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Análisis del cambio en las comunidades microbianas en un suelo salino-alcalino del exlago de Texcoco en presencia de antraceno.

Que Presenta

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RESUMEN

En los último años, la contaminación con hidrocarburos aromáticos policíclicos y la salinización del suelo ha incrementado considerablemente debido a actividades antropogénicas, por tal motivo es necesario realizar estudios para implementar técnicas de remediación de suelos.

La investigación se realizó para identificar los cambios en las comunidades bacterianas en presencia de antraceno, cuando este compuesto era derramado artificialmente en un suelo salino alcalino del exlago de Texcoco, a una concentración de 550 mgkg^{-1} suelo seco. Las evidencias demostraron que era posible remover hasta un 65% de antraceno en el suelo salino alcalino del exlago y que esto ocurría posiblemente por procesos bióticos. Por tanto, se encontró que el antraceno modificó las comunidades bacterianas al incrementar el porcentaje del *phylum* Actinobacteria. Además, se identificó que la acetona (utilizada como disolvente del antraceno) no modificó significativamente a la comunidad bacteriana.

También, se implementaron métodos de extracción no exhaustiva de antraceno para conocer la cantidad biodisponible en el suelo del exlago de Texcoco. Las evidencias demostraron que el pH y la capacidad de retención de agua del suelo tienen un efecto sobre la cantidad de antraceno extraído por técnicas no exhaustivas.

ABSTRACT

In the last years, soil contamination with polycyclic aromatic hydrocarbons and soil salynity have increased considerably due to anthropogenic activities. For this reason, developing of soil biorremediation techniques is urgent.

In this work, a research to identify changes in the soil microbial communities, when soil samples from the former Texcoco lake were spiked with anthracene, at a final concentration of 550 mg kg^{-1} dry soil. It was seen that a biotically anthracene removal, of 65% was possible in this soil. The soil bacterial communities were modified increasing the percentage of the *phylum* Actinobacteria by the presence of anthracene. No significant effect of the acetone, which was used to dissolve the anthracene, on the bacterial communities was observed.

Non-exhaustive methods of anthracene extraction to know the bioavailability of anthracene in soil were also developed. It was demonstrated that the soil pH and the water holding capacity had a significant effect on the efficiency of anthracene extraction by non-exhaustive techniques.

INTRODUCCIÓN

GENERALIDADES DEL SUELO

Definición de suelo

Se le llama suelo al material exterior poco compacto de la corteza terrestre, el cual es un estrato característicamente diferente al lecho rocoso subyacente. Está constituido por las siguientes fracciones: fracción mineral, espacio poroso ocupado por agua o aire, materia orgánica inerte y organismos vivos. La fracción mineral de éste se origina de la desintegración y descomposición (intemperización) de las rocas a través de cientos de años, siendo la fuente de nutrientes y soporte físico para las plantas, la matriz para capturar agua, aire y el hábitat para un sinnúmero de organismos vivos (Alexander, 1994).

Funciones de los suelos

El suelo tiene diversas funciones y muy importantes para los ecosistemas terrestres y el ambiente del planeta: es el sustento para la vida vegetal, del cual las plantas obtienen soporte físico y muchos de sus nutrientes; es el hábitat para una gran diversidad tanto de microorganismos (bacterias, actinomicetos, arqueas, hongos, algas, protozoarios y virus) como de macroorganismos (coleópteros, miriápodos, hormigas, colémbolos, nemátodos, ácaros, larvas, e incluso, mamíferos y reptiles pequeños); es el lugar donde se llevan a cabo la mayor parte de los ciclos biogeoquímicos de los ecosistemas terrestres. Dada la gran diversidad biótica del suelo, se presentan interacciones muy complejas entre los diferentes organismos y, junto con los ecosistemas acuáticos,

representan la base de la vida en este planeta; actúa como filtro regulador durante la recarga de acuíferos y protección de éstos; es la base física para la construcción de edificaciones y servicios (SEMARNAT, 2009; Alexander, 1994).

Estructura de los suelos

El suelo está conformado por varios horizontes que difieren en su estructura, color y composición. Según Alexander los horizontes se clasifican:

Horizonte A. Zona superficial de restos orgánicos en descomposición, sujeto a una marcada lixiviación. En este estrato se presenta la mayor actividad biológica aerobia; hay una gran incidencia de raíces, animales pequeños y microorganismos.

Horizonte B. Estrato subyacente al horizonte A, del cual se han eliminado algunos constituyentes inorgánicos hay poca materia orgánica, escasas raíces y microflora; se acumulan compuestos de Fe y Al.

Horizonte C. Contiene el material parental del suelo; la materia orgánica se encuentra en cantidades muy pequeñas y hay escasas formas de vida (Alexander, 1994).

La degradación del suelo pone en riesgo la viabilidad de actividades agropecuarias, forestales y disminuye su biodiversidad. La fertilidad del suelo depende de las características físicas y químicas de éste. En México el 64% de los suelos presenta algún tipo de deterioro (SAGARPA, 2013).

La degradación del suelo en México se debe principalmente a 4 causas: erosión hídrica, erosión eólica, degradación química y degradación física

Tanto la erosión hídrica y eólica incluyen procesos en los cuales hay desplazamiento de material del suelo; mientras que en la degradación química y física hay procesos que ocasionan el deterioro interno del suelo.

La degradación química es el proceso mas extendido en México representa el 17.8% de la superficie nacional y está presente en todas las entidades federativas, de éste, el 3.2% se debe a procesos de salinización y alcalinización de suelos, 3.5 a procesos de polución, 0.6 a procesos de eutrofización y 92.7 a procesos de disminución de fertilidad.

Dentro de los tipos de degradación química está la salinización y alcalinización del suelo en donde se presenta un incremento en el contenido de sales que provoca entre otras cosas la disminución del rendimiento de los cultivos. Otro tipo de degradación química es por polución y se debe a la presencia, o incremento en la concentración de contaminantes.

SUELOS SALINO-ALCALINOS

Los procesos de salinización y alcalinización se encuentran principalmente juntos, un suelo salino según la Norma Oficial Mexicana NOM-021-SEMARNAT-2000, que establece las especificaciones de fertilidad, salinidad y clasificación de suelos, estudios, muestreo y análisis, es aquel que contiene suficientes sales solubles como para reducir el crecimiento de las plantas, con conductividad electrolítica (CE) en la pasta de saturación igual o mayor a 4 dS m^{-1} y un suelo sódico es en donde el porcentaje de sodio intercambiable es de 15% o más, o el que contiene el sodio suficiente como para afectar adversamente las propiedades físicas y la permeabilidad. Por lo tanto, un suelo salino-sódico es el que contienen suficiente sodio intercambiable para interferir

con el crecimiento de la mayoría de los cultivos y que contiene cantidades apreciables de sales solubles.

Los suelos sódicos presentan un pH alcalino entre 8 y 9 en el extracto de saturación.

El pH es una de las características fisicoquímicas más importantes de un ecosistema, ya que determina las especies químicas y por ende, la biodisponibilidad de los nutrientes en el sistema. Valores de pH en intervalos extremos <5.5 o >9.5 representan condiciones extremas que afectan el crecimiento óptimo de varios organismos. En la figura 1 se muestra un ejemplo de cómo afecta el pH al crecimiento de un cultivo de trigo las deficiencias aparecen como barras angostas.
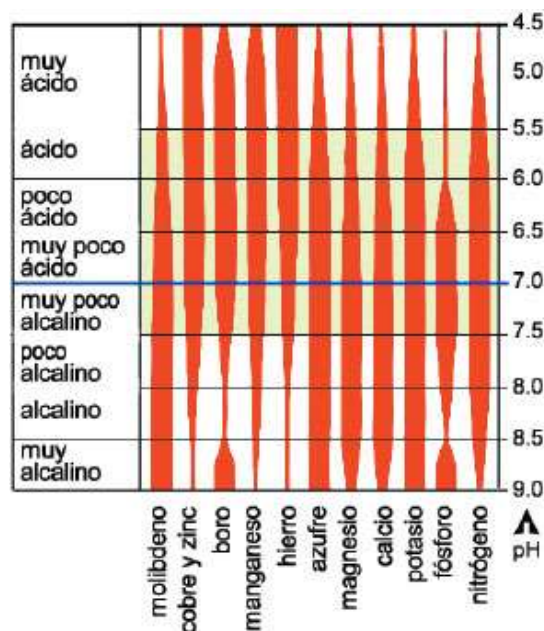

Figura 1. Efecto del pH del suelo en la disponibilidad de nutrientes en cultivo de trigo. (Fuente: Pratt (1965) http://www.fao.org/DOCREP/006/ X8234S/x8234s08.htm)

Para fines agrícolas y de forestación, los suelos salino-alcalinos representan un problema debido a que el exceso de sales solubles produce un efecto dañino en los cultivos.

Los suelos salinos se clasifican de acuerdo a su conductividad electrolítica (Tabla 1) (FAO, 2009).

Tabla 1 Clasificación de suelos de acuerdo a su conductividad electrolítica.

Clasificación	Conductividad electrolítica (dSm^{-1})
No-salino	0-2
Ligeramente salino	2-4
Moderadamente salino	4-8
Fuertemente salino	8-16
Muy fuertemente salino	>16

Los suelos salinos se encuentran principalmente en zonas de clima árido o semiárido. En condiciones húmedas, las sales solubles originalmente presentes en los materiales del suelo y las sales que se forman por la intemperización de minerales, se mueven a las capas inferiores, hacia el agua subterránea y finalmente a los océanos. Por lo tanto, los suelos salinos no existen en regiones húmedas, excepto cuando el suelo ha estado expuesto al agua del mar, como en los deltas de los ríos y en tierras bajas cercanas al mar. En las regiones áridas el lavado del suelo es de naturaleza local y las sales solubles no pueden transportarse muy lejos. Esto ocurre no solamente porque hay menos precipitación para la lixiviación y el transporte de las sales, sino también

a consecuencia de las elevadas tasas de evaporación, característica del clima árido, que tiende a concentrar las sales de los suelos y del agua superficial.

El drenaje restringido es un factor que frecuentemente contribuye a la salinización de los suelos y que puede llevar consigo la presencia de una capa freática poco profunda o una baja permeabilidad del suelo. La capa freática poco profunda casi siempre guarda estrecha relación con la topografía del terreno. Debido a la baja precipitación en las regiones áridas, las corrientes del drenaje superficial están poco desarrolladas y, en consecuencia, existen depresiones sin drenaje por no tener salida a corrientes permanentes. El drenaje de las aguas con sales de las tierras arriba de la depresión puede elevar el nivel de la capa freática hasta la superficie en las tierras bajas, causar un flujo temporal o formar lagos salados permanentes. Bajo tales condiciones, el movimiento ascendente del agua subterránea o la evaporación del agua superficial da origen a la formación de suelos salinos. La magnitud de las áreas salinas así formadas varía desde unas cuantas hectáreas hasta cientos de kilómetros cuadrados (Gutiérrez-Castorena, 1997; Primo-Yúfera y Carrasco-Dorrien, 1973).

La baja permeabilidad del suelo es causa del mal drenaje, impidiendo el movimiento descendente del agua. La baja permeabilidad puede deberse a la textura, a una estructura desfavorable o a la presencia de capas endurecidas que pueden estar constituidas por arcilla compacta o una capa silíca dura.

El proceso de salinización de los suelos se puede dividir en dos clases:

1. El proceso de salinización primaria, involucra una acumulación de la sal en el suelo a través de un proceso natural como puede ser que el tipo de suelo no permita el drenaje al subsuelo y por consiguiente se presente el acúmulo de las sales. La acumulación de sales se ve favorecida

topográficamente en sitios bajos del terreno, donde tienden acumularse las aguas que se escurren de grandes áreas y que disuelven y arrastran consigo grandes cantidades de sales en el proceso; este fenómeno se da con mayor intensidad cuando se presentan cuencas de tipo endorreico, carentes de drenaje natural. Otro tipo de acumulación primaria es donde los suelos pueden estar expuestos a invasiones o intrusiones directas del agua de mar, o en áreas interiores de los continentes en contacto directo con depósitos fósiles (FAO, 2009).

2. El proceso secundario de salinización de suelos (es decir cuando un suelo no salino se vuelve salino), es causado por actividades antropogénicas principalmente por prácticas inapropiadas de irrigación. Estos suelos frecuentemente se encuentran en valles cercanos a las corrientes y por la facilidad con que pueden irrigarse, se escogen los más planos para el cultivo. Aun cuando estos suelos estén bien drenados y no sean salinos bajo condiciones naturales, puede ser que el drenaje no sea adecuado para la irrigación. Cuando se someten nuevas tierras al riego, los agricultores casi siempre olvidan la necesidad de establecer drenajes artificiales que regulen el agua adicional y las sales solubles. A consecuencia de esto, la capa freática puede surgir de profundidades considerables y llegar hasta cerca de la superficie del suelo en pocos años (FAO, 2009).

Los altos niveles de sales y de iones presentes en los suelos afectados por sales provocan distintos tipos de efectos negativos sobre el desarrollo de las plantas como: efectos osmóticos, efectos tóxicos y efectos nutricionales. Estos fenómenos afectan principalmente a los cultivos agrícolas y ocasionan una disminución en sus rendimientos. Por otro lado, los microorganismos se ven igualmente sujetos a los efectos anteriores, con lo cual se ven alterados los ciclos biogeoquímicos en los cuales intervienen, y estos afectan de manera indirecta a las plantas. Por ejemplo, en el caso del ciclo biogeoquímico del nitrógeno, la nitrificación y la fijación de nitrógeno se ven disminuidas a causa

del incremento en la concentración de sales (Ghandi y Paliwal, 1976); mientras que, la desnitrificación se ve incrementada, lo que provoca una disminución del contenido de nitrógeno disponible en el suelo (Gandhi y Paliwal, 1976).

En los suelos alcalinos se presenta además, una reducción en la permeabilidad del suelo, con lo que se generan condiciones de anaerobiosis, con todos los efectos negativos que esto provoca en la plantas, por ende, hay una disminución en la producción agrícola.

Alrededor de una décima parte de la superficie irrigada del planeta está saturada de sal (FAO, 2009). México tiene serios problemas de salinidad (Rhoades *et al*., 1998). Las zonas áridas y semiáridas, ocupan cerca del 40% de la superficie nacional. Fuera de estas zonas existen también diversas cuencas cerradas donde se presentan fuertes acumulaciones de sal y amplias zonas costeras ocupadas por suelos salinos. No existe una evaluación precisa y actual de las superficies de los suelos con problemas de salinidad y alcalinidad en México, sobre todo en las áreas agrícolas. Sin embargo, pueden señalarse, de manera aproximada, la presencia de 3.5 millones de hectáreas afectadas en las zonas áridas y semiáridas, 1 millón en otras zonas no agrícolas, 800 000 en las áreas costeras, 1 millón en áreas agrícolas de temporal y 500 000 en zonas agrícolas de riego, lo cual totaliza unos 6.8 millones de hectáreas de suelos afectados por sales en todo el país (SEMARNAT, 2009). El suelo del exlago de Texcoco es un suelo salino alcalino que se tomará de modelo en este estudio.

Suelo del exlago de Texcoco

El municipio Texcoco se encuentra en el Estado de México, cuenta con una superficie de 404 Km^2 y una población total de 209 308 habitantes en el año 2000 (INEGI, 2009). La actual zona federal del exlago de Texcoco, se encuentra dentro de la cuenca llamada Valle de México, al noreste de la

Ciudad de México y queda comprendida entre los paralelos 19º 25" y 19º 35" de latitud norte y los meridianos 98º 58" y 99º 52" de longitud oeste. El área es una planicie de pendientes menores al 1%; presenta algunas variaciones altimétricas, pero en promedio se ubica a una altitud sobre el nivel del mar de 2 236 m sobre el nivel del mar. Los suelos encontrados, donde antes estaba gran parte del lago de Texcoco, se encuentran clasificados como suelos salino-alcalinos. El suelo que forma el exlago de Texcoco es un ecosistema único, que se caracteriza por sus elevados niveles de sales y sodio intercambiables, los cuales limitan seriamente el desarrollo vegetal, con lo cual su uso agrícola se ve impedido por completo. Los suelos del exlago de Texcoco pueden llegar a tener un pH mayor a 10 y conductividad electrolítica mayor a 150 dS m^{-1} (Luna-Guido *et al.*, 2000). Los microorganismos en este ecosistema se han adaptado a condiciones de salinidad y alcalinidad, por lo cual su estudio representará una predicción de cuál sería el comportamiento de las comunidades microbianas cuando hayan estado expuestas a procesos de salinización secundaria.

