller, I., 2011. Effects of elevated CO_2 concentrations on the vegetation and microbial populations at a terrestrial CO_2 vent at Laacher See, Germany. International Journal of Greenhouse Gas Control 5, 1093-1098.

- Krüger, M., West, J., Oppermann, B., Dictor, M.-C., Frerichs, J., Jouliand, C., Jones, D., Coombs, P., Green, K., Pearce, J., May, F., Möller, I., 2009. Ecosystem effects of elevated CO_2 concentrations on microbial populations at a terrestrial CO_2 vent at Laacher See, Germany. Energy Procedia 1, 1933-1939.

- Lindeberg, E. and Bergmo, P., 2003. The long-term fate of CO_2 injected into an aquifer. In Gale, J. and Kaya, Y. (Eds), Sixth International Conference on Greenhouse Gas Control Technologies, Kyoto, vol. I, Pergamon, Amsterdam, pp. 489–494.

- Maček, I., 2013. A decade of research in mofette areas has given us new insights into adaptation of soil microorganisms to abiotic stress. Acta Agriculturae Slovenica 101(2), 209–217.

- Maček, I., Dumbrell, A.L., Nelson, M., Fitter, A.H., Vodnik, D., Helgason, T., 2011. Local adaptation to soil hypoxia determines the structure of an

arbuscular mycorrhizal fungal community in roots from natural CO_2 springs. Applied and Environmental Microbiology 77 (14), 4770-4777.

- Madoni, P., 1984. Estimation of the size of freshwater ciliate populations by a subsampling technique. Hydrobiologia 111, 201-206.

- MAGRAMA, 2013. National Inventory Report: Spain 2013, Ministry of Agriculture, Food and Environment, Madrid.

- Maier, R.M., Neilson, J.W., 2015. Extreme environments. In: Pepper, I.L., Gerba, C.P., Gentry, T.L. (Eds), Environmental Microbiology 3rd edition. Academic Press, San Diego CA, pp 139-153.

- McFarland, J.W., Waldrop, M.P., Haw, M., 2013. Extreme CO_2 disturbance and the resilience of soil microbial communities. Soil Biology and Biochemistry 65, 274-286.

- Mehlhorn, J., Beulig, F., Küsel, K., Planer-Friedrich, B., 2014. Carbon dioxide triggered metal(loid) mobilisation in a mofette. Chemical Geology 382, 54–66.

- Ministerio de Agricultura, Pesca y Alimentación, 1994. Métodos oficiales de análisis. Tomo III. Secretaria General de Alimentación, Madrid, Spain.

- Morales, S.E., Holben, W.E., 2014. Simulated geologic carbon storage leak reduces bacterial richness and alters bacterial community composition in surface soil. Soil Biology and Biochemistry 76, 586-296.

- Morales, S.E., Holben, W.E., 2013. Impacts of carbon dioxide gas leaks from geological storage sites on soil ecology and above-ground vegetation. PLoS ONE 8(11): e81742.

- Morozova, D., Zettlitzer, M., Daniela Let, D., Würdemann, H., CO SINK group, 2011. Monitoring of the microbial community composition in deep subsurface saline aquifers during CO_2 storage in Ketzin, Germany. Energy Procedia 4, 4362-4370.

- NOAA, 2015. Ed Dlugokencky and Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) (Accessed on 20.08.15)

- Noble, R.R.P., Stalker, L., Wakelin, S. A., Pejcic, B., Leybourne, M. I., Hortle, A. L., Michael, K., 2012. Biological monitoring for carbon capture and storage - A review and potential future developments. International Journal of Greenhouse Gas Control 10, 520–535.

- Nübel, U., Engelen, B., Felske, A., Snaidr, U., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus H., 1996. Sequence heterogeneities of genes encoding

16s rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol. 178 (19), 5636–5643.

- Nybroe, O., Brandt, K.K., Nicolaisen, M.H., Sørensen, J., 2007. Methods to detect and quantify bacteria in soil in: Van Elsas J.D., Jansson J.K., Trevors, J.T. (Eds), Modern Soil Microbiology, Second Edition. CRC Press, Boca Raton, USA, pp 284-316.

- Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L., Schleper, C., 2003. Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. Environmental Microbiology 5 (9), 787-797.

- OECD, 2015a. Air and GHG emissions (indicator). (Accessed on 18.08.15)

- OECD, 2015b. OECD Environmental Performance Reviews: Spain 2015, OECD Publishing.

- Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2010. Vegan: Community Ecology Package. R Package Version 1, pp. 17–24.