El lago de Texcoco fue drenado en el siglo XVII para evitar las inundaciones en la ciudad colonial de México. Con ello, apareció un nuevo suelo con escasa vegetación debido a que, el suelo de origen volcánico, era salino alcalino con un pH promedio de 8.5 (CNA, 1996). La salinidad se mantiene en la solución del suelo debido al poco drenaje natural del área, además de tener elevadas tasas de evaporación (2000 mm año^{-1}) y una lluvia anual de 705 mm. Debido a la proximidad de la erosionada área del exlago de Texcoco a la mancha urbana de la Ciudad de México, graves problemas de polvaredas se detectaron, por lo que a principios del año 1970 comenzó un programa de forestación para controlar la erosión (Beltrán-Hernández *et al.*, 1999). El programa de forestación incluyó la instalación de un sistema de drenado y la adición de aguas residuales para disminuir la cantidad de sales presentes en el suelo y la introducción de especies vegetales como un pasto nativo halotolerante: *Distichlis spicata* y el árbol tamarix (*Tamarix spp.*) también conocido como pino

salado. Con estas medidas, comenzó la forestación del área federal del exlago de Texcoco, y por consiguiente, surgió la necesidad de realizar estudios del ciclo del carbono y del nitrógeno que explicaran el efecto de la salinidad y la alcalinidad sobre los pastos.

Microorganismos de ambientes extremos

El suelo del exlago de Texcoco es un ambiente extremo en cuanto a alcalinidad y salinidad, por tanto, su diversidad microbiana está comprendida por microorganismos extremófilos. Los extremófilos han desarrollado características fisiológicas adaptativas que se han especializado para permitir su supervivencia y funcionamiento dentro de las constricciones fisicoquímicas de esos ecosistemas

Condiciones extremas de alcalinidad

Los microorganismos que toleran o que necesitan altas concentraciones salinas se llaman halotolerantes (toleran) y halófilos (necesitan) (Gilmour, 1990). En concentraciones elevadas de sal el ambiente hipertónico deshidrata a los microorganismos no halotolerantes. Además de afectar la presión osmótica, la concentración elevada de sal desnaturaliza las proteínas; es decir, altera su estructura terciaria que es esencial para su actividad enzimática.

Los microorganismos halotolerantes y halófilos suelen excluir de sus células las altas y relativamente tóxicas concentraciones de ion sodio que, por lo general, predominan en sus ambientes. Consiste en llevar a un equilibrio osmótico con su medio por mecanismos similares a los que emplean las osmófilas. Por ejemplo, *Halobacterium* consigue su equilibrio osmótico mediante altas concentraciones de cloruro de potasio. Los ribosomas y enzimas necesitan de

altas concentraciones de sales para conservar su estabilidad, además de que carecen de mureína y su pared celular necesita de iones sodio para su funcionamiento.

Algunas cepas de *Halobacterium* tienen bacteriorrodopsina en su membrana, que funciona como bomba de protones fuera de la célula y genera así un potencial electroquímico. Esto a su vez conduce a la síntesis de ATP, *Halobacterium* necesita un mínimo de 3.0 M de NaCl. La holatolerancia es una característica de los géneros *Halomonas* y *Staphylococcus* (Atlas y Bartha, 2006).

Condiciones extremas de la concentración del ion Hidrógeno

Una característica común de los microorganismos que toleran, o incluso necesitan, un pH extremo para su crecimiento es que su citoplasma se mantiene próximo a valores neutros. La pared y membrana celular de dichos microorganismos necesitan adaptarse para que su integridad se conserve a esos valores de pH extremos, para que mantenga el interior de la célula cercano a la neutralidad y para realizar síntesis quimiosmótica de ATP en esas condiciones poco frecuentes. Muchas bacterias y hongos toleran pH alcalino de hasta 9.0 entre auténticas alcalófilas encontramos algunas cepas de *Bacillus* como *B. alcalophilus* y *B. pasteurii*. Los microorganismos que se consideran halófilos como *Halobaterium*, *Natronobaterium*, *Natrococcus*, también son alcálofilos y viven con elevado pH (Atlas y Bartha, 2006).

El suelo por sus múltiples funciones es de vital importancia para sustentar la vida terrestre y las actividades económicas de un país, por tanto, debe protegerse para evitar su degradación y su contaminación. La contaminación

del suelo conduce a la pérdida de los valores económico, cultural y ambiental asociados al uso del suelo, por ejemplo, si se contamina el suelo de un predio disminuye considerablemente o desaparece totalmente el valor monetario de este sitio. No proteger el suelo significa también la desaparición de servicios ambientales en especial el de constituir el filtro para la recarga de acuíferos y con ello se pone en riesgo el suministro de agua potable. Cuando la contaminación del suelo y la degradación de sus funciones ocurren debe realizarse, en lo posible, su remediación. En la Tabla 2 se muestran algunos ejemplos de los microorganismos extremos.

Tabla 2 Ejemplos de microorganismos extremos

Parámetro	Tipo	Condición óptima de crecimiento	Ejemplo
pH	Alcalófilo	pH > 8	*Bacillus Spirulina*
Salinidad	Ligeramente halófilo Moderadamente halófilo Halófilo extremo	1 a 5% NaCl 5 a 15% NaCl 15 a 30% NaCl	*Halomonas, Delega, Volcaniella, Flavobacterium, Paracoccus, Pseudomonas, Halovibrio y.*

Hasta el momento se ha tratado la degradación química por procesos de salinización y alcalinización, sin embargo México al ser uno de los principales productores de hidrocarburos en el Mundo, sufre por ello un deterioro ambiental por procesos extracción, refinación, tomas clandestinas, transporte, almacenamiento etc.

HIDROCARBUROS

Los hidrocarburos son compuestos de gran abundancia en la naturaleza están formado por carbono e hidrógeno y están presentes principalmente en el petróleo. Dependiendo del número de átomos de carbono y de la estructura de los hidrocarburos que integran el petróleo, se tienen diferentes propiedades que los caracterizan y determinan su comportamiento como combustibles, lubricantes, ceras o solventes.

Las cadenas lineales de carbono asociadas a hidrógeno constituyen las parafinas; cuando las cadenas son ramificadas se tienen las isoparafinas; al presentarse dobles uniones entre los átomos de carbono se forman las olefinas; las moléculas en las que se forman ciclos de carbono son los naftenos, y cuando estos ciclos presentan dobles uniones alternas (anillo bencénico) se tiene la familia de los hidrocarburos aromáticos policíclicos.

Además hay hidrocarburos con presencia de azufre, nitrógeno y oxígeno formando familias bien caracterizadas, y un contenido menor de otros elementos. Al aumentar el peso molecular de los hidrocarburos las estructuras se hacen verdaderamente complejas y difíciles de identificar químicamente con precisión. Un ejemplo son los asfaltenos que forman parte del residuo de la destilación al vacío; estos compuestos además están presentes como coloides en una suspensión estable que se genera por el agrupamiento envolvente de las moléculas grandes por otras cada vez menores para constituir un todo semicontinuo.
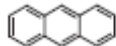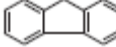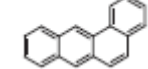
HIDROCARBUROS POLICÍCLICOS AROMÁTICOS
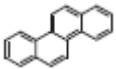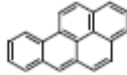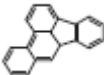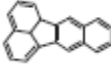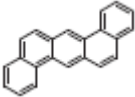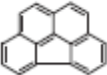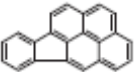
Los hidrocarburos policíclicos aromáticos (PAHs por siglas en inglés) constituyen un grupo de alrededor 100 compuestos orgánicos tóxicos, están

formados por dos a más anillos bencénicos o estructuras de anillos pentacíclicos, fusionados de manera lineal, angular o con arreglos más complicados y contienen por definición solo átomos de carbono e hidrógeno, se encuentran presentes principalmente en el petróleo. Los PAHs son termodinámicamente estables y tienen baja solubilidad en agua y bajas presiones de vapor. Las propiedades químicas, hidrofobicidad, recalcitrancia y toxicidad de los PAHs, dependen tanto del número de anillos presentes, como del tipo de enlace que los une (Leahy y Colwell, 1990). Los PAHs se dividen generalmente en PAHs de bajo peso molecular (los hidrocarburos que contienen de uno a tres anillos aromáticos) y PAHs de alto peso molecular (contienen cuatro o más anillos aromáticos).

La Agencia de Protección al Ambiente de los Estados Unidos de América (EPA por sus siglas en inglés), y de la Comunidad Europea tienen a 16 PAHs como contaminantes prioritarios (Tabla 3).

Tabla 3 Los 16 PAHs reportados por la EPA como prioridad por su toxicidad

Nombre	Estructura	Fórmula	Peso Molecular	Solubilidad (mg l^{-1})
Naftaleno		$C_{10}H_8$	128	31
Acenafteno		$C_{12}H_{10}$	156	3.8
Acenaftileno		$C_{12}H_8$	152	16.1
Antraceno		$C_{14}H_{10}$	178	0.045
Antraceno		**$C_{14}H_{10}$**	**178**	**1.1**
Fluoreno		$C_{13}H_{10}$	166	1.9
Fluoranteno		$C_{16}H_{10}$	203	0.26
Benzo[a]antraceno		$C_{18}H_{12}$	228	0.011

Criseno		$C_{18}H_{12}$	228	0.0015
Pireno		$C_{16}H_{10}$	202	0.132
Benzo[a]pireno		$C_{20}H_{12}$	252	0.0038
Benzo[b]fluoranteno		$C_{20}H_{12}$	252	0.0015
Benzo[k]fluoranteno		$C_{20}H_{12}$	252	0.0008
Dibenzo[a, h]antraceno		$C_{22}H_{14}$	278	0.0005
Benzo[g,h,i]perileno		$C_{22}H_{12}$	276	0.00026
Indeno[1,2,3-c,d]pireno		$C_{22}H_{12}$	276	0.062
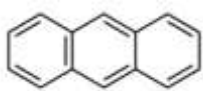
SUELOS CONTAMINADOS CON PAHS

El acelerado incremento de la población mundial ha dado como resultado la demanda de mayores cantidades de combustibles fósiles (hidrocarburos) (Álvarez, 2008). Aunque muchos de estos compuestos se utilizan para generar energía, un alto porcentaje se libera al ambiente, por procesos de extracción, refinado, transporte y almacenamiento, lo cual representa un riesgo potencial para la integridad de los ecosistemas, así como para la preservación de los recursos naturales en los lugares donde se producen (SEMARNAT, 2009). Aproximadamente el 90% de los ambientes contaminados con PAHs, son ecosistemas terrestres, y más específicamente, los primeros 20 cm del horizonte del suelo (Doyle *et al.*, 2008).

México es el séptimo país en el mundo con mayor producción de petróleo (PEMEX, 2012) por consiguiente existen extensas áreas contaminadas con hidrocarburos del petróleo debido principalmente a derrames, así como a las actividades propias de la industria petrolera. Este tipo de compuestos, se acumulan en ecosistemas marinos y en suelos, siendo responsables de su deterioro. Algunos suelos contaminados, principalmente en el sureste de México, contienen concentraciones de hidrocarburos hasta de 450 000 mg kg^{-1} (Gallegos-Martínez *et al.*, 2000).

El antraceno son cristales de color amarillo y en Tabla 4 se pueden ver algunas de sus características.

Tabla 4 Propiedades físicas del hidrocarburo aromático (PAHs) empleado en esta investigación.

PAHs	Estructura Química	Alteraciones que causa	Características
Antraceno		Irrita piel y ojos	Peso molecular: 178.28 Gravedad específica a 20º C: 1.25 Punto de fusión: 217º C Punto de ebullición: 340º C

Los PAHs son contaminantes de origen natural o de origen antropogénico. La cantidad y tipo de contaminación con PAH tiene efectos sobre la estructura de las comunidades microbianas en el suelo y la presencia de una estructura

particular es determinante para que las estrategias de bioremediación tengan éxito (Lorraine *et al.*, 2007).

El uso de métodos biológicos (biorremediación) para el tratamiento de sitios contaminados por hidrocarburos, es una tecnología que representa varias ventajas con respecto a los métodos físicos y químicos tradicionales (Leahy y Colwell, 1990; Doyle *et al.*, 2008; Johnsen *et al.*, 2005), en la siguiente figura se enlistan algunos de los factores bióticos y abióticos que afectan la degradación de los PAHs en el suelo.
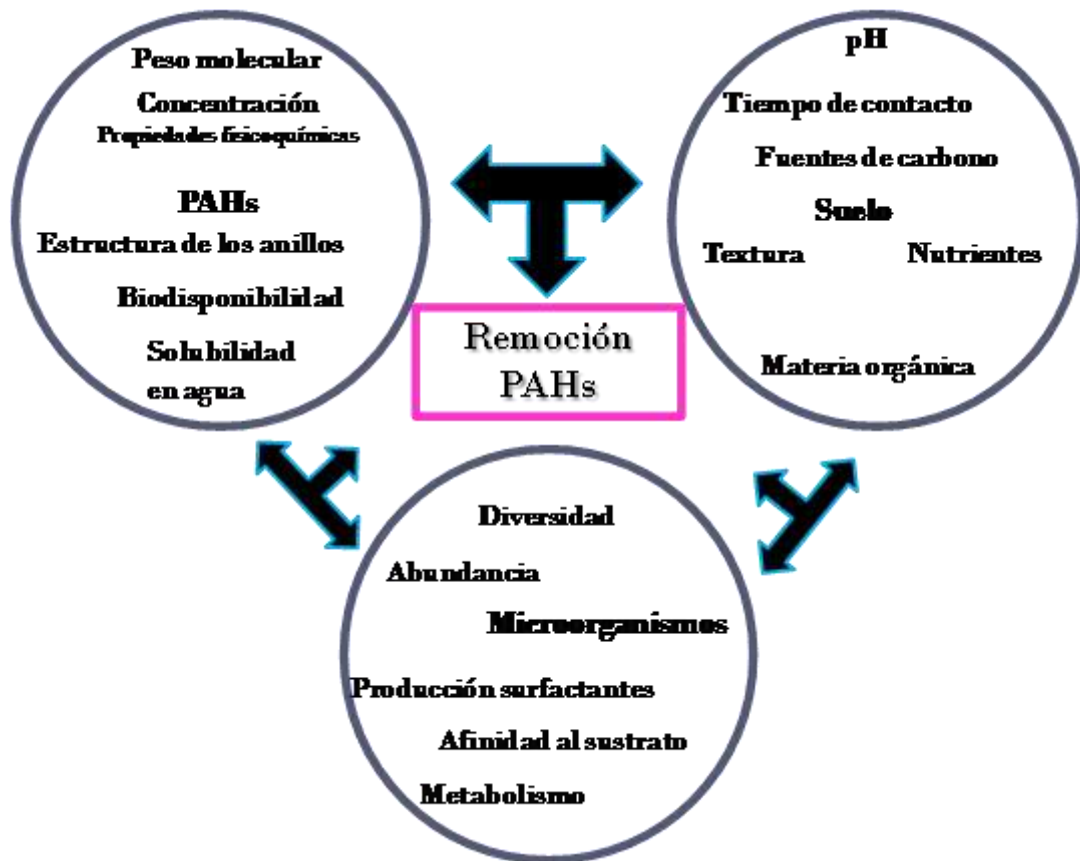
Figura 2. Factores bióticos y abióticos que afectan la degradación de PAHs (Doyle *et al.,* 2008; Lorenzo, 2008)

BIODISPONIBILIDAD

La remoción de hidrocarburos está influenciada por diversos factores, uno de ellos es la biodisponibilidad del hidrocarburo cuando está en la matriz del suelo, ya que el hidrocarburo es de naturaleza hidrofóbica (no polar) y tiene baja solubilidad en el agua, además de tender a adsorberse fuertemente a las partículas del suelo y de que la biodisponibilidad de los contaminantes disminuye a través del tiempo.

La contaminación por hidrocarburos se ha detectado en suelos, sedimentos y agua y por esto se han desarrollado muchas estrategias de análisis para su extracción y su análisis.

El término extracción significa sacar o retirar de la forma más efectiva y lo más completa posible las sustancias activas contenidas. En este proceso existen diversos factores que influyen: solvente, agitación, temperatura, pH, etc.

Técnicas de extracción

- Extracción mecánica: se denomina también como prensanda.
- Extracción por arrastre de vapor donde implica aquellos compuestos químicos que son solubles a temperaturas de ebullición.
- Extracción líquido-líquido, este método se basa en el empleo de disolventes, inmiscibles entre sí.
- Extracción sólido-líquido, consiste en poner en contacto el sólido con el disolvente, obteniéndose el líquido extractor.

El tipo de extracción puede ser parcial o exhaustiva, la extracción parcial es cuando no existe una extracción completa de los principios activos y puede ser por: maceración, digestión, infusión, etc. La extracción exhaustiva es cuando existe una extracción completa de los principios activos y puede ser por

percolación, soxhlet etc.

La cantidad de hidrocarburo biodisponible en el suelo ha sido relacionada con la cantidad de hidrocarburo extraído por técnicas no exhaustivas (Gan *et al*. 2009) como la técnica de hidroxipropil-beta-ciclodextrina (HPCD) (Reid *et al*. 2000) o n-butanol (Kelsey *et al*. 1997).

Kelsey y colaboradores (1997) probaron varios ensayos químicos de extracción de hidrocarburos para relacionarlos con la cantidad de hidrocarburo biodisponible en suelo. La cantidad de hidrocarburo biodisponible lo midieron con ensayos con lombrices y encontraron que la cantidad de hidrocarburo extraído con la técnica de de n- butanol fue similar a la cantidad de hidrocarburo extraído con ayuda de las lombrices. Sin embargo en el suelo del exlago de Texcoco sería difícil hacer ensayos con lombrices debido a la alta salinidad.

EL USO DE HERRAMIENTAS MOLECULARES EN EL ESTUDIO DE LA ECOLOGÍA DE SUELOS

Aproximadamente del 1 al 3% de los microorganismos del suelo se han cultivado , por lo cual, la ecología microbiana de suelos todavía representa un reto para los microbiólogos que intentan establecer los mecanismos por los cuáles los microorganismos metabolizan sustratos activamente, que a su vez se relacionan con redes alimenticias y residuos animales, y con la provisión de nutrientes esenciales a las plantas, es decir, la función de los organismos dentro de los ecosistemas, en su habitad natural. A partir de la década de 1990 el uso de la biología molecular ha permitido explorar la biodiversidad y avanzar en el conocimiento de los microorganismos de ambientes extremos, que no habían podido cultivarse por los métodos de la microbiología clásica (Atlas y Bartha, 2006).

En la búsqueda de solucionar estos problemas, se han desarrollado diversas técnicas para obtener resultados más cercanos a la realidad de la biodiversidad de los ecosistemas. El aislamiento de ácidos nucleícos de ambientes naturales

es una de estas técnicas y ha mostrado ser una herramienta útil en la detección e identificación de bacterias que no se han cultivado por técnicas de microbiología clásica, esto se ha hecho para determinar las dinámicas de determinadas bacterias bajo condiciones naturales y revelar la diversidad de genotipos en diferentes ecosistemas microbianos (Borneman y Triplett, 1997; Picard *et al.*, 1992). Una de las herramientas para el conocimiento de las comunidades bacterianas en suelos es la elaboración de bibliotecas genómicas del rRNA de la subunidad pequeña de los ribosomas bacterianos.

La diversidad es un término abstracto que requiere especificarse en cada contexto. Dentro de este concepto se debe englobar toda clase de variedad natural, desde diversidad de comunidades a nivel de paisaje (diversidadγ), diferencia entre comunidades (diversidadβ) y diversidad de especies dentro de una comunidad (diversidadα).

La diversidad de las especies suele ser baja en los ecosistemas controlados físicamente, porque las adaptaciones al estrés fisicoquímico imperante son prioritarias y dejan poco espacio para la evolución de las interacciones entre las especies que se encuentran integradas y en equilibrio. La distribución ecológica y el funcionamiento de las poblaciones microbianas están fuertemente influidos por factores abióticos (Atlas y Bartha, 2006).

Existen numerosos índices para medir la diversidad de especies en un determinado ecosistema siendo los más frecuentes la riqueza, el índice de Shannon, el índice de Simpson y el índice de uniformidad. Estos índices se basan en la presencia y proporción de las distintas especies.

Riqueza de especies

La riqueza es el número de especies presentes en el sistema, aumenta con la superficie de la parcela, siendo la incorporación de especies muy pequeña a partir de cierto tamaño de la parcela

Índice de Shannon

El índice de Shannon viene dado por la expresión: $H' = -\Sigma\, pi \log_2 (pi)$ donde pi es la abundancia relativa de cada especie (en número de individuos). Este índice aumenta con el número de especies presente y toma mayores valores cuando las proporciones de las distintas especies son similares.

Índice de uniformidad.

En el índice de Shannon se da un gran peso al número de especies presentes, pero en ocasiones es más interesante conocer el reparto de las especies en proporciones sin que influya el número de especies (n). Este es el caso del índice de uniformidad (Magurran, 1988): $Ev = H' (\log (n))^{-1}$. Este índice varía entre 0, valor que toma cuando todos los individuos pertenecen al mismo grupo, y 1, si los individuos se reparten homogéneamente en los distintos grupos.

Índice de Simpson

El índice de Simpson mide la probabilidad de que dos individuaos de la población extraídos al azar sean de la misma especia (Σpi^2); valores altos indican la dominancia de alguna especie. Para medir la diversidad se utiliza el complemento del índice de Simpson, ya que varía de 0 a 1 indicando valores próximos a 1 mayor diversidad: $1-D = 1- (\Sigma pi^2)$. Este índice ofrece información intermedia entre el índice de Shannon y el de uniformidad, ya que aumenta con

el número de especies y refleja a su vez el reparto de las especies en proporciones.

Tabla 5 Resumen de los principales índices de diversidad aplicados a diversidad de especies.

Índice	Referencia	Aspecto en que se incide
Shannon	Shannon (1949) Berger y Puettmann (2000)	Diversidad de especies
Simpson	Simpson (1949) Magurran (1988)	Diversidad de especies
Uniformidad	Magurran (1988)	Dominancia de especies
Segregación de Pielou	Pielou (1977)	Mezcla de especies considerando un patrón espacial

Pero cómo saber si nuestra descripción de diversidad representa a la totalidad de la comunidad, a menudo resulta imposible registrar la totalidad de las especies presentes. Las curvas de rarefacción de especies representan el número de especies acumulado frente al esfuerzo de muestreo empleado y son una eficaz metodología para estandarizar la estimación de riqueza obtenida en distintos trabajos de compilación. Cuanto mayor sea el muestreo, mayor será el número de especies colectadas, al principio se encuentran especies comunes; sin embargo, después esta adición disminuye hasta que se haya registrado todas las especies presentes.

La rarefacción parte de consideraciones básicas, asume que todos los individuos se distribuyen al azar en el ecosistema y que la toma son muestras aleatorias de esos individuos; dicho de otra forma, que todos los individuos tienen la misma probabilidad de ser identificados aleatoriamente.

El término representativo significa que la muestra refleja la diversidad y la densidad de organismos de la totalidad del ambiente del cual se ha extraído; sin embargo debe tenerse presente que no se puede completar nunca la diversidad total de especies, por lo que la estimación final depende de la resolución temporal y espacial que empleemos en el muestreo.

ANTECEDENTES

Desde el año 1999 (Beltrán-Hernández *et al.*, 1999) hasta la fecha, el grupo de trabajo del Doctor Luc Dendooven ha investigado el suelo del exlago de Texcoco. El suelo del exlago tiene características propias de un ambiente extremo, se puede encontrar pH alcalino superior a 10 y conductividad electrolítica (CE) de 2 a 150 dS m^{-1} (Fernández-Luqueño 2009).

En el año de 2006 Betancur-Galvis y colaboradores encontraron que era posible remover hidrocarburos aromáticos policíclicos en el suelo del exlago de Texcoco y esta remoción ocurría rápidamente en los primeros siete días de incubación. Más tarde, Fernández-Luqueño y colaboradores en el 2009, encontraron una disminución de aproximadamente el 30% de hidrocarburos aromáticos policíclicos en los primeros tres días, en una incubación aerobia en suelos del exlago de Texcoco.

Por otro lado, Valenzuela-Encinas y colaboradores (2009) realizaron bibliotecas de amplicones de la región del DNA que codifica al rRNA 16S de la subunidad pequeña de los ribosomas bacterianos, a partir del DNA extraído de suelos salino-alcalinos del exlago de Texcoco con diferente CE y encontraron géneros bacterianos que ya habían sido reportados como bacterias degradadoras de hidrocarburos aromáticos policíclicos.

Con estos antecedentes surgió la pregunta de ¿qué sucedía con las bacterias cuando estaban en presencia del contaminante?, ¿o si habría un grupo bacteriano que predominada? Así que en el presente trabajo se estudió el cambio en las comunidades bacterianas de un suelo salino alcalino del exlago de Texcoco en presencia de un hidrocarburo aromático policíclico (antraceno), empleando bibliotecas de DNA, basadas en el marcador rRNA 16S.

JUSTIFICACIÓN

El suelo del exlago de Texcoco es salino-alcalino extremo con alta conductividad electrolítica de hasta 150 dS m^{-1} y pH > 10 (Castro *et al.*, 2008). En estudios previos se ha mostrado que en este suelo es posible la remoción de hidrocarburos aromáticos policíclicos (PAHs) (Betancur-Galvis *et al.*, 2006). Los PAHs se encuentran dentro de los contaminantes orgánicos más distribuidos en el suelo, en agua natural y en agua residual, aproximadamente el 90% del total de los ambientes contaminados con PHAs se encuentra en ecosistemas terrestres (Doyle, 2008). A pesar de que existen cientos de PAHs, la mayoría de los estudios se enfocan en un número limitado de ellos, por ejemplo los 16 PAHs listados como contaminantes por la Agencia de Protección al Ambiente de los Estados Unidos (US EPA) y la Comunidad Europea (Samanta *et al.*, 2002; Puglisi *et al.*, 2007). Siete de ellos, incluyendo el antraceno, podrían ser carcinogénicos (Cai *et al.*, 2007). El Antraceno es un hidrocarburo tricíclico aromático, conocido irritante de piel y ojos (IARC, 1983). A pesar de que existen diversos estudios sobre los microorganismos que degradan estos contaminantes en suelos, solo el 1% de los microorganismos que habitan en el suelo son cultivados por técnicas de microbiología clásica (Torsvik y Øvreås, 2002), sin embargo, el uso de las herramientas de biología molecular, como la reacción en cadena de la polimerasa (PCR), han servido para el desarrollo del conocimiento de estas especies, por tanto, el uso de estas herramientas nos servirá para determinar cómo se modifican las poblaciones microbianas cuando están en presencia del antraceno.

HIPÓTESIS

1. La biodisponibilidad del antraceno disminuirá por la salinidad del suelo salino-alcalino del exlago de Texcoco.

2. La diversidad microbiana del suelo del exlago de Texcoco disminuirá debido a la presión selectiva que ejercerá el antraceno.

OBJETIVO GENERAL

Analizar el cambio de las comunidades microbianas en un suelo salino-alcalino del exlago de Texcoco en presencia de antraceno y determinar la biodisponibilidad del antraceno en suelos con diferente salinidad, durante una incubación de 56 días.

OBJETIVOS PARTICULARES

- Analizar la cinética de remoción del antraceno en el suelo del exlago de Texcoco.
- Evaluar el efecto de la adición de antraceno sobre las poblaciones microbianas en un suelo del exlago de Texcoco.
- Analizar el efecto de la adición de antraceno sobre la dinámica de nitrógeno en el suelo del exlago de Texcoco.
- Determinar el antraceno biodisponible en cuatro suelos del exlago de Texcoco mediante dos técnicas no exhaustivas de extracción.

MATERIALES Y MÉTODOS

Con el objetivo de evitar la repetición de información, los materiales y métodos se mencionan en el apartado correspondiente de cada artículo. La estrategia experimental del proyecto doctoral se muestra en un diagrama en la Figura 3.

Figura 3. Diagrama general del proyecto.

RESULTADOS Y DISCUSIÓN

Con el objetivo de evitar duplicar la información, los resultados y la discusión están en el apartado correspondiente en cada artículo.

Changes in the bacterial community structure in an alkaline saline soil spiked with anthracene

Running title: Bacteria in anthracene-amended soil

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Abstract

Background: The application of a PAH will affect the bacterial community structure as some groups will be favoured and others not. An alkaline saline soil with electrolytic conductivity (EC) 56 dS m^{-1} was spiked with anthracene and acetone while their effect on bacterial community structure was investigated.

Results: The percentage of Acidobacteria and Actinobacteria decreased over time, while the percentage of Proteobacteria, mostly Xanthomonadales, increased. The percentage of the phylotypes belonging to the *Nocardioides*, *Rhodococcus* and *Streptomyces*, known degraders of PAHs, was larger in the anthracene-amended soil than in the acetone-amended and unamended soil at day 14, but that of the phylotypes belonging to the genera *Sphingomonas*, also a known degrader of PAHs, was lower. Weighted and unweighted PCoA with UniFrac indicated that phylotypes were similar in the different treatments at day 0, but changed at day 1. After 14 days, phylotypes in the unamended and acetone-amended soil were similar, but different from those in the anthracene-spiked soil.

Conclusions: It was found that incubating the soil and contaminating it with anthracene changed the bacterial community structure, but spiking soil with acetone had little or no effect on the bacterial community structure compared to the unamended soil.

Keywords: C dynamics; N mineralization; phylogenetic analysis; polycyclic aromatic hydrocarbons; principal component analysis; UniFrac.

INTRODUCTION

Mexico is an important petroleum producing country so contamination during extraction and transport occurs frequently (U.S. EIA, 2011, http://www.eia.gov/). In Mexico, 27 971 ton oil contaminated the environment due to leaks and spills in 2010 (http://www.pemex.com/informes/pdfs/anuario_estadistico_2010.pdf). Polycyclic aromatic hydrocarbons (PAHs) are important components of petroleum. They are resistant to degradation and have been listed as priority pollutants by both the US Environmental Protection Agency and European Union (Doyle *et al*. 2008). Consequently PAHs, such as anthracene, have often been used as a model in the study of factors controlling the removal of hydrocarbons from soil (Vázquez-Núñez *et al*. 2009; Zhang *et al*. 2011). Anthracene is a tricyclic aromatic hydrocarbon and it has been detected in fumes from vehicle exhaust, coal, coal tar, tobacco smoke and at hazardous waste sites. Humans exposed to anthracene experienced headaches, nausea, loss of appetite, inflammation or swelling of the stomach and intestines (ATSDR, 1990).

Microorganisms remove PAHs from soil and even complex compounds, such as anthracene with three benzene rings, are dissipated within weeks (Amezcua-Allieri *et al*. 2012). Numerous bacteria have been described that can degrade PAHs so they will be favoured (Jones *et al*. 2011), but contaminating soil will inhibit other groups (Wang *et al*. 2011). As such, changes in the bacterial community structure will be indicative of the effect of anthracene.

In studies with PAHs, a carrier or organic solvent (e.g. acetone) is used to contaminate the soil. Acetone is used as C substrate by soil microorganisms as evidenced by the increases in CO_2 (Vázquez-Núñez *et al*. 2009). Consequently, spiking a soil with acetone, might also affect the bacterial community structure.

An alkaline saline soil of the former lake Texcoco (Mexico) with electrolytic conductivity (EC) 56 dS m^{-1} and pH 9 was spiked with acetone, contaminated with anthracene dissolved in acetone or left unamended. The C and N dynamics, and anthracene concentrations were monitored in an aerobic incubation while the bacterial community structure was determined after 0, 1 and 14 days. The objective of this study was to investigate how incubation time, and acetone and anthracene affected the bacterial community structure in an alkaline saline soil.

MATERIALS AND METHODS

Site description and soil sampling

The sampling site is located in the former lake Texcoco in the valley of Mexico City. The soil was sampled at random by augering the 0-10 cm layer of two 0.5 ha plots. The soil from each plot was pooled so that two soil samples were obtained (n = 2). This field-based replication was maintained in the incubation study. The pH$_w$ in the sandy soil (clay 22 g kg^{-1}, silt 106 g kg^{-1} and sand 872 g kg^{-1}) was 9.0, electrolytic conductivity 56 dS m^{-1}, water holding capacity (WHC) 504 g kg^{-1} soil and organic carbon content 12.9 g kg^{-1} soil. Techniques used to characterize the soil are described in Fernández-Luqueño *et al.* (2008).

Treatments and aerobic incubation

Four different treatments were applied to the soil. First, 20 g sub-samples were amended with 2 mL acetone (acetone-amended soil). Second, 20 g sub-samples were spiked with anthracene dissolved in 2 mL acetone (anthracene-spiked soil). As such, 550 mg anthracene kg^{-1} was added. It was found that this concentration was sufficient to study dynamics of the contaminant in an aerobic incubation experiment. Third, 20 g sub-samples that were sterilized on three consecutive days were spiked with anthracene dissolved in 2 mL acetone under sterile conditions (anthracene-sterile soil). Fourth, 20 g sub-samples were left untreated (unamended soil). All soil samples were mixed and placed under vacuum in desiccator for 45 min to evaporate the acetone.

The aerobic incubation experiment was conducted in the same way as described in Fernández-Luqueño *et al.* (2008). Briefly, the soil was 5-mm sieved, adjusted to 40% WHC and incubated for 7 days. The earlier mentioned treatments were then applied and the soil incubated aerobically for 56 days, while CO$_2$ emissions, mineral N and anthracene concentrations were

monitored. After 0, 1, 3, 7, 14, 28 and 56 days, a sub-sample of 5 g soil was taken and stored at -80°C until DNA extraction.

Anthracene in the soil was determined using an exhaustive ultrasonic extraction method developed by Song *et al.* (1995). Details of the extraction technique and the setting for the gas chromatograph (GC) can be found in Contreras-Ramos *et al.* (2008).

DNA extraction and PCR amplification of bacterial rDNA genes

The DNA was directly extracted from soil. The technique used was based on the techniques described by Valenzuela-Encinas *et al.* (2008). Primers 46F (5´GCC TAA CAC ATG CAA GTC 3´) and 1540R (5´GGT TAC CTT GTT ACG ACT T 3´), were used for amplification of *ca.* 1500 bp-long 16S rRNA gene segments from the metagenomic DNA (Edwards *et al.* 1989; Yu and Morrison, 2004). The PCR was done using a Touchgene Gradient thermal cycler (Techne, Cambridge United Kingdom).