- Oppermann, B.I., Michaelis, W., Blumenberg, M., Frerichs, J., Schulz, H.-M., Schippers, A., Beaubien, S.E., Krüger, M., 2010. Soil microbial community changes as a result of long-term exposure to a natural CO_2 vent. Geochimica et Cosmochimica Acta 74, 2697-2716.

- Oros-Sichler, M., Costa, R., Heuer, H., Smalla, K., 2007. Molecular fingerprinting techniques to analyze soil microbial communities in: Van Elsas J.D., Jansson J.K., Trevors, J.T. (Eds), Modern Soil Microbiology, Second Edition. CRC Press, Boca Raton, USA, pp 355-386.

- Over, J.A., de Vries, J.E., and Stork, J., 1999. Removal of CO_2 by Storage in the Deep Underground, Chemical Utilization and Biofixation. Options for the Netherlands, Novem, Sittard, p. 64.

- Paoletti, E., Pfanz, H., Raschi, A., 2005. Pros and cons of CO_2 springs as experimental sites. In Omasa, K., Nouchi, I., De Kok L.J. (Eds.), Plant Responses to Air Pollution and Global Change. Springer Japan, Tokyo, pp. 195–202.

- Patil, R.H., Colls, J.J., Steven, S.D., 2010. Effects of CO_2 gas as leaks from geological storage sites on agro-ecosystems. Energy 35, 4587-4591.

- Peinado, M.; García Rayego, J.L.; González Cárdenas, E. & Ruiz Pulpón, A.R., 2009. Itinerarios geográficos y paisajes por la provincia de Ciudad Real. Guía

de salidas de campo del XXI Congreso de Geógrafos Españoles. Imprenta Provincial, Ciudad Real.

- Pfanz, H., Vodnik, D., Wittmann, C., Aschan, G., Raschi, A., 2004. Plants and Geothermal CO_2 Exhalations — Survival in and Adaptation to a High CO_2 Environment. In: K. Esser, U. Lüttge, W. Beyschlag, J. Murata (Eds.), Progress in Botany Volume 65. Springer Berlin Heidelberg, pp 499-538

- Pierce, S., Sjörgersten, S., 2009. Effects of below ground CO_2 emissions on plant and microbial communities. Plant and Soil 325, 197–205.

- Prosser, J.I. 2007. Microorganisms cycling soil nutrients and their diversity in: Van Elsas J.D., Jansson J.K., Trevors, J.T. (Eds), Modern Soil Microbiology, Second Edition. CRC Press, Boca Raton, USA, pp 237-261.

- PTECO$_2$, 2012. Almacenamiento de CO_2: tecnologías, oportunidades y expectativas. 1ª Edición. Plataforma Tecnológica Española del CO_2. (www.pteco2.es)

- Rillig, M.C., Wright, S.F., Allen, M.F., Field, C.B., 1999. Rise in carbon dioxide changes soil structure. Nature 400, 628.

- RISCS, 2014. A Guide to potential impacts of leakage from CO_2 storage. Pearce, J, Blackford, J, Beaubien, S, Foekema, E, Gemeni, V, Gwosdz, S, Jones, D, Kirk, K, Lions, J, Metcalfe, R, Moni, C, Smith, K, Steven, M, West, J and Ziogou, F. British Geological Survey. Available from www.riscs-co2.eu 70 pages.

- Rønn, R., Ekelund, F., Christensen, S., 2003. Effects of elevated atmospheric CO_2 on protozoan abundance in soil planted with wheat and on decomposition of wheat roots. Plant and Soil 251, 13-21.

- Rousk, J., Brookes, P.C., Bååth, E., 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Applied and Environmental Microbiology 75, 1589-1596.

- Russell, D.J., Hohberg, K., Pfanz, H., 2011. Occurrence of collembolan fauna in mofette fields (natural carbon- dioxide springs) of the Czech Republic. Soil organisms 83(3), 489–505.

- Rygiewicz, P.T, Monleon, V.J., Ingham, E.R., Martin, K.J., Johnson, M.G., 2010. Soil life in reconstructed ecosystems: Initial soil food web responses after rebuilding a forest soil profile for a climate change experiment. Applied Soil Ecology 45(1), 26-38.

- Sáenz de Miera, L.E., Arroyo, P., de Luis Calabuig, E., Ansola G. Effects of varying CO_2 flows on bacterial communities in mesocosms created from two soils. International Journal of Greenhouse Gas Control (under revision).