Cloning and sequencing PCR products

The 1500 bp-long segments were used to clone and construct 16S rRNA gene libraries. The TOPO TA cloning kit with the pCR® II-TOPO® vector (Invitrogen, Carlsbad, CA) was used to clone the PCR products. Details of the cloning procedure can be found in Valenzuela-Encinas *et al.* (2008). The 16S rDNA gene sequences were obtained with a 3730X DNA Analyzer (Applied Biosystems, Foster City, CA) using M13 primers at the LANGEBIO (Cinvestav, Mexico).

Phylogenetic and statistical analysis

A total 2966 of sequences were aligned using the NAST tool from Greengenes (26 April 2011, DeSantis *et al.* 2006) and chimeras were detected using Bellerophon v. 3.0 (Huber *et al.* 2004). The screened sequences (2898) were classified using the naïve Bayesian rRNA classifier from the Ribosomal Data Project (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Wang *et al.* 2007).

Reference sequences were obtained from the Ribosomal Database Project 10 website (http://rdp.cme.msu.edu/) using seqmatch (type and non-type strains, isolates, > 1200 bp, good quality) to construct the phylogenetic trees (Cole *et al*. 2009). Sequences were aligned using the NAST tool from Greengenes (DeSantis *et al*. 2006). Phylogenetic trees were constructed by Maximum Likelihood with the online program PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) (Guindon *et al*. 2010) using the general time reversible model (GTR) (Tavaré, 1986).

Rarefaction, richness and diversity indices were calculated using mothur (Schloss *et al*. 2009). The input files were in the form of distance matrices generated by using phylip program dnadist (Felsenstein, 1989). The Good's coverage of our libraries was calculated (Good, 1953). Operational taxonomic units (OTUs) for community analysis were 20%, 10%, 5%, 3% distance cut-off (Rosselló-Mora and Amann, 2001).

The effect of the different treatments on the different genera was analyzed by principal component analysis (PCA) using the orthogonal/varimax rotation. Data were transformed using the Hellinger transformation (Ramette, 2007).

Differences among microbial communities were also characterized using UniFrac (Lozupone and Knight, 2005). A neighbour-joining tree was constructed with a maximum-likelihood approximation method using FastTree (Price *et al*. 2009) with a GTR substitution model (Tavaré, 1986). UniFrac and weighted UniFrac were used to determine the β-diversity of the different treatments at day 0, 1 and 14 (Lozupone *et al*. 2006). PCoA, abundance weighted Jackknife environment cluster analysis and lineage specific analysis were used to determine effects of treatment and time on the bacterial community structure. Lineage specific analysis to determine whether the sequences have a different distribution among environments was done with a branch length threshold 0.80 and minimum descendants of six. The abundance weighted Jackknife environment cluster analysis was done with 255 sequences and 1000 permutations.

Nucleotide sequence accession numbers

The sequences were deposited in the GenBank database and assigned the accession numbers JQ425854-JQ428819.

RESULTS

Dynamics of anthracene in soil

The anthracene concentration in the sterilized soil was not significantly different over time (Fig. 1). In the unsterilized soil, the anthracene concentration dropped sharply within 14 days, but changes were small thereafter. After 14 days, 64% of the anthracene was removed from soil and 65% after 56 days.

The CO_2 emission immediately increased when soil was amended with acetone or anthracene compared to the unamended soil (Fig. 2a). After 56 days, the CO_2 emission increased 7.5 times when soil was amended with acetone compared to the unamended soil, but only 3.1 times when spiked with anthracene.

The NH_4^+ and NO_2^- concentrations were not affected by the application of acetone or anthracene (Fig. 2b, c). The NO_3^- concentration was significantly lower in the soil amended with acetone or anthracene than in the unamended soil (Fig. 2d).

Bacterial diversity and richness analyses

Large amounts of anthracene were removed from soil after 1 day and no significant changes occurred after 14 days. Consequently, DNA was extracted from soil 0, 1 and 14 days after the anthracene was added.

A marked decrease in the rates of OTUs from rarefaction curves was observed only at the 10% and 20% cut-off, but not at the level of genus (5% cut-off) or species (3% cut-off) (No data shown). This indicated that at the order and phylum level, the major bacterial groups were detected, but not at the level of genus or species.

The Chao1 richness of the three treatments showed different values (from 148 to 673 OTUs), but was generally highest in the acetone-amended soil (from 409 to 673 OTUs) (Table 1). The Shannon index (H') ranged from 3.23 to 4.47 in the unamended soil, from 3.32 to 4.51 in acetone-amended soil, and from 3.05 to 4.33 in anthracene-spiked soil. The Simpson index (D) in all the studied

bacterial communities ranged from 0.02 to 0.16. The lower *D* values were founded at day 0 and the higher ones at day 1 (except in the acetone-amended soil).

Bacterial community structure

Eleven different phyla were found in the different treatments (Table 2). Bacteria belonging to the Proteobacteria were the most abundant. Their abundance increased in the unamended and acetone-amended soils over time, but not in the anthracene-spiked soil (Table 2). The abundance of the Acidobacteria (contributing between 4.7% and 14.5%) decreased over time in all treatments, while the abundance of the Actinobacteria (contributing between 1.2% and 17.3%) decreased in the unamended and acetone-amended soils, but increased in the anthracene-spiked soil. Of the other phyla detected, only phylotypes belonging to Chloroflexi were found in all soil samples.

The class of the Gammaproteobacteria was the most abundant in all treatments at all sampling times and the Alphaproteobacteria the second most abundant (Table 2). The abundance of the Alphaproteobacteria was larger in the unamended and acetone-amended treatments at day 14 than at day 0, but the opposite was found in the anthracene-spiked soil. The Acidobacteria-Gp6 was the third most abundant class of bacteria, but the abundance decreased over time in all treatments.

Phylotypes belonging to the order of Xanthomonadales (Gammaproteobacteria) were the most abundant. They often represented > 50% of all phylotypes with a maximum of 58.3% in the anthracene-spiked soil at day 1 (Table 2). Sphingomonadales (Alphaproteobacteria) were the second most abundant order. They were more abundant in the unamended and acetone-amended treatments at day 14 than at day 0, but the opposite was found in the anthracene-spiked soil.

Phylotypes belonging to the genus *Lysobacter* (Xanthomonadales, Gammaproteobacteria) were the most abundant in each of the treatments at each of the sampling times (Table 3). They represented 31.26% of all the sequences analyzed (2898). The second most abundant group (12.46%) was the genus *Sphingomonas* (Sphingomonadales, Alphaproteobacteria). Only four more genera of the 87 found, represented ≥ 1% of all phylotypes (*Altererythrobacter* (1.69%), *Steroidobacter* (1.59%), *Gemmatimonas* (1.59%) and *Iamia* (1.00%)), while 38.13% of the phylotypes could not be assigned to a genus.

The abundance of the genus *Iamia* was higher in the acetone and anthracene-amended soil at day 0 and day 1 compared to the unamended soil, but was similar at day 14 (Table 3). The abundance of the genera *Nocardioides*, *Rhodococcus* and *Streptomyces*, known degraders of PAHs, was larger in the anthracene-spiked soil than in the acetone-amended and unamended treatments at day 14. The abundance of the genus *Sphingomonas* was lower in the anthracene-spiked soil than in the acetone-amended and unamended treatments at day 14.

Principle component analysis

At day 0, the different treatments showed a large difference in the PC1 value, but not in PC2 (Fig. 3). PC1 was loaded mostly by the genera *Acidovorax*, *Bacillariophyta*, *Catellatospora*, *Cellulomonas*, *Corallococcus*, *Enterobacter*, *Ignavibacterium*, *Porticoccus*, *Pseudofulvimonas*, *Skermanella* and *Smaragdicoccus*. After 1 and 14 days, PC1 and PC2 were similar and negative in all treatments, except for the anthracene-amended soil after 14 days, which was characterized by a large positive PC2.

The PCoA based on the absence or presence of sequences (qualitative β-diversity) gave a different picture of the effect of treatment and time on the bacterial community structure (Fig. 4). The different treatments at time zero were grouped together in the lower left quadrant and had similar negative values for PC1 and PC2. At day 1 and day 14, the different treatments were grouped together and were characterized by a positive PC1 and a small negative PC2, except for the acetone spiked soil at day 1 characterized by a negative PC1 and the anthracene-contaminated soil at day 14 characterized by a large negative PC1 and large positive PC2. The PCoA based on the abundance of sequences (quantitative β-diversity) showed a similar effect of treatment and time on the bacterial community structure as the qualitative β-diversity (Fig. 5).

Lineage specific analysis with UniFrac indicated that sequences belonging to the genera *Nocardioides* and *Marmoricola* were highly significant ($P < 0.001$) and those belonging to *Arthrobacter* were significant (*P*-value between 0.001 and 0.01) with branch length threshold of 0.8.

On the one hand, the unamended soil and anthracene-spiked soil clustered together with the acetone-amended soil at day 0 and day 1 (Fig. 6). On the

other hand the anthracene-spiked soil and the unamended soil at day one cluster together with the acetone-amended soil and the unamended soil at day 14. The anthracene-spiked soil lay in between those two clusters although more related to the first than the latter.

DISCUSSION

Removal of anthracene and emissions of CO_2

The amount of anthracene extractable with the exhaustive extraction technique appeared not to be affected by abiotic processes as the concentration of the contaminant did not change significantly over time in the anthracene-amended sterile soil. In this study approximately 65% of the anthracene was removed from the soil after 56 days. Lower amounts were reported by Fernández-Luqueño *et al.* (2008) as they found that 52% of the applied anthracene was removed from soil after 56 days. Differences in the amount of anthracene that are removed from soil depend on soil characteristics and the capacity of the soil microorganisms to degrade the pollutant (Silva *et al.* 2009).

It has been reported that acetone increases the emission of CO_2 from soil, while anthracene reduced it (Vázquez-Núñez *et al.* 2009). Acetone can liberate soil organic C or can serve itself as a C substrate for soil microorganisms thereby increasing emissions of CO_2. PAHs can be toxic for microorganisms thereby reducing emissions of CO_2, but not always (Silva *et al.* 2009).

Bacterial diversity and richness analyses

The highest values of *H'* (4.47, 4.51 and 4.33) and the lowest of *D* (0.02, 0.02 and 0.03) found at day 0 revealed that no immediate effect was detected in the bacterial community in terms of heterogeneity (*H'*) and evenness (*D*). Negative effects on the bacterial diversity of the treatments were observed after day 1. Lower evenness in the soil bacterial communities could be due to the dominancy of *Lysobacter*, *Sphingomonas* and Acidobacteria Gp6, which comprises 40%, 16% and 11% of the clones, respectively. Apparently, contaminating the soil and incubation conditions stimulated the proliferation of these three genera. A recovery in heterogeneity and evenness was found at day 14 in the unamended and anthracene-spiked soils. The highest values for *H'* at a genetic distance of 3% found in the acetone-amended soil confirmed that acetone had little or no effect on bacterial diversity.

Phylogenetic analysis of the bacterial population

Phylotypes belonging to the Proteobacteria were the most dominant in the different treatments. Phylotypes belonging to the Proteobacteria are often dominant in soil (Roesch *et al.* 2007). For instance, Martin *et al.* (2012) found that phylotypes belonging to the Proteobacteria were dominant (35-66%) in soil amended with phenanthrene and Wang *et al.* (2011) in an uncontaminated soil amended with anthracene (96.4%).

Gammaproteobacteria were the most abundant class of the Proteobacteria in this study ranging from 27.4 to 61.7%. Zhang *et al.* (2011) found similar results in municipal solid waste composting soil amended with anthracene after 15 days and Lors *et al.* (2010) reported that 73% of the sequences belonged to the Gammaproteobacteria in soil contaminated with PAHs. The order of the Xanthomonadales (20.4% to 54.9%) were the most dominant Gammaproteobacteria and included the most abundant genus, i.e. *Lysobacter* (9.77% to 43.8%). *Lysobacter* spp. are versatile. They are rhizospheric and chitinolytic free-living diazotroph (Someya *et al.* 2011), and have a high antagonistic potential against phytopathogens (Furnkranz *et al.* 2012). They have the capacity to degrade chlorophenols (2,4,6-trichlorophenol and pentachlorophenol) (Caliz *et al.* 2011) and hydrocarbons (Cervantes-Gonzalez *et al.* 2008).

Numerous genera belonging to the Gammaproteobacteria found in this study are known to have the capacity to degrade PAHs and anthracene [e.g. *Enterobacter* (Bautista *et al.* 2009), *Pseudoxanthomonas* (Yutthammo *et al.* 2010), *Pseudomonas* (Bautista *et al.* 2009; Yutthammo *et al.* 2010; Gonzalez *et al.* 2011; Jurelevicius *et al.* 2012) and *Stenotrophomonas* (Gonzalez *et al.* 2011)], but the percentage of phylotypes belonging to these genera did not increase in the anthracene-amended soil.

Phylotypes belonging to the Alphaproteobacteria were the second most abundant class of bacteria in soil, with the Sphingomonadales (5.3 ± 4.4% to 20.5 ± 18.6%) and Rhizobiales (2.9% to 7.5%) the most important orders. Numerous genera belonging to the Alphaproteobacteria are capable to metabolize PAHs or are associated with their degradation, e.g. all the genera detected in this soil belonging to the Caulobacterales (*Brevundimonas* (Phillips *et al.* 2008) and *Caulobacter* (Chang *et al.* 2007) and Sphingomonadales). However, the percentage of phylotypes belonging to the genus *Caulobacter* were not affected by the application of anthracene and the abundance of phylotypes belonging to the genus *Sphingomonas* even decreased, although they are know degraders of anthracene (Jurelevicius *et al.* 2012). It appears that in this soil anthracene inhibited them. The phylotypes belonging to the Betaproteobacteria (order Burkholderiales), i.e. the least abundant class of

Proteobacteria, were not affected in this study by the application of anthracene although all the genera detected are known to degrade PAHs [*Achromobacter* (Tiwari *et al.* 2010), *Acidovorax* (Jurelevicius *et al.* 2012), *Herbaspirillum* (Louvel *et al.* 2011), *Methylibium* (Zhang *et al.* 2012), *Polaromonas* (Jurelevicius *et al.* 2012) and *Variovorax* (Zhang *et al.* 2011)].

Acidobacteria were the second most important phylum in this study. Wang *et al.* (2011) found the same in a soil spiked with anthracene. Acidobacteria are characterized as versatile heterotrophs and among the most abundant bacteria in soil (Eichorst *et al.* 2011). However, the percentage of phylotypes belonging to the Acidobacteria decreased over time in all treatments. This might be due to changes in the soil organic matter as the easily decomposable fraction is mineralized. Sequences belonging to eight subgroups of the Acidobacteria were detected. Phylotypes belonging to Acidobacteria Gp6 were the most abundant while phylotypes belonging to the other groups were represented only sporadically. Only a few of the known Acidobacteria have been isolated until now, although they are so abundant in soil (George *et al.* 2011), and consequently little is known about their functionality.

Actinobacteria were the third most important phylum in this study. Their importance varies from study to study, independent of the contamination with PAHs. Roesch *et al.* (2007) using pyrosequencing found that phylotypes belonging to the Actinobacteria were the most abundant after Proteobacteria and Bacteroidetes in uncontaminated soils. Wang *et al.* (2011) found the major phylum was Actinobacteria in an uncontaminated soil spiked with anthracene, but they were not detected after 45 days. In contaminated soil amended with phenanthrene, however, they found that the percentage of Actinobacteria was low and varied between 0 and 3%. However, Ros *et al.* (2010) found that the percentage of Actinobacteria (50%) was similar to that of Proteobacteria in uncontaminated soil, but six times higher (67%) in soils contaminated with PAHs. The percentage of phylotypes belonging to the *Euzebya* (Euzebyales, Actinobacteria) was higher in all treatments at day 0 than day 14. Little is known about them, and only one species *Euzebya tangerina* was isolated from the sea cucumber *Holothuria edulis* (Kurahashi *et al.* 2010).

Application of acetone increased the emission of CO_2 7.5 times, but this increase in microbial activity had little effect on the soil bacterial community structure. However, one group of bacteria appeared to be affected by the application of acetone. The percentage of *Iamia* (Iamiaceae, Acidimicrobiales) increased when acetone was applied to soil (day 0 and 1), and although they have not been reported as acetone degraders, they appear to be favoured by acetone application to soil. Little is known about Iamiaceae, but the first known species *Iamia majanohamensis* was isolated from the abdominal epidermis of a sea cucumber *(Holothuria edulis)* (Kurahashi *et al.* 2009).

The percentage of phylotypes belonging to three genera of the Actinomycetales (Actinobacteria), i.e. *Nocardioides*, *Rhodococcus* and *Streptomyces*, known degraders of PAHs, increased in the anthracene-spiked soil at day 14 compared to day 0 and 1. These genera have been studied intensively and have been shown to metabolize PAHs or have genes that encode for enzymes involved in the degradation of PAHs (Cébron *et al.* 2008). The percentage of other known degraders of PAHs, such as *Microbacterium* (Cébron *et al.* 2011), however, did not increase in the anthracene-spiked soil at day 14.

Chloroflexi were found in all treatments. Ros *et al.* (2010) reported that phylotypes belonged to Chloroflexi made up 6.7% of bacterial population found in soil contaminated with PAHs. However, little direct evidence exist that they are capable of degrading PAHs. Gemmatimonadetes appeared in all libraries except one. They have been found in coal-tar-contaminated soil (Kumar and Khanna, 2010), but there is no evidence that they degrade PAHs.

Although in this study phylotypes belonging to the Bacteroidetes and Firmicutes made up <1%, they are sometimes well presented in soil. Petrić *et al.* (2011) found that phylotypes belonging to the Bacteroidetes were one of the three most abundant phyla in soils contaminated with PBCs. Species belonging to the genus *Bacillus* (e.g. *Bacillus pumilus* and *Bacillus subtilis*) are well known metabolisers of PAHs (Toledo *et al.* 2006). Additionally, many species belonging to the genus *Bacillus* are acetone tolerant (e.g. *Bacillus aquimaris*, Trivedi *et al.* 2011), but they were not favoured by the application of acetone to the soil in this study.

Phylotypes belonging to the Chlorobi, Cyanobacteria/Chloroplast, Nitrospira and TM7 represented on average < 1%. These phyla are often considered rare phyla (Militon *et al.* 2010). None of the reported genera belonging to the Chlorobi, Nitrospira and TM7 are known degraders of PAHs, but it has to be remembered that most of them have not yet been isolated and/or studied in detail. Bacteroidetes, TM7 and Cyanobacteria/Chloroplast have been found in municipal solid waste composting soil amended with anthracene (Zhang *et al.* 2011).

The PCA using weighted Hellinger transformed number of phylotypes belonging to the different genera indicated an immediate effect of spiking the soil with acetone. However, the UniFrac, weighted UniFrac and clustering the environments (Lozupone *et al.* 2006), which is more robust as it deals with sequences directly and their relationship, did not confirm this result, as the different treatments at day 0 were grouped together in the PCoA. The UniFrac

and weighted UniFrac also confirmed the fact that incubating the soil for even just one day had an effect on the bacterial population. This might have been due to handling the soil and the decomposition of organic material made available by mixing the soil. The effect on the bacterial community structure of applying acetone appeared to be retarded as after 1 day the acetone amended soil grouped with the different treatments at day 0 (UniFrac and weighted UniFrac), but after 14 days the unamended soil and the soil spiked with acetone were grouped together. The three analyses (PCA, unweighted PCoA and weighted PCoA (UniFrac)) and clustering the environments, confirmed the fact that contaminating the soil with anthracene had a profound effect on the bacterial community structure at day 14, i.e. anthracene increased the phylotypes belonging to the Actinomycetales.

CONCLUDING REMARKS

It is clear from the results obtained that mixing and incubating the soil, and application of anthracene had an effect on the soil bacterial structure, but a possible effect of acetone was less clear. Incubating the soil nearly halved the percentage of sequences belonging to the Acidobacteria after 14 days. The changes in the sequences belonging to the Acidobacteria was mostly due to a decrease in the sequences belonging to the Acidobacteria-Gp6 class. Application of acetone had little effect on the bacterial community structure in this soil, but the percentage of sequences belonging to the genus *Iamia* (Acidimicrobiales) increased at the onset of the incubation. Application of anthracene increased the percentage of Actinobacteria compared to the unamended soil or soil applied with acetone, while it reduced the percentage of Proteobacteria after 14 days. Changes within the Actinobacteria were mostly due to an increase in the percentage of sequences belonging to the genera *Nocardioides*, *Rhodococcus* and *Streptomyces* (Actinomycetales), all known degraders of PAHs. Increases in the percentages of sequences belonging to the Proteobacteria in the unamended and acetone-amended soil were mostly due to increases in sequences belonging to the genus *Lysobacter* (Xanthomonadales, Gammaproteobacteria), while the decrease in the anthracene-spiked soil was mostly due to a decrease in phylotypes belonging to the Rhizobiales (Alphaproteobacteria) and the genus *Sphingomonas* (Sphingomonadales, Alphaproteobacteria).

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Legends to the figures

Fig. 1 Concentration of anthracene (mg kg^{-1} dry soil) in unsterilized (■) or sterilized soil (□) incubated aerobically at $22 \pm 2^{o}C$ for 56 days. Bars are ± one standard deviation.

Fig. 2 a) Emission of CO_2 (mg C kg^{-1} dry soil) and concentration of b) NH_4^+, c) NO_2^- and d) NO_3^- (mg N kg^{-1} dry soil) in unamended soil (■) or soil spiked with acetone (○) or anthracene (□) incubated aerobically at $22 \pm 2^{o}C$ for 56 days. Bars are ± one standard deviation.

Fig. 3 Principal component analysis with the different genera found in unamended soil (CON), in the aceton-amended soil (ACE) and spiked with anthracene (ANT) at day 0 (d0), day 1 (d1) and after 14 days (d14) of the aerobic incubation. Data were transformed using the Hellinger transformation before analysis (Ramette, 2007).

Fig. 4 Principal coordinate analysis using UniFrac with the different sequences found in unamended soil (CON), in the aceton-amended soil (ACE) and soil spiked with anthracene (ANT) at day 0 (d0), day 1 (d1) and after 14 days (d14) of the aerobic incubation.

Fig. 5 Principal coordinate analysis using weighted Unifrac with the different sequences found in unamended soil (CON), in the aceton-amended soil (ACE) and soil spiked with anthracene (ANT) at day 0 (d0), day 1 (d1) and after 14 days (d14) of the aerobic incubation.

Fig. 6 Jackknife cluster analysis of sequences of the unamended soil (CON), acetone-amended soil (ACE) and soil spiked with anthracene (ANT) at day 0 (d0), day 1 (d1) and after 14 days (d14) of the aerobic incubation. Supported values are show in the branch. The scale bar represents the UniFrac distance over all sites analyzed.

Fig. 1

Concentration of anthracene

Fig. 2

a) Emission of CO_2

b) Concentration of NH_4^+

c) Concentration of NO_2^-

d) Concentration of NO_3^-

Time (days)

53

Fig. 3

Fig. 5
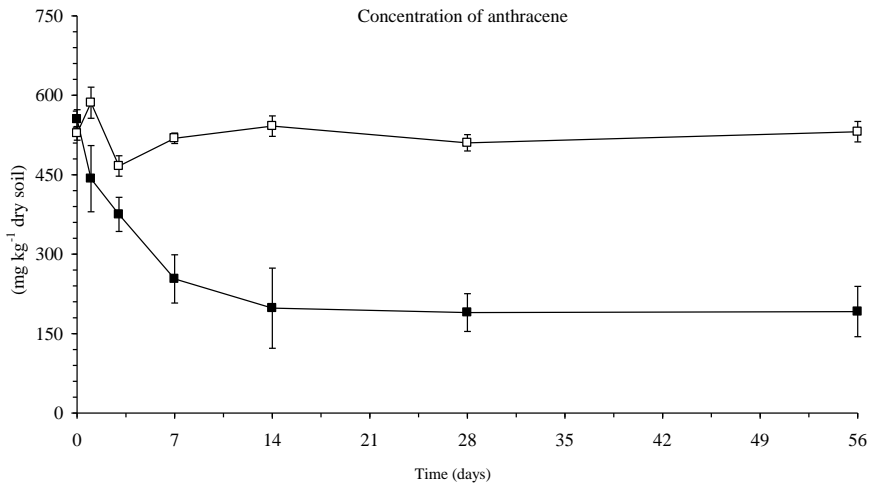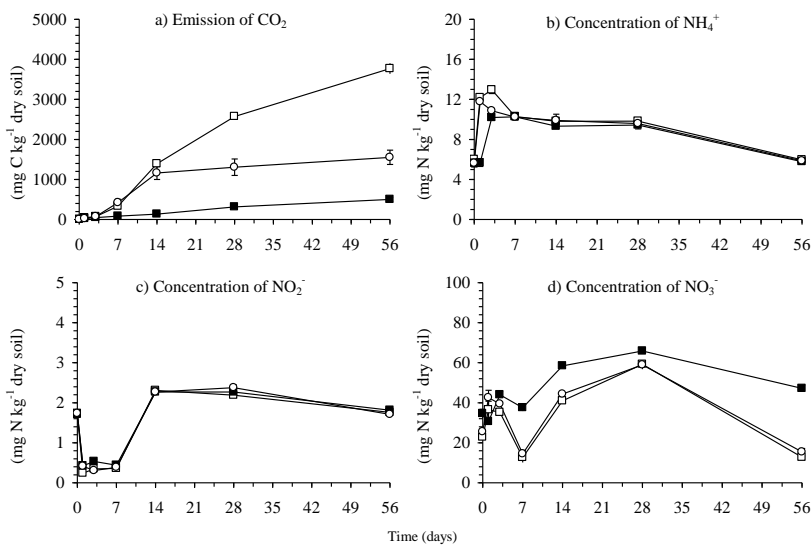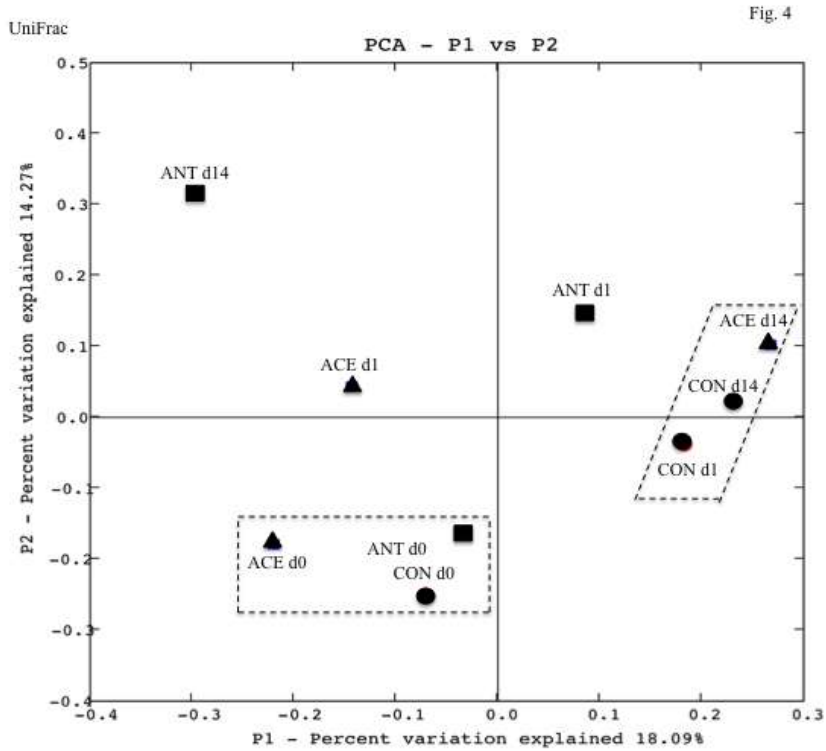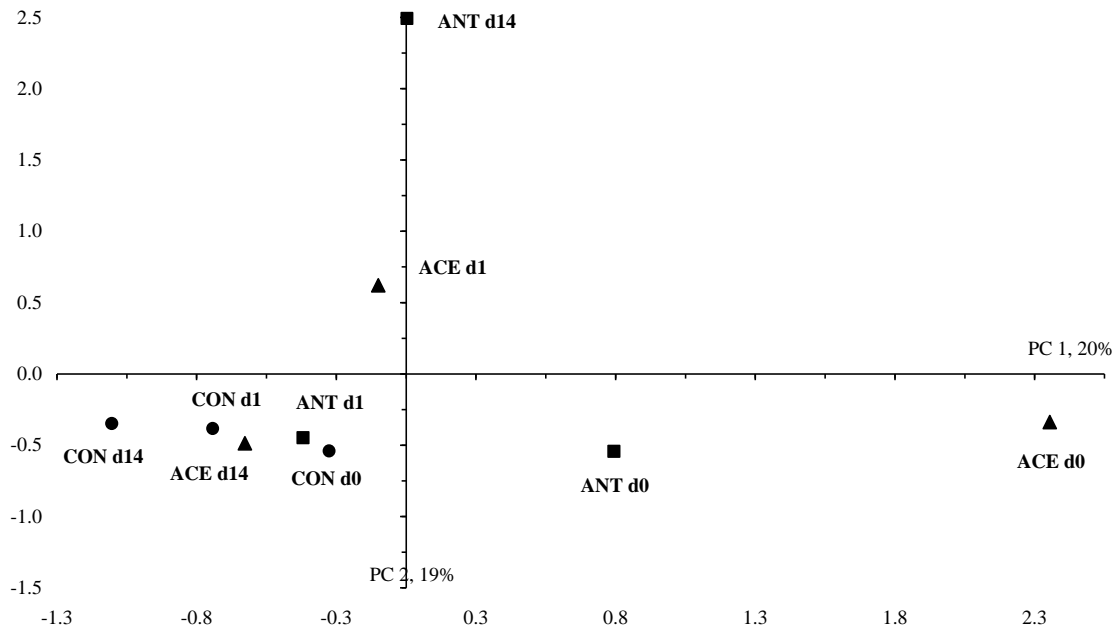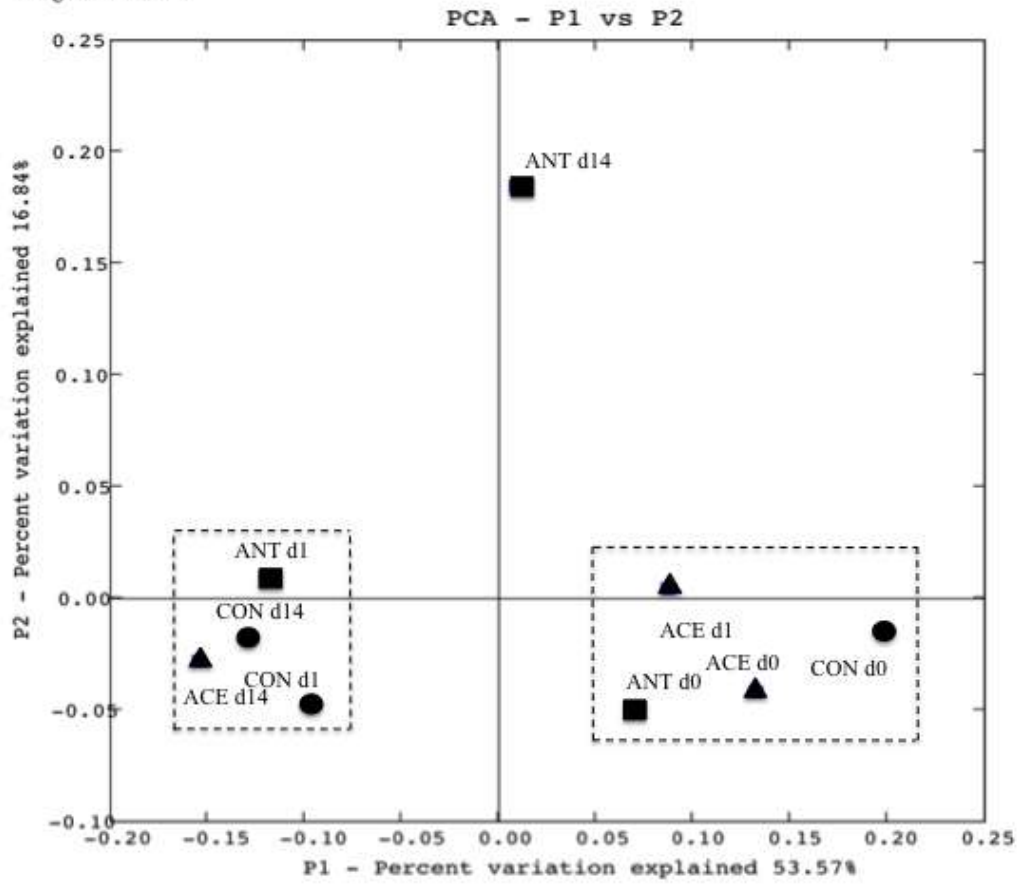
Fig. 6
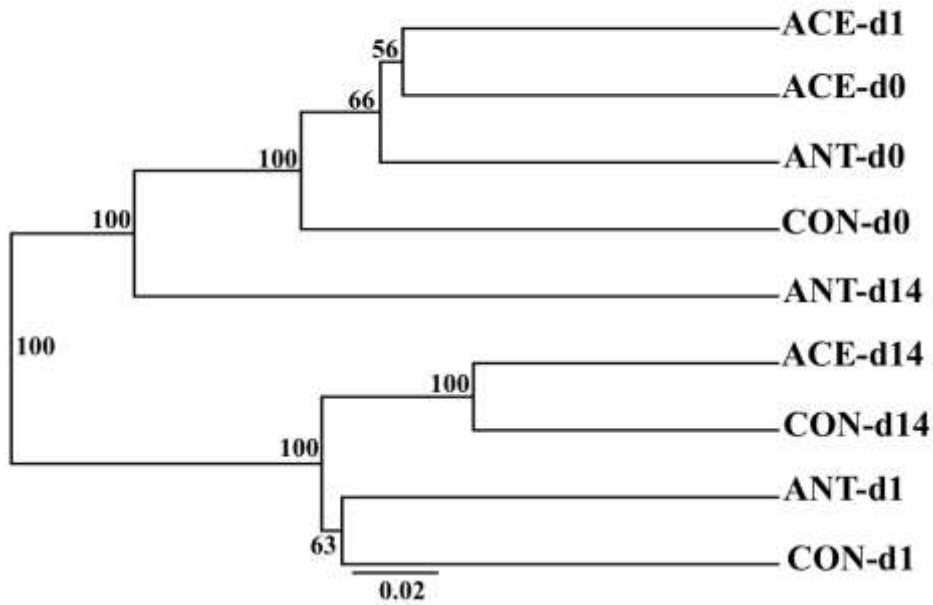
Table 1. Comparison of microbial richness and diversity of 16S rRNA gene libraries in the unamended soil, soil amended with acetone or spiked with anthracene as calculated with mothur (Schloss *et al*. 2009) after 0, 1 or 14 days days.