- Sáenz de Miera, L. E., Arroyo, P., de Luis Calabuig, E., Falagán, J., Ansola, G., 2014. High-throughput sequencing of 16S RNA genes of soil bacterial communities from a naturally occurring CO_2 gas vent. International Journal of Greenhouse Gas Control 29, 176–184.

- Schütze, C., Sauer, U., Beyer, K., Lamert, H., Bräuer, K., Strauch, G., Flechsig, Ch., Kämpf, H., Dietrich, P., 2012. Natural analogues: A potential approach for developing reliable monitoring methods to understand subsurface CO 2 migration processes. Environmental Earth Sciences 67(2), 411–423.

- Shi, C.E. and Huang, B. Effects of pinewood nematode (Bursaphelenchus xylophilus) infected-Pinus massoniana on soil bacterial communities. Unpublished.

- Šibanc, N., Dumbrell, A.J., Mandič-Mulec, I., Maček, I., 2014. Impacts of naturally elevated soil CO_2 concentrations on communities of soil archaea and bacteria. Soil Biology and Biochemistry 68, 348-356.

- Smith, K.L., Steven, M.D., Jones, D.G., West, J.M., Coombs, P., Green, K.A., Barlow, T.S., Breward, N., Gwosdz, S., M Krüger, S E Beaubien, Annunziatellis, A., Graziani, S., and S Lombardi,S., 2013. Environmental impacts of CO_2 leakage: recent results from the ASGARD facility, UK. Energy Procedia 37, 791-799.

- Standing, D., Killham, K., 2007. The soil environment in: Van Elsas J.D., Jansson J.K., Trevors, J.T. (Eds), Modern Soil Microbiology, Second Edition. CRC Press, Boca Raton, USA, pp 1-22.

- Steven, M., Smith, K., Colls, J., 2010. Environmental risks and impacts of carbon dioxide (CO_2) leakage in terrestrial ecosystems in: Maroto-Valer M. (Ed.), Developments and Innovation of CO_2 capture and storage, Volume 2. Woodhead Publishing, Cambridge, pp 324-343.

- Stoppa, F., Rosatelli, G., Schiazza, M., Tranquilli, A., 2012. Hydrovolcanic vs magmatic processes in forming maars and associated pyroclasts: The Calatrava – Spain –Case History. In: Stoppa, F. (Ed.), Updates in Volcanology – A ComprehensiveApproach to Volcanological Problems. InTech, pp. 5–26.

- Tarkowski, R., Królik, W., Uliasz-Misiak, B., Barabasz, W., 2009. Indicative microorganisms as a tool for testing the underground storage of carbon dioxide, In: Grobe, M., Pashin, J.C., Dodge, R.L. (Eds). Carbon dioxide

sequestration in geological media—State of the science: AAPG Studies in Geology 59, pp. 637–642.

- Tarkowski, R., Barabasz, W., Królik, W., Uliasz-Misiak, B., 2008. Preliminary results of the microbiological research on CO_2 natural exhalations executed for geo- biomonitoring needs. Slovak Geological Magazine, 23-28.

- Timms-Wilson, T.M., Griffiths, R.I., Whiteley, A.S., Prosser, J.I., Bailey, M.J., 2007. Detection of active bacterial populations in soil in: Van Elsas J.D., Jansson J.K., Trevors, J.T. (Eds), Modern Soil Microbiology, Second Edition. CRC Press, Boca Raton, USA, pp 387-407.

- Torsvik, V., Øvreås, L., Thingstad, T.F., 2002. Prokaryotic Diversity--Magnitude, Dynamics, and Controlling Factors. Science 296 (5570), 1064-1066.

- Treonis, A.M., Lussenhop, J., 1997. Rapid response of the bacterial-based soil food chain to elevated CO_2. Biol. Fertil. Soils 25, 60-62.

- Turner, S., Pryer, K.M., Miao, V.P.W., and Palmer, J.D., 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol 46, 327–338.

- United States Department of Agriculture, 1999. Soil Taxonomy. A Basic System of Soil Classification for Making and Interpreting Soil Surveys, second ed. In: Agriculture Handbook Natural Resources Conservation Service. Number 436.

- Vainio, E.J., Hantula, J., 2000. Direct analysis of Wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycological Research 104, 927-936.

- Vaselli, O., Nisi, B., Tassi, F., Giannini, L., Grandia, F., Darrah, T., Capecchiacci, F., Pérez del Villar, 2013. Water and gas geochemistry of the Calatrava Volcanic Province (CVP) hydrothermal system (Ciudad Real, central Spain). Geophysical Research Abstracts Vol. 15, EGU2013-11102, 2013. EGU General Assembly 2013.