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Treatment	Time (days)	Number of sequences obtained	Number of different OTUs[a]	Richness estimator		Diversity index	
				Ace[b]	Chao1[c]	H'[d]	D[e]
Unamended soil	0	255	123	165 (158, 174) [f]	148 (135, 172)	4.47 (4.34, 4.61)	0.02 (0.01, 0.02)
	1	321	89	156 (124, 219)	154 (120, 225)	3.23 (3.02, 3.44)	0.14 (0.10, 0.18)
	14	284	89	333 (255, 450)	200 (144, 311)	3.31 (3.10, 3.51)	0.10 (0.08, 0.13)
Acetone amended	0	275	156	1103 (875, 1403)	673 (441, 1092)	4.51 (4.35, 4.67)	0.02 (0.01, 0.03)
	1	337	157	409 (314, 563)	411 (305, 595)	4.24 (4.06, 4.42)	0.05 (0.03, 0.07)
	14	426	109	514 (415, 646)	264 (189, 411)	3.32 (3.15, 3.50)	0.11 (0.08, 0.13)
Anthracene spiked	0	301	146	365 (280, 502)	356 (266, 514)	4.33 (4.16, 4.50)	0.03 (0.02, 0.05)
	1	308	94	262 (183, 411)	213 (155, 326)	3.05 (2.83, 3.28)	0.16 (0.12, 0.19)
	14	391	146	715 (580, 892)	365 (272, 528)	3.86 (3.68, 4.04)	0.07 (0.05, 0.09)

———

[a] OTUs defined by using the furthest-neighbor algorithm in mothur at 97% similarity.

[b] Ace, abundance based coverage estimator.

[c] Chao1, bias corrected Chao1.

[d] H': Shannon-Weaver diversity index.

[e] D: Simpson diversity index.

[f] Confidence intervals (95%) are given between parenthesis.

Table 2. Percentage of identified clones belonging different phyla, classes and orders based on the ribosomal data project in soil of the former lake Texcoco (CONTROL) amended with acetone (ACETONE) or spiked with anthracene (ANTHRA) incubated aerobically for 14 days.

Phylum
  Class
    Order
	CONTROL			ACETONE			ANTHRACENE		

	Day 0	Day 1	Day 14	Day 0	Day 1	Day 14	Day 0	Day 1	Day 14
Acidobacteria	13.8 (6.0) [a]	9.7 (6.8)	5.2 (0.2)	12.4 (0.5)	13.9 (1.5)	4.5 (3.3)	15.0 (5.5)	7.1 (8.0)	6.1 (0.4)
Acidobacteria-Gp1	0.3 (0.5)	0	0	0.4 (0.6)	0.3 (0.4)	0	2.3 (3.3)	0	1.0 (0.1)
Acidobacteria-Gp3	0	0	0	0	0	0	0.2 (0.4)	0	0.3 (0.4)
Acidobacteria-Gp4	1.9 (0.2)	0.6 (0.8)	0	0	0.6 (0.9)	0	0	0.6 (0.8)	0
Acidobacteria-Gp5	0	0.4 (8.8)	0	0.4 (0.6)	0	0	0.2 (0.4)	0	0
Acidobacteria-Gp6	10.2 (4.7)	8.8 (5.6)	4.9 (0.2)	10.5 (0.5)	12.6 (0.5)	3.6 (1.9)	12.2 (8.1)	6.3 (6.8)	4.3 (0.1)
Acidobacteria-Gp10	0	0	0	0.4 (0.6)	0	0	0	0	0
Acidobacteria-Gp17	1.4 (1.9)	0	0	0.6 (0.9)	0.3 (0.5)	0.7 (1.0)	0	0.3 (0.4)	0.5 (0.7)
Acidobacteria-Gp21	0	0	0.3 (0.5)	0	0	0	0	0	0
Actinobacteria	10.0 (5.2)	1.2 (0.7)	2.5 (0.6)	8.9 (1.8)	6.0 (3.4)	2.3 (0.5)	5.5 (6.8)	8.0 (2.8)	17.1 (19.3)
Actinobacteria	10.0 (5.2)	1.2 (0.7)	2.5 (0.6)	8.9 (1.8)	6.0 (3.4)	2.3 (0.5)	5.5 (6.8)	8.0 (2.8)	17.1 (19.3)
Acidimicrobiales	0.8 (0.2)	0.9 (0.3)	0.7 (1.0)	3.7 (4.1)	2.5 (0.8)	1.4 (0.1)	3.0 (2.9)	2.8 (3.2)	2.0 (0.1)

Actinomycetales	5.1 (7.3)	0.3 (0.4)	1.1 (1.5)	3.1 (2.7)	2.3 (3.2)	0.2 (0.3)	1.2 (1.0)	0	13.5 (19.1)
Euzebyales	3.3 (1.7)	0	0.3 (0.5)	1.6 (1.1)	1.0 (1.4)	0.7 (0.3)	2.4 (0.6)	0	0.5 (0.7)
Nitriliruptorales	0.3 (0.5)	0	0	0.4 (0.6)	0	0	0.2 (0.4)	0	0
Solirubrobacterales	0	0	0.4 (0.5)	0	0.3 (0.4)	0	0	0	0.7 (1.0)
Bacteroidetes	0.5 (0.6)	0	0	0.8 (1.2)	1.9 (1.9)	0	0	0.3 (0.4)	0.5 (0.7)
Flavobacteria	0	0	0	0	0	0	0	0.3 (0.4)	0
Flavobacteriales	0	0	0	0	0	0	0	0.3 (0.4)	0
Sphingobacteria	0.5 (0.6)	0	0	0.8 (1.2)	1.9 (1.9)	0	0	0	0.5 (0.7)
Sphingobacteriales	0.5 (0.6)	0	0	0.8 (1.2)	1.9 (1.9)	0	0	0	0.5 (0.7)
Chlorobi	0	0	0.4 (0.5)	1.2 (1.7)	0	0	0	0	0.4 (0.5)
Ignavibacteria	0	0	0.4 (0.5)	1.2 (1.7)	0	0	0	0	0.4 (0.5)
Ignavibacteriales	0	0	0.4 (0.5)	1.2 (1.7)	0	0	0	0	0.4 (0.5)
Chloroflexi	5.0 (4.5)	0.3 (0.4)	1.0 (0.4)	3.2 (0.2)	4.1 (4.3)	1.4 (0.5)	1.7 (1.1)	1.6 (0.2)	2.8 (0.5)
Anaerolineae	2.2 (1.8)	0.3 (0.4)	1.0 (0.4)	2.8 (0.3)	2.5 (2.9)	0.7 (0.3)	0.2 (0.4)	1.3 (0.2)	1.5 (0.1)
Anaerolineales	2.2 (1.8)	0.3 (0.4)	1.0 (0.4)	2.8 (0.3)	2.5 (2.9)	0.7 (0.3)	0.2 (0.4)	1.3 (0.2)	1.5 (0.1)
Caldilineae	1.0 (1.5)	0	0	0	0.3 (0.4)	0.2 (0.3)	1.0 (1.4)	0.3 (0.4)	0.5 (0.7)
Caldilineales	1.0 (1.5)	0	0	0	0.3 (0.4)	0.2 (0.3)	1.0 (1.4)	0.3 (0.4)	0.5 (0.7)
Unclassified	1.8 (1.3)	0	0	0.4 (0.6)	1.3 (1.8)	0.5 (0.1)	0.5 (0.7)	0	0.8 (0.3)
Cyanobacteria/ Chloroplast	0	0	0	0.4 (0.6)	0	0	0	0	0

Chloroplast	0	0	0	0.4 (0.6)	0	0	0	0	0
Firmicutes	0	0	0	0	0.6 (0.9)	0	0	0	0
Bacilli	0	0	0	0	0.3 (0.5)	0	0	0	0
Bacillales	0	0	0	0	0.3 (0.5)	0	0	0	0
Clostridia	0	0	0	0	0.3 (0.5)	0	0	0	0
Gemmatimonadetes	4.2 (0.8)	0.6 (0.8)	0	1.7 (0.1)	2.1 (1.6)	0.2 (0.3)	1.4 (0.6)	1.1 (1.5)	1.3 (0.4)
Gemmatimonadetes	4.2 (0.8)	0.6 (0.8)	0	1.7 (0.1)	2.1 (1.6)	0.2 (0.3)	1.4 (0.6)	1.1 (1.5)	1.3 (0.4)
Gemmatimonadales	4.2 (0.8)	0.6 (0.8)	0	1.7 (0.1)	2.1 (1.6)	0.2 (0.3)	1.4 (0.6)	1.1 (1.5)	1.3 (0.4)
Nitrospira	0.9 (1.3)	0	0	0	0	0	0	0	0
Nitrospira	0.9 (1.3)	0	0	0	0	0	0	0	0
Nitrospirales	0.9 (1.3)	0	0	0	0	0	0	0	0
Proteobacteria	60.3 (3.9)	86.5 (9.2)	89.2 (0.9)	66.5 (4.6)	66.2 (13.7)	91.1 (2.7)	71.3 (7.9)	84.1(1.9)	66.5 (25.3)
Alphaproteobacteria	25.4 (4.9)	25.0 (0.0)	29.2 (5.5)	28.4 (0.2)	21.9 (2.0)	33.7 (6.5)	28.2 (3.3)	20.4 (0.2)	14.8 (1.6)
Caulobacterales	0.3 (0.5)	1.0 (0.6)	1.0 (1.4)	1.0 (0.3)	0.8 (0.3)	0.5 (0.7)	1.0 (0.1)	1.3 (0.2)	0.5 (0.7)
Rhizobiales	5.7 (7.2)	5.4 (1.8)	4.2 (1.8)	5.6 (2.8)	3.7 (0.6)	7.5 (2.6)	7.0 (2.1)	4.0 (2.5)	2.9 (2.7)
Rhodobacterales	0.7 (1.0)	0.6 (0.1)	0.4 (0.5)	1.4 (0.3)	0.6 (0.9)	0.9 (0.6)	1.6 (1.6)	0.4 (0.5)	0.3 (0.4)
Rhodospirillales	1.8 (2.5)	0.3 (0.4)	0.4 (0.5)	0.7 (0.2)	0.3 (0.5)	0	0.5 (0.7)	0	0
Sphingomonadales	14.0 (4.5)	17.4 (1.0)	23.0 (3.8)	18.9 (3.0)	14.6 (0.4)	24.1 (9.0)	15.8 (2.6)	14.2 (2.7)	10.3 (3.4)
Unclassified	2.8 (1.1)	0.3 (0.5)	0.4 (0.5)	0.7 (0.2)	1.8 (1.1)	0.7 (0.3)	2.4 (0.6)	0.6 (0.8)	0.8 (1.1)

Betaproteobacteria	1.7 (2.4)	0	0.4 (0.5)	1.6 (1.4)	2.5 (2.9)	0	0.2 (0.4)	0.6 (0.1)	2.6 (3.0)
Burkholderiales	1.7 (2.4)	0	0.4 (0.5)	0.7 (0.2)	1.3 (1.8)	0	0	0	0.5 (0.7)
Unclassified	0	0	0	0.8 (1.2)	1.2 (1.0)	0	0.2 (0.4)	0.6 (0.1)	2.1 (2.3)
Deltaproteobacteria	5.8 (0.5)	3.0 (2.7)	3.4 (2.8)	2.6 (1.1)	2.9 (0.5)	0.5 (0.7)	3.2 (1.9)	1.4 (2.0)	2.6 (1.6)
Myxococcales	4.0 (0.8)	1.3 (1.1)	0.7 (1.0)	2.3 (1.5)	2.3 (0.4)	0	2.2 (0.4)	0.6 (0.8)	2.1 (1.6)
Syntrophobacterales	0	0	0	0.3 (0.5)	0	0	0	0	0
Unclassified	1.8 (1.3)	1.7 (1.6)	2.7 (3.8)	0.3 (0.4)	0.3 (0.4)	0.5 (0.7)	1.0 (1.4)	0.8 (1.2)	0.5 (0.1)
Gammaproteobacteria	27.4 (2.0)	58.3 (7.0)	55.7 (8.2)	33.9 (2.0)	38.8 (19.1)	56.7 (2.7)	39.6 (6.8)	61.7 (4.0)	46.3 (31.1)
Alteromonadales	0	0.6 (0.8)	0	0.4 (0.6)	0	0	0	1.1 (1.5)	0.2 (0.3)
Chromatiales	0	0	0.7 (0.1)	0	0.3 (0.4)	0.2 (0.3)	0	0	0
Enterobacteriales	0	0	0	1.7 (2.4)	0	0	0	0	0
Gammaproteobacteria-incertae-sedis	0.5 (0.6)	0	0	1.6 (1.4)	1.2 (1.0)	0	0	0	0
Legionellales	0	0	0.4 (0.5)	0.3 (0.4)	0.3 (0.4)	0	0	1.1 (1.5)	0
Oceanospirillales	0	0.3 (0.5)	0	1.3 (1.8)	1.0 (1.4)	0.2 (0.3)	1.2 (1.8)	0	0
Pseudomonadales	3.2 (0.6)	1.7 (1.6)	0.7 (1.0)	2.1 (3.0)	0.9 (0.6)	1.0 (0.7)	0.2 (0.4)	0	0.8 (0.4)
Xanthomonadales	20.4 (2.1)	54.9 (6.9)	52.9 (6.1)	21.8 (8.3)	31.8 (23.9)	54.8 (1.2)	34.5 (8.8)	58.4 (2.5)	42.5 (30.9)
Unclassified	3.4 (2.9)	0.8 (1.2)	1.1 (0.5)	4.7 (1.6)	3.4 (2.6)	0.5 (0.1)	3.6 (0.2)	1.1 (1.6)	2.8 (0.2)
Unclassified	0	0.3 (0.4)	0.4 (0.5)	0	0	0.2 (0.3)	0	0	0.2 (0.3)
TM7	0	0	0	0	0.3 (0.5)	0	0	0	0

Unclassified	5.3 (1.2)	1.8 (0.6)	1.7 (1.4)	4.9 (3.3)	4.8 (0.4)	0.5 (0.1)	3.8 (0.2)	3.0 (1.9)	5.2 (3.9)
Unclassified	7.2 (2.5)	2.1 (1.0)	2.1 (0.9)	5.3 (2.7)	6.5 (1.9)	1.2 (0.5)	4.3 (0.5)	3.0 (1.9)	6.4 (3.6)
Unclassified	29.4 (6.8)	14.6 (6.9)	11.4 (3.9)	24.7 (0.8)	27.3 (8.2)	7.3 (4.2)	26.5 (6.1)	13.3 (9.5)	19.0 (6.8)

[a] Values between parenthesis are standard deviation of the mean ($n = 2$).

Table 3. Abundance of clones belonging different genera based on the ribosomal data project that represent ≥ 0.1% of the total number of clones in the unamended soil of the former lake Texcoco (CONTROL) or in soil amended with acetone (ACETONE) or spiked with anthracene (ANTHRA) incubated aerobically for 14 days.