- Videmšek, U., Hagn, A., Suhadolc, M., Radl, V., Knicker, H., Schloter, M., Vodnik, D., 2009. Abundance and diversity of CO_2-fixing bacteria in grassland soils close to natural carbon dioxide springs. Microbial Ecology 58(1), 1–9.

- Vodnik, D., Videmšek, U., Pintar, M., Maček, I., Pfanz, H. 2009. The characteristics of soil CO_2 fluxes at a site with natural CO_2 enrichment. Geoderma 150, 32–37.

- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane D.J., 1991. 16s ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173(2), 697-703.

- West, J.M., Jones, D.G, Annunziatellis, A., Barlow, T.S., Beaubien, S.E., Breward, N., Coombs, P., de Angelis, D., Gardner, A., Gemeni, V., Graziani, S., Green, K.A., Gregory, S., Gwosdz, S., Hannis, S.,1, Koukouzas, N., Krüger, M., Libertini, S., Lister, T.R., Lombardi, S., Smith, K.L., Steven M.D., Ziogou, F., 2013. Comparison of the impacts of elevated CO_2 soil gas concentrations on selected european terrestrial environments. 7th "Trondheim Conference on CO_2 Capture, Transport and Storage" (TCCS-7), June 4-6, 2013. Trondheim, Norway. http://www.sintef.no/Projectweb/TCCS-7/Program/ (consulted in 29.01.14)

- West, J.M., Pearce, J.M., Coombs, P., Ford, J.R., Scheib, C., Colls, J.J., Smith, K.L., Steven, M.D., 2009. The impact of controlled injection of CO_2 on the soil ecosystem and chemistry of an English lowland pasture. Energy Procedia 1, 1863-1870.

- West, J.M., Pearce, J.M., Bentham, M., Rochelle, C., Maul, P., Lombardi, S., 2006. Environmental Issues and the geological storage of CO_2 – A European Perspective. 8th International Conference on Greenhouse Gas Control Technologies, Trondheim, Norway, 19–22 June 2006.

- West, J.M., Pearce, J., Bentham, M., Maul, P., 2005. Issue profile: environmental issues and the geological storage of CO_2. European Environment 15 (4), 250–259.

- Williamson, K.E., Kan, J., Polson, S.W. and Williamson, S.J. (2011) Optimizing the indirect extraction of prokaryotic DNA from soils. Soil Biology and Biochemistry 43 (4), 736-748.

- Winding, A., Hund-Rinke, K., Rutgers, M., 2005. The use of microorganisms in ecological soil classification and assessment concepts. Ecotoxicology and Environmental Safety 62, 230-248.

- Yeates, G., Newton, P., Ross, D. 1999. Response of soil nematode fauna to naturally elevated CO_2 levels influenced by soil pattern. Nematology 1 (3), 285–293.

- ZERO, 2015. Zero Emission Resource Organization. Carbon Capture and Storage. U.Berge, M. Gjerset, B- Kristoffersen, M.Lindberg, T. Palm, T. Risberg, C.S. Skriung. Oslo, Norway. (Accessed on 18.08.15)

- Zhang, H. B., Yang, M. X. and Tu, R. (2008). Unexpectedly high bacterial diversity in decaying wood of a conifer as revealed by a molecular method. International Biodeterioration and Biodegradation 62 (4), 471-474.
- Zhou, X., Apple, M. E., Dobeck, L. M., Cunningham, A. B., Spangler, L. H., 2013. Observed response of soil O_2 concentration to leaked CO_2 from an engineered CO_2 leakage experiment. International Journal of Greenhouse Gas Control 16, 116-128.
- Ziogou, F., Gemenia, V., Koukouzasa, N., de Angelis, D., Libertini, S., Beaubien, S.E, Lombardi, S., West, J. M., Jones, D. G., Coombs, P., Barlow, T.S, Gwosdz, S., Krüger, M., 2013. Potential environmental impacts of CO_2 leakage from the study of natural analogue sites in Europe. Energy Procedia 37, 3521-3528.

Acknowledgements_Agradecimientos

La verdad que han pasado volando estos cuatro años y cuando me doy cuenta…c'est fini! Y son muchos los que han pasado por este camino y me han ayudado a completarlo.

En primer lugar, muchísimas gracias a mis directores de tesis, Eloy Bécares y Anna Pedescoll. Por todo lo que me han transmitido, su experiencia, su motivación, su continuo apoyo y comprensión. A Eloy, por empujarme a salir fuera y conocer, por sus "no te compliques" y a la vez sus vueltas de tuerca para que siga mejorando y aprendiendo cada día. A Anna, por soportarme todos los días durante cuatro años, ayudándome a dar lo mejor de mí misma y no dejar que me rinda nunca.

Quería también expresar mi agradecimiento al Departamento de Educación, Universidades e Investigación del Gobierno Vasco por concederme una beca del Programa de Formación de Personal Investigador (RBFI-2012-465), gracias a la cual pude completar mis estudios doctorales. Gracias a DAAD pude realizar una estancia de tres meses en Alemania para finalizar esta tesis. Este estudio es parte del Proyecto Compostilla OXYCFB300, ya ha sido financiado por La Fundación CIUDEN a través del convenio de colaboración PISCO-BIO, entre la Fundación y la Universidad de León, y cofinanciado por el Programa Europeo Energético para la Recuperación (PEER), de Comisión Europea. Gracias en especial a David García, de la CIUDEN, por su ayuda en las mediciones de CO_2, en el aporte de información y por estar siempre dispuesto a resolver mis dudas.

Gracias a todos los miembros del departamento de Ecología (y cía) por compartir tantos cafés y buenos momentos: Marga, Camino, Gemma, Paco, Leo, Susana, Elena,

Reyes, Tanis, Geli, Raquel, Luisfe…A Héctor, que me escuchaba maldecir porque se me contaminaban las PCR mientras me animaba con su música en aquel peculiar pseudolaboratorio. A Merce, mi Mertxiñas… que cuida de todos y cada uno de nosotros sin perder la sonrisa, alegrándonos cada día, que me transmite su paz cuando me dan "neuras asesinas" y que inspiró la portada de esta tesis con lo que yo llamo "azul merce". Sin ti este departamento no se tendría en pie, ¡que lo sepas! A Luz, nuestra lucecita, que siempre tiene una palabra para darme ánimos y seguir adelante. Y siguiendo con los BBTeros, a Toni, Marta, Xavi por compartir esas geniales excursiones y quedadas para "filosofar". A mi querido Gus, no sólo por descubrirme las mejores rutas de naturocio gastronómico de la provincia sino por ser el mejor guía que uno puede tener para alegrarse el alma.

A los compañeros que han ido pasando y a los recién llegados (¡suerte a todos!): a Carlos Martínez, Carlos Ciudad, Ángela, Gema Núñez, Sofía, Javi, Víctor, Paula García, Rut…gracias por compartir cafés, cenas, penas y alegrías, y por animar el departamento con vuestras risas. A Marta y Natalia, estudiantes de biotecnología que me ayudaron mucho con las terribles DGGEs…A Bea y Manu, por organizar las mejores excursiones guiadas de León, y por enseñarnos lo muchísimo que sabéis de "más que pájaros". A Ivanin, nuestro tuno, que se fue para allá donde quiera que esté ahora, a ver si te vienes más a menudo, ¡leches! A Ale, gracias por volver y traernos esa maravillosa sonrisa que tú tienes, por los buenos momentos que hemos pasado, desde el final de tu tesis hasta el final de la mía…¡¡que siga así!! Y no podría faltar aquí la "más mejor" compañera de despacho que una puede tener. La Pedescoll. Millones de gracias por estar ahí cada día desde que empecé esta andadura, ayudarme mil veces con los muestreos, por darme consejos, compartir conmigo tu experiencia y enseñarme tantísimas cosas: a perseverar, a mejorar cada día, a hacer gráficos molones y manejar el fotochó, a intentarlo una y otra vez y no dejarse vencer. De mayor quiero ser como tú.

A los compañeros del IMA, por las magníficas cenas de navidad y esas quedadas de cortos, Paula Arrollo, Jorge, Gema, Natalia, Antonio; en especial a Irene que siempre anima a todos con sus bailes y esa alegría que la caracteriza (que sigas igual de feliz por los madriles). A Regina y Amandito, por vuestra ayuda en los muestreos, por hacer que fuesen memorables, ¡qué buenos viajes! Y al ya casi doctor Ricardo (estamos compitiendo) por ser el primero que me acogió y enseñó en esta familia de ecólogos. Eskerrik asko Richard, *abrelabotellaahí!*

No me gustaría olvidarme de Luis Sáenz de Miera, gracias por echarme una mano con las secuenciaciones. Gracias también a Xavier Casqueiro y Esther Nistal, que me ayudaron con las clonaciones cuando estaba más que perdida, Esther me enseñó cómo manejar la máquina del demonio que llaman DGGE.