Genus	CONTROL Day 0	Day 1	Day 14	ACETONE Day 0	Day 1	Day 14	ANTHRACENE Day 0	Day 1	Day 14	Saline [a]	Acetone [b]	PAHs [c]
Lysobacter										*		*
Sphingomonas										*		*
Altererythrobacter										*		*
Steroidobacter												
Gemmatimonas										*		(*)
Iamia										*		
Euzebya										*		
Arthrobacter										*	*	*
Pseudomonas										*	*	*
Nocardioides										*		*
Rhodococcus										*	*	*
Pseudoxanthomonas										*		*
Brevundimonas										*		*
Luteimonas										*		
Hyphomicrobium										*		(*)
Halomonas										*		*
Caldilinea												
Mesorhizobium										*		*
Streptomyces											*	*
Arenimonas												(*)
Cellvibrio												
Novosphingobium										*		*

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Genus											Saline[a]	Acetone[b]	PAHs[c]
Azotobacter				▓							*		*
Pedomicrobium		▓											
Bauldia							▓						
Aciditerrimonas							▒						
Aquicella								▒					
Conexibacter													
Devosia		▓									*		
Gracilimonas					▒						*		
Ignavibacterium				▓									
Rhodobacter							▒				*	*	*
Agromyces			▒								*		(*)
Haliangium							▒				*		
Rhodoplanes					▒								
Stenotrophomonas					▒		▒				*		*
Aeromicrobium											*		*
Caulobacter													*
Methylohalomonas				▒							*		
Nitriliruptor											*		
Paracoccus											*	*	*
Pseudofulvimonas				▒									

■ >20%	■ >10%	▓ >5%	▒ >1%	░ >0.5%	□ <0.5%
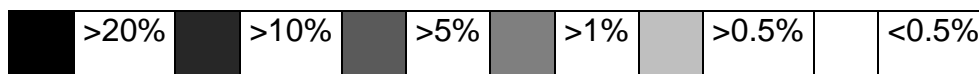
[a] * Bacteria encountered in saline environments.

[b] * Evidence exist that the bacteria belonging to this genus are capable of degrading acetone.

[c] * Evidence exist that the bacteria belonging to this genus are capable of degrading PAHs, (*) Bacteria isolated from hydrocarbon contaminated environment, but no direct evidence that they can degrade PAHs.

Bioavailability and dissipation of anthracene from soil with different alkalinity and salinity

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Abstract

Bioavailability of contaminants, such as anthracene (Anthra) a polycyclic aromatic hydrocarbon (PAHs), and their removal from soil has been related to their extractability with non-exhaustive techniques, such as hydroxypropyl-beta-cyclodextrin (HPCD) or *n*-butanol. Anthra was extracted with HPCD or *n*-butanol, and an exhaustive ultrasonic extraction method from sterilized and unsterilized soil of Texcoco with alkalinity ranging from pH 8.2 to 10.1 and electrolytic conductivity (EC) from 1.2 dS m^{-1} to 95.2 1.2 dS m^{-1} during an aerobic incubation of 56 days. Between 24.4 and 37.6% of the anthracene was removed biologically from soil as determined with the exhaustive technique after 56 days. The percentage of Anthra that was removed from soil as determined with the exhaustive technique was not related to the amount that was extractable with HPCD or *n*-butanol after 56 days. None of the soil characteristics was correlated to the percentage of Anthra removed from soil. However, water holding capacity and pH were negatively and highly significantly correlated with the percentage of Anthra extractable from soil with *n*-butanol or HPCD after 56 days. It was found that the Anthra extractable with *n*-butanol or HPCD did not correlate well with the removal of the contaminant from soil. The extractability of Anthra with *n*-butanol or HPCD was determined by soil characteristics, but not its removal from alkaline saline soil.

Keywords: CO_2 emissions; Exhaustive ultrasonic extraction method; hydroxypropyl-beta-cyclodextrin (HPCD); *n*-butanol; polycyclic aromatic hydrocarbon; soil characteristics

1. Introduction

Contamination of soil with polycyclic aromatic hydrocarbons (PAHs) occurs often and their removal is difficult and time consuming (Gan et al., 2009). Polycyclic aromatic hydrocarbons are produced naturally, such as in forest fires and volcanic eruptions, or the result of human activity, such as fossil fuel burning, the production of gas and coal tar and wood processing (Mumtaz and George, 1995). It is well known that soil microorganisms can degrade PAHs (Haritash and Kaushik, 2009). Both bacteria and fungi have been described that degrade PAHs, but the mineralization rate is often low. In general, the rate of degradation of PAHs decreases with increased number of aromatic rings (Jonsson et al., 2007), but is also affected by soil characteristics and that in two different ways (Chung and Alexander, 1998; Bogan and Sullivan, 2003). First, soil properties can inhibit microbial activity and as such the degradation of PAHs. For instance, high salinity is known to inhibit carbon and nitrogen mineralization and soil enzyme activities, which are crucial for the decomposition of organic matter (Tripathi et al., 2007, Ghollarata and Raiesi, 2007). This reduction in activity might reduce removal of PAHs from soil. Second, soil characteristics determine the bioavailability of contaminants in soil (Semple et al., 2003; Stokes et al., 2006). It is well known that contaminants can be physically immobilized as they diffuse into micropores of soil particles (Nam and Alexander, 1998) and can be fixed or react with soil organic matter (Chiou et al., 1983) rendering them unavailable for microbial degradation (Macleod and Semple, 2000).

Soil in the former lake Texcoco is an extreme alkaline saline with pH that can reach 10.5 and salinity often above 100 dS m^{-1} (Dendooven at al., 2010). Removal of PAHs from these soils occurred, but was highly variable and not related to the alkalinity or salinity of the soil or microbial activity as evidenced by emission of CO_2. It appeared that physical or chemical soil characteristics other than alkalinity or salinity, such as organic material, might have determined the bioavailability and removal of the PAHs from soil (Chung and Alexander, 2002; Bogan and Sullivan, 2003).

Different techniques have been used to define the bioavailability of PAHs in soil, such as extraction with organic solvents, which will control their removal (Gan et al., 2009; Gomez-Eyles et al., 2010). Two non-exhaustive extraction techniques, hydroxypropyl-beta-cyclodextrin (HPCD) (Reid et al., 2000) and *n*-butanol were used as solvent (Kelsey et al., 1997) to determine the bioavailability of an organic contaminant, i.e. anthracene, in four soils of Texcoco with varying pH and EC. *n*-Butanol was selected as the amount of PAHs extracted with it has been found to be related to the availability of PAHs for earthworms and bacteria (Kelsey et al., 1997; Liste and Alexander, 2002), while HPCD has been shown to predict the microbial degradation of a range of compounds in a range of soils (Swindell and Reid, 2006). An exhaustive ultrasonic extraction method based on the technique of

Song et al. (1995) was used to measure the removal of Anthra from these soils and related to the bioavailibity of the organic contaminant in soil as determined with *n*-butanol or HPCD. The objective of this study was to investigate the relationship between the removal of Anthra from soils with different salinity and alkalinity as determined with an exhaustive technique and its extractability with two non-exhaustive techniques.

2. Material and Methods

2.1. Chemicals used

Anthracene with purity > 98% and hydroxypropyl-β-cyclodextrin (HPCD molar substitution 0.8) were obtained from Sigma-Aldrich (USA). Acetone with purity > 99.7% and *n*-butanol with purity > 99.4% were from obtained J.T Baker (USA).

2.2. Site description and soil sampling

The sampling sites were located in the former lake Texcoco in the valley of Mexico City. Details of the sampling site can be found in Luna-Guido et al. (2000). Five 0.5 ha plots were defined at four sites with different electrolytic conductivity (EC). Soil with EC 1.2 dS m^{-1} was considered soil A, with EC 3.2 dS m^{-1} soil B, with EC 80.2 dS m^{-1} soil C and 95.2 dS m^{-1} as soil D (Table 1). Soil was sampled at random by augering the 0-15 cm top-layer of the five plots at each of the four sites. The soil from each plot was pooled so that five soil samples were obtained ($n = 5$) for each site. As such, 20 soil samples were obtained. This field based replication was maintained in the incubation study.

2.3. Soil preparation

The soil was taken to the laboratory and treated as follows. The soil from each site was passed separately through a five mm sieve and adjusted to 40% water holding capacity (WHC) by adding distilled water. The soil was conditioned at ambient room temperature (22 ± 2°C) in drums containing a beaker with sodium hydroxide (NaOH) to trap the carbon dioxide (CO_2) evolved and a beaker with 1 l water to avoid desiccation for a week.

Fourteen sub-samples of 20 g of soil from each plot ($n = 5$) and site with different EC ($n = 4$) were added to a 120 ml glass flask. Half of them (seven) were sterilized on three successive days. The sterilized and unsterilized soil samples

were amended with Anthra dissolved in 2 ml acetone under sterile conditions. As such, approximately 550 g Anthra kg^{-1} was added to soil. After spiking the soil with Anthra, all samples were placed under vacuum in desiccator for 45 min to evaporate the acetone.

The flasks were placed in 945 ml glass jars containing 10 ml distilled water to avoid desiccation of the soil and a vessel with 20 ml NaOH to trap the evolved CO_2. The jars were sealed and stores in the dark at 22 ± 2^oC. An additional 18 jars containing a vessel with 20 ml NaOH and 10 ml distilled water, but without soil, were sealed and served as control to account for the CO_2 trapped from the air. After 1, 3, 7, 14, 28 and 56 days a flask of each plot was chosen at random from each treatment and opened. The vessel with NaOH was removed and 1.5 g soil was weighted and extracted for Anthra with an exhaustive extraction procedure (Song et al., 1995), 1.5 g was weighted and extracted for Anthra with HPCD and 10 g was weighted and extracted for Anthra with *n*-butanol.

2.4. Soil chemical analysis and extraction of anthracene

Soil pH, total N, organic C and the soil particle size distribution were determined as described in Contreras-Ramos et al. (2006). Concentration of Anthra in the soil was analyzed using an exhaustive ultrasonic extraction method developed by Song et al. (1995) and described in Contreras-Ramos et al. (2006).

Concentration of Anthra was determined in the soil using a non-exhaustive extraction with HPCD (Reid et al., 2000) and *n*-butanol (Liste and Alexander, 2002). Briefly, 1.5 g sub-sample of soil was amended with 25 ml 60 mM HPCD, shaken in the dark for 20 h and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with 20 ml dichloromethane and shaken for 10 min. The HPCD was decanted and the solution centrifuged at 3000 rpm for 10 min. The remaining HPCD was discarded and the dichloromethane was evaporated at room temperature in the dark overnight. Two ml of acetone was added to the residue, mixed and analyzed for the Anthra. A solution of HPCD with a known concentration of Anthra was used to determine the amount of Anthra that was lost during the procedure at each sampling day. The amount of Anthra lost during the procedure was approximately 20%, so data reported were adjusted for these losses.

A 10 g sub-sample of soil was extracted with 15 ml *n*-butanol and shaken for 5 min. The soil *n*-butanol mixture was left to stand for 30 min and centrifuged at 3000 rpm for 10 min. The supernatant was decanted and evaporated in the dark at room temperature. Two ml acetone was added to the residue and analyzed for Anthra. A solution of *n*-butanol with a known concentration of Anthra was used to determine the amount of Anthra that was lost during the procedure at each sampling day. Although, the amount of Anthra lost during the procedure was < 2%, data reported were adjusted for these losses.

2.5. Statistical analysis

Cumulative production of CO_2 was regressed on elapsed time using a liner regression model which was forced to pass origin, but allowed different slopes (production rate) for each treatment. Concentrations of Anthra as determined with the different extraction techniques in the sterilized and unsterilized soil were subjected to one-way analysis of variance using PROC GLM (SAS Institute, 1989) to test for significant differences between the soils and the least significance difference was then calculated.

The relationships between the different soil properties, i.e. pH, EC, WHC, and sand, clay, loam and soil organic C content, CO_2 emission rate, and the Anthra extractable with the exhaustive technique and *n*-butanol or HPCD from the unsterilized soil after 56 days, were visualized by principal component analysis (PCA). Details of the PCA analysis can be found in Vásquez-Murrieta et al. (2006). Briefly, only principal components with Eigenvalues > 1 and that explained > 10% of the total variance were retained. The matrix of 20 columns (four soils each with five plots) and 11 lines (variables: WHC, pH, EC, organic C, sand clay and loam content, emission of CO_2 and the percentage of Anthra extracted from the unsterilized soil with the three extraction techniques at day 56) was used for PCA. All analyses were performed using the SAS statistical package (SAS, 1989).

3. Results

3.1. Concentration of anthracene in the sterilized soil

Between 92.4±20.1% and 100.5±6.1% of the 550 mg Anthra kg^{-1} added to the sterile soil was recovered with the exhaustive extraction technique just after contaminating the soil and was not significantly different between the soils (Table 2). The amount of Anthra extracted from the sterile soil with the exhaustive technique did not change significantly over time (Fig. 1a). After 56 days, between 90.2±15.0 and 105.5±16.6% of added Anhtra was extracted from the sterilized soil and was not significantly different between soils (Table 2).

The concentrations of Anthra extracted from the unsterilized soil with the exhaustive technique decreased sharply in soil C within the first day and showed little difference thereafter (Fig. 1b). In soil D, concentrations of Anthra decreased until day 7, but showed little difference thereafter. In soils A and B, the concentration of Anthra decreased only after day 28. After 56 days, between

62.4±8.3 and 75.6±14.1% of the added Anthra was removed from the unsterilized soils (Table 2).

The concentrations of Anthra extracted from the sterilized soil with *n*-butanol were similar for the different soils and did show the same pattern (Fig. 2a). The amount of Anthra extractable with *n*-butanol nearly halved within the first 14 days and showed little difference afterwards in soils A and C, but further decreased in soils B and D. The amount of Anthra extracted with *n*-butanol from the sterilized soil after 56 days ranged from 32.6±14.9% in soil A to 57.2±13.4% in soil C (Table 2).

The concentrations of Anthra extracted from the unsterilized soil with *n*-butanol more than halved between the onset of the experiment and day 7 (Fig. 2b). Subsequent decreases were small with the least found in soil A and the largest in soil B and D. At day 56, the amount of Anthra extracted with *n*-butanol from the unsterilized soil ranged from 18.4±5.5% in soil B to 50.3±13.2% in soil A and was significantly different between the soils ($P<0.05$) (Table 2).

The concentrations of Anthra extracted with HPCD were significantly different between the sterilized soils just after application and ranged from 20.4±3.6 to 73.6± 12.2% ($P<0.05$) (Table 2, Fig. 3a). After 56 days, the amounts of Anthra extracted with HPCD from the sterilized soils were significantly different and ranged from 11.1±4.5% in soil C to 33.3±9.5% in soil A ($P<0.05$).

The concentrations of Anthra extracted from the unsterilized soil with HPCD were < 160 mg Anthra kg^{-1} and significantly different between the soils ($P<0.05$) (Fig. 2c). After 56 days, the lowest amount of Anthra was extracted from soil C (2.0±4.0%) and the most from soil A (27.5±3.3%). The amount of Anthra extracted with HPCD was less than that extracted with *n*-butanol or with the exhaustive technique.

3.3. Correlations between soil characteristics and extraction of Anthra

None of the soil characteristics was correlated significantly with the percentage of Anthra extracted with the exhaustive technique from the sterile soil at day 0 and 56 and from the unsterilized soil at day 56 (Table 3). However, the percentage of Anthra extracted with *n*-butanol from the unsterilized soil after 56 days was negatively significantly correlated with pH, WHC, clay content and emission of CO_2 and positively with sand content ($P<0.05$). The percentage of Anthra extracted with HPCD from the unsterilized soil after 56 days was negatively significantly correlated with pH, WHC and emission of CO_2 ($P<0.05$).

The percentages of Anthra extracted with HPCD, *n*-butanol or the exhaustive technique from the sterilized soil after 0 and 56 days and from the

unsterilized soil after 56 days were not significantly correlated with each other (Table 4).

3.4. Principal component analysis

Loading parameters obtained after VARIMAX rotation are given in Table 5. A first PC (PC1) explained 51% of variation. PC1 had positive loading from pH, WHC, EC, and clay and sand content while a negative loading from organic C and sand content. The second PC (PC2) explained another 19% of variation. The PC2 had positive loading from pH, WHC, emission of CO_2 and a negative loading from Anthra extracted with HPCD and *n*-butanol after 56 days.

On the scatter plot (Fig. 4a, b), samples of the same soil were grouped together and visually distinct from samples of the other soils. Samples of soil A and B lie in the left quadrants as they have a negative PC1 (Fig. 4a, b). The soils were characterized by a low pH, EC, WHC and clay and loam content, and high sand and organic C content. Soil B was characterized by a positive PC2, i.e. emission of CO_2 was high and the percentage of Anthra extracted with HPCD and *n*-butanol was low, while soil A had a negative PC2, i.e. the percentage of extracted Anthra with HPCD and *n*-butanol was high (Fig. 4a). Samples of soil C lie in the right upper quadrant as they have a positive PC1 and PC2. The value of PC1 for soil C was between that of soils B and D, while the value for PC2 was similar to that of soil B. Samples of soil D were characterized by a large PC1 value, i.e. high pH, EC, WHC and clay and loam content, and low sand and organic C content, and generally a negative PC2.

4. Discussion

The exhaustive method as suggested by Song et al. (1995) is a robust technique as most of the Anthra added was extracted immediately after application (Table 2). Similar results were reported by Song et al. (2002). They found recoveries of 93% for Anthra from soil with 98% sand.