Y siguiendo con la técnica que me trajo de cabeza, millones de gracias a todos los que me acogieron en el IIAG de Santiago de Compostela, donde aprendí a hacer PCRs y geles como churros. Moitas graciñas a Ángeles por ayudarme tanto a enfocar esta tesis desde su inicio, a Petra, Carmen, Marian, y todo el grupo de Microbiología de Suelos y, en especial, a la mejor profa de DGGE do mundo inteiro: María Touceda, sin tu gran paciencia no lo habría conseguido. Gracias a los que me descubrieron todos los rincones de Santiago (con nocturnidad y alevosía) y me hicieron sentir una más: Vanesa, Maribel, Cristina, María (de Esteiro), Félix, Xela…y a Analia, mi compañera de piso macanuda, que me ayudó muchísimo allí.

I would like to thank to Stefan Scheu for giving me the chance to stay in his research group at Göttingen, and for being ready to solve my doubts. Thanks also to Mark Maraun for helping me so much with statistical issues, and to my great office mate, Tanja. My flatmates at Kepler-WG, Beatrice, Sabja and Rajius were incredibly kind with me and showed me the most delicious recipes from their countries. Gracias a Pablo, Laurita, Arturo, Magda, Bea, Cristian, al otro Christian, y a mi querida Odettinchis por hacer que me sintiera como en casa, y por acompañarme a conocer un pequeño trocito de Alemania.

A todos los falamosos (no cito porque no me llegan las hojas) por hacer que León siga siendo tan divertido como siempre y a Joselito, por acogerme en su pueblo, por invitarme aquel primer año a la Chopera, por tus alfredos, tus post-it y demás chorradas que nos hacen quererte tontaco como eres… por ser siempre un gran amigo.

Y para completar la "trinidad", moltes gràcies a L'Annita, que lo es todo: advisor, psicoterapeuta, la tieta más molona, vecina, *janmaider*, mi profa de setas, compañera do camiño y del güertín, de buenos y malos momentos…pero sobre todo amiga inseparable (mi mili, o mi pili…aún no está claro). T'estimo molt!!

Gracias a los compañeros más gamberros que tengo, la Charanga Manía, por compartir estos dos últimos veranos de fiestas interminables, por ayudarme a desconectar del estrés "tesinil" y sobre todo por hacer que me reencontrase con mi fiel saxofón. Y gracias a mi profe de saxo y compañeros de la escuela por hacer que cada descanso del curro sea para alegrar mis oídos.

Muchas gracias a los amigos de "casa", Sendoa, Jon, en especial a Itxaso por esperarme siempre aunque aparezca de pascuas a ramos y hacerme saber que siempre estarás allí, a mi querida ex marida Patri, a Itziar que ya es medio alemana pero siempre será mi compi de bus, y a Ane que me conoce desde los 4? años y que por muchos más que pasen, y por muchos kilómetros que nos separen

A Cristian, por confiar en mí, darme todo su apoyo cada día, ayudarme con su buena mano de *graphic designer*, gracias por animarme y regalarme las más preciosas sonrisas, y por querer acompañarme allá donde vaya. ¿Próximo destino…?

Y gracias a mi otro amor incondicional, mi Grouchi. Que me alegra el día con sus "fiestitas" cada vez que abro la puerta de casa, y me despierta una sonrisa cuando más lo necesito. Le debo muchísimos paseos para compensar lo mucho que ha soportado mis horarios y viajes.

Mil gracias a toda mi familia por apoyarme durante todo este periodo y mimarme cuando volvía a casa. A los aitás, porque sin su confianza en mí no podría haber seguido adelante en muchas ocasiones. Gracias mami, por llamarme todos los días y aguantar los plomazos sobre temas inentendibles, por todo tu cariño, por creer en mí y por todos esos "nena, tu vales mucho" que para mí significan lo muchísimo que tu vales. Gracias papitxu, por enseñarme a ser constante, perfeccionista, a dar siempre lo mejor de mí, por hacerme querer ser un reflejo tuyo, ya que el único bastón que puede guiar mis pasos es el tuyo.

Muchisimas gracias a todos

Thanks a lot to all of you

Mila esker denoi

Para el que mira sin ver, la tierra es tierra no más
Atahualpa Yupanqui

Área de Ecología
Departamento de Biodiversidad
y Gestión Ambiental
universidad de león