The amounts of Anthra extracted from the sterilized soil with the method of Song et al. (1995) showed only small changes over time. This indicated that only small amounts of Anthra were sequestered in soil and the effect of abiotic processes on the concentrations of the contaminant was negligible. Kottler and Alexander (2001) reported similar results. They found that $101.7\pm9.6\%$ of Anthracene was recovered with an exhaustive extraction technique (Soxthlet) from a sterilized soil shortly after contamination and $97.8\pm5.2\%$ after 28 days. Some authors reported an increased sequestration and a decreasing extractability of PAHs with aging of contaminated

soil (Nam and Alexander, 2001). Northcott and Jones (2000) found that extraction of BaP decreased 17% after 525 days aging while Johnson et al. (2002) found that the extractability of pyrene decreased 10.3% and benz[a]anthracene 22.7% after 240 days. Extractability and bioavailability of PAHs in soil decreases as the soil-PAH contact increases over time (Chaker, 2007). This phenomenon has been termed the 'ageing effect' (Hatzinger and Alexander, 1995; Johnson et al., 2002). The aging effect is determined mostly by soil characteristics and the length of conditioning (Chung and Alexander 2002; Ling et al., 2010). In the study reported here, the incubation time was shorter than in some experiments mentioned and this might have reduced the amount of Anthra sequestered in the Texcoco soil (Semple et al., 2003).

The amount of Anthra sequestered in the different soils was low so most of the Anthra was biologically removed from them. It is well known that soil microorganisms can remove hydrocarbons from soil and numerous bacteria and fungi have been reported that can degrade PAHs (Fernández-Luqueño et al., 2011). Biological removal of PAHs depends on its composition with the more aromatic rings the more resistant the compound is. Soil characteristics are also known to affect removal of Anthra from soil. It was hypothesized the high pH and the large salt content would inhibit biotic removal of Anthra from the Texcoco soil. However, no such effect was found in this experiment. The amount of Anthra biologically removed was similar in soils A and D, although the pH and EC was much higher in soil D than soil A.

Although the extraction of Anthra from soil with n-butanol is considered less exhaustive than the method as suggested by Song et al. (1995), nearly all the Anthra added to the four Texcoco soils was extractable immediately after application. Similar results were reported by Swindell and Reid (2006). After 1 day, nearly all of the added phenanthrene was extracted from the contaminated soil with n-butanol. However, Kottler and Alexander (2001) only recovered 64.7±1.0% of Anthra added to a sterilized soil.

The dynamics of Anthra extracted with n-butanol from the sterilized soil was different from the Anthra extracted with the exhaustive method. The concentration Anthra extracted with n-butanol from the sterile soil showed a sharp drop within the first days and after 56 days, approximately half of the Anthra added was not extractable from the sterile soil with n-butanol although nearly 100% was with the exhaustive technique. A mild extraction with n-butanol has been suggested as an appropriate way to determine the bioavailability of a hydrocarbon in soil (Kelsey et al., 1997; Liste and Alexander, 2002). The sharp drop in the amount of Anthra extractable with n-butanol within the first two weeks as found in the experiment reported here might thus be an indication of a drop in its bio-availability.

The amount of Anthra extracted from the sterilized soil with HPCD was low (Table 2). Swindell and Reid (2006) reported a near 100% recovery of phenanthrene 1 day after application in two soils with < 10% clay, but only a 53.8±17.8% recovery from soil with 20% clay. They suggested that organic matter and soil type influenced the amount of phenanthrene extracted using HPCD. Reid

et al. (2000) found that in a sandy soil the extractability was 89.1±1.2% while only 74.8±7.9% in a clay soil rich in organic matter. However, in the study reported here, no relationship with clay or organic matter content and extractability of Anthra with HPCD was found.

The ability of *n*-butanol extractability of Anthra to predict the removal of Anthra from soil as determined with the exhaustive technique was poor (Table 4). These results confirm the results obtained by Chung and Alexander (1998), Tang and Alexander (1999) and Bogan and Sullivan (2003). The latter stated that 'although *n*-butanol extraction under rapid, mild conditions may represent an excellent means for estimating PAH availability to earthworms, and possible other receptor macro-organisms (Tang and Alexander, 1999), it is considerably less useful for estimating bacterial biodegradability'. The ability of HPCD extractability of Anthra to predict its removal from soil as determined with the exhaustive technique was also poor (Table 4). As such, although the HPCD extraction of Anthra might represent an excellent means for estimating the microbial degradable fraction, it was less successful for estimating the amount of Anthra removed from soil.

None of the measured soil characteristics was correlated to biological removal of Anthra from soil as determined with the exhaustive technique (Table 3). However, soil organic matter has often been found to affect dissipation of PAHs from soil, but not in our experiment (Yang et al., 2010). It can be speculated that soil characteristics other than those measured defined the removal of Anthra from soil. Consequently, it might be difficult to determine which factor affected the dissipation of the organic contaminant from soil (Breedveld and Karlsen, 2000).

The percentages of Anthra extracted with *n*-butanol and HPCD from the unsterilized soil after 56 days, however, were highly significantly and negatively affected by pH, and WHC ($P<0.001$) (Table 3). As such, extractability of Anthra with *n*-butanol or HPCD reduced with increased WHC of the soils. The WHC of a soil is normally related to clay content, aggregate formation and soil organic matter content and those factors are known to affect the extractability of organic contaminants from soil. An increase in pH reduces microbial activity and this might have inhibited the removal of Anthra from soil as defined with HPCD and *n*-butanol.

It can be debated whether the Anthra that was not extractable with *n*-butanol or HPCD poses a treat to the environment. It can be speculated that the Anthra not extractable with *n*-butanol or HPCD was strongly physically and/or chemically protected. As such, the amount of Anthra extractable with *n*-butanol or HPCD might be a better indicator of the Anthra that might pose a treat to the environment than the amount extracted with an exhaustive technique. However, it was also clear from this study that some of the Anthra that was not extractable with *n*-butanol or HPCD became available for degradation and was removed from soil.

5. Conclusion

It was found that the removal of Anthra was most accentuated in the first days of the incubation. Most of the removal was due to biological degradation, as abiotic sequestration was low or non-existent. There was no correlation between the Anthra biological removed from soil and the amount that was available as defined with *n*-butanol or HPCD. None of the measured soil characteristics was correlated with the removal of Anthra from the alkaline saline soil of Texcoco. However, pH, water holding capacity and microbial activity affected the amount of Anthra extracted from soil with *n*-butanol or HPCD.

Acknowledgements

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Legends to the figures

Fig. 1. Amount of anthracene (mg kg^{-1} dry soil) extracted from a) sterilized or b) unsterilized soil with electrolytic conductivity 1.2 dS m^{-1} (□), 3.2 dS m^{-1} (■), 80.2 dS m^{-1} (●) or 95.2 dS m^{-1} (○) with an exhaustive technique (Song et al., 1995). Soil was incubated at 22±2 ^{o}C for 56 days. Bars are ± 1 standard deviation ($n = 5$).

Fig. 2. Amount of anthracene (mg kg^{-1} dry soil) extracted from a) sterilized or b) unsterilized soil with electrolytic conductivity 1.2 dS m^{-1} (□), 3.2 dS m^{-1} (■), 80.2 dS m^{-1} (●) or 95.2 dS m^{-1} (○) with n-butanol. Soil was incubated at 22±2 ^{o}C for 56 days. Bars are ± 1 standard deviation ($n = 5$).

Fig. 3. Amount of anthracene (mg kg^{-1} dry soil) extracted from a) sterilized or b) unsterilized soil with electrolytic conductivity 1.2 dS m^{-1} (□), 3.2 dS m^{-1} (■), 80.2 dS m^{-1} (●) or 95.2 dS m^{-1} (○) with hydroxypropyl-beta-cyclodextrin. Soil was incubated at 22±2 ^{o}C for 56 days. Bars are ± 1 standard deviation ($n = 5$).

Fig. 4. Principal component analysis (PCA) performed on soil characteristics (sand, clay, loam and organic C content, electrolytic conductivity (EC), water holding capacity (WHC), pH and emissions of CO_2) and extraction of anthracene with a exhaustive technique, n-butanol or hydroxypropyl-beta-cyclodextrin (HPCD) after a varimax rotation. Soil A with EC 1.2 dS m^{-1} (□), soil B with 3.2 dS m^{-1} (■), soil C with EC 80.2 dS m^{-1} (○) and soil D with EC 95.2 dS m^{-1} (●). The two axes explained 75% of the variation.

Fig. 1

a) Sterile soil

b) Unsterilized soil

A=1.2 dS m-1   B=3.2 dS m-1
C=80 dS m-1   D=95 dS m-1

Concentration of antharcene extracted with the exhaustive technique (mg kg^{-1} dry soil)

Fig. 2

a) Sterile soil

b) Unsterilized soil

A=1.2 dS m-1   B=3.2 dS m-1
C=80 dS m-1   D=95 dS m-1

Concentration of antharcene extracted with n-butanol (mg kg^{-1} dry soil)

78
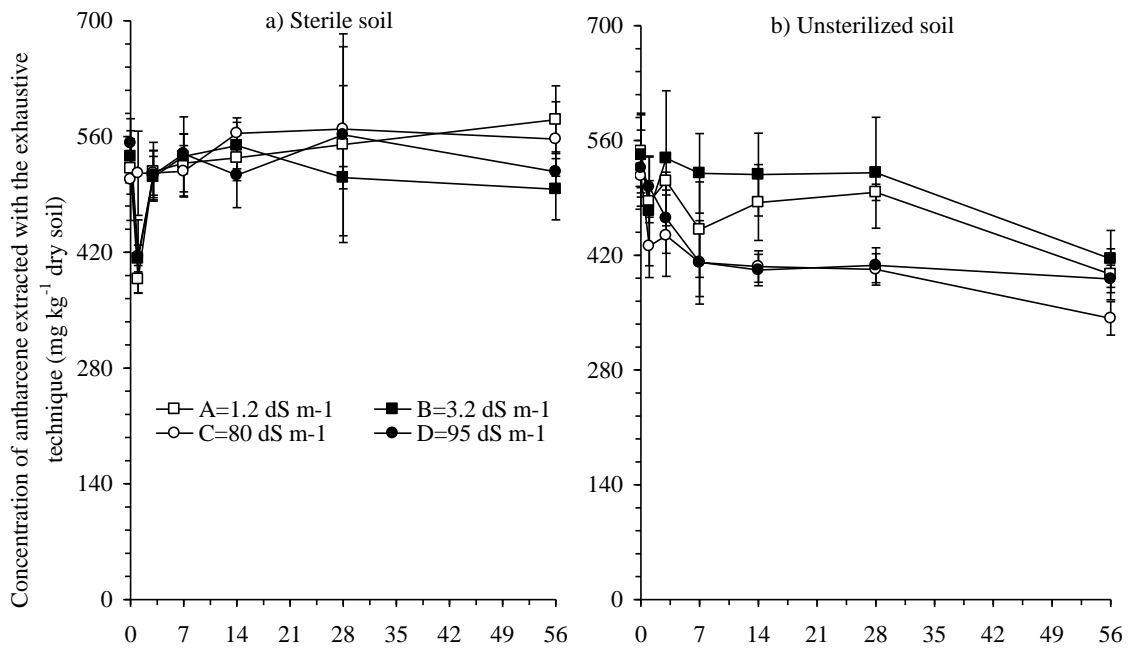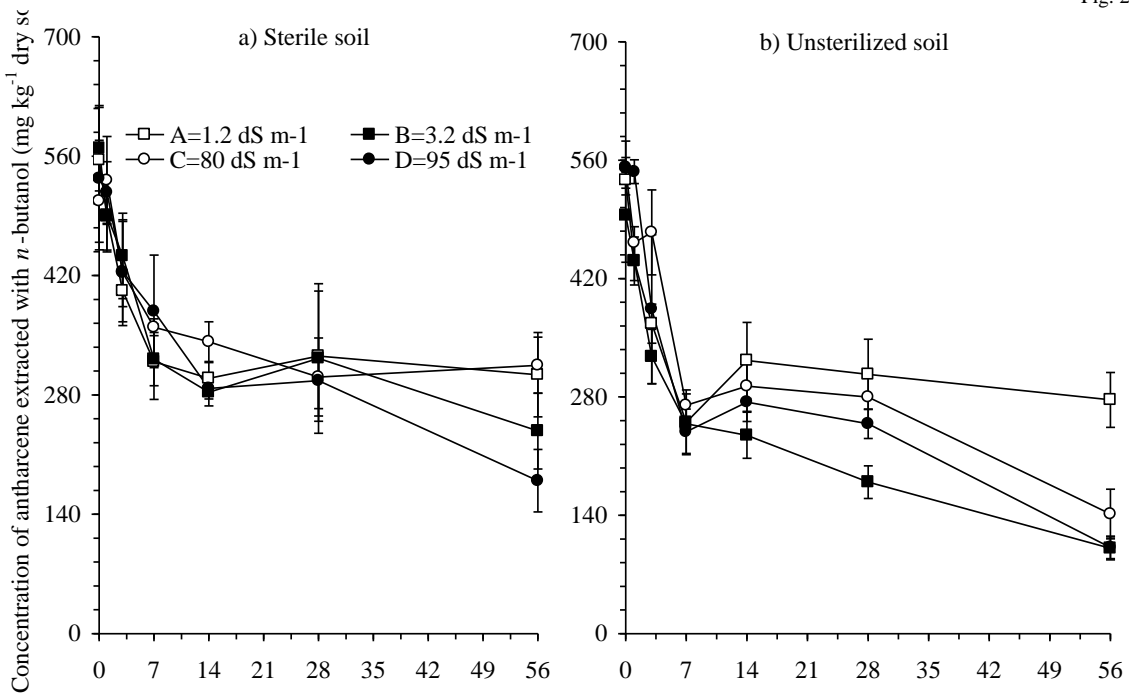
Fig. 3
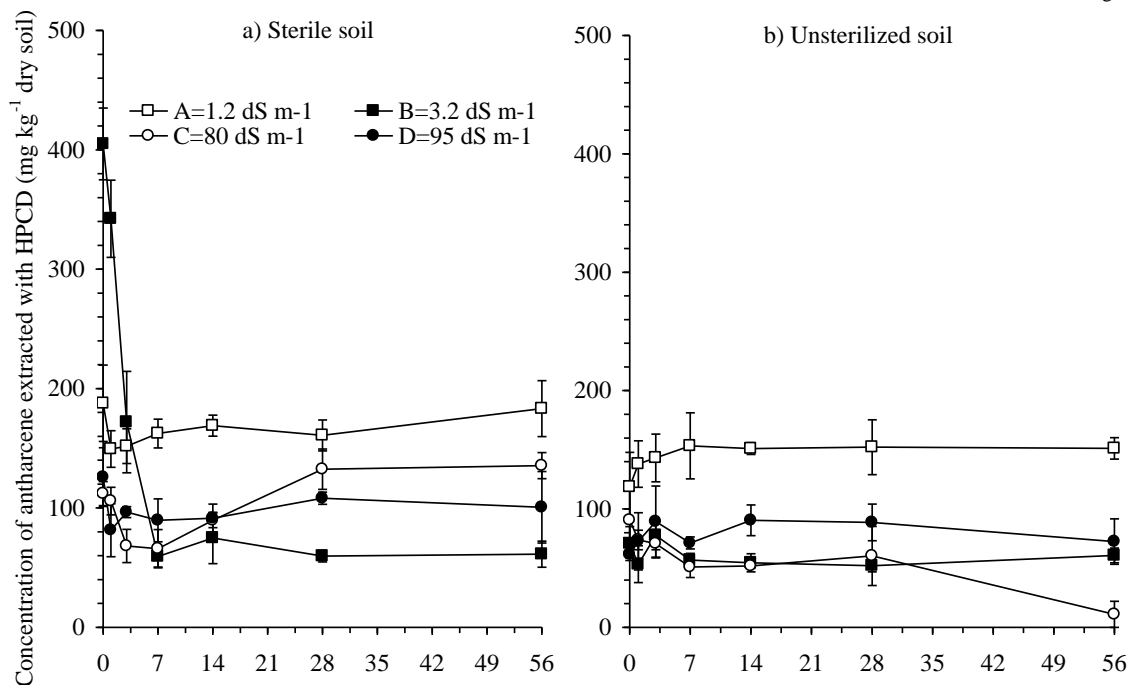
Table 1. Some characteristics of the Texcoco soils and emissions of CO_2.

Soil	EC [a] (dS m^{-1})	pH	Organic C	WHC [b]	Clay	Silt	Sand	Textural classification	CO_2 emission rate (mg CO_2-C kg^{-1} soil day^{-1})
			———————— (g kg^{-1} soil) ————————						
Soil A	1.2 C [c]	8.2 C	76 B	526 C	40 D	70 C	890 A	Sand	17.63 B
Soil B	3.2 C	9.8 B	101 A	802 B	60 C	100 C	840 A	Loamy sand	35.66 A
Soil C	80.2 B	10.1 A	35 C	1011 A	100 B	170 B	730 B	Sandy loam	39.77 A
Soil D	95.2 A	10.1 A	35 C	932 A	150 A	270 A	580 C	Sandy loam	19.77 B
LSD [d]	12.6	0.1	19	96	30	40	60	SEE [e]	3.81

[a] EC: Electrolytic conductivity, [b] WHC: Water holding capacity, [c] mean of five different soil samples ($n = 5$), [d] LSD: Least significant difference (P<0.05), [e] SEE: standard error of the estimates (P<0.05).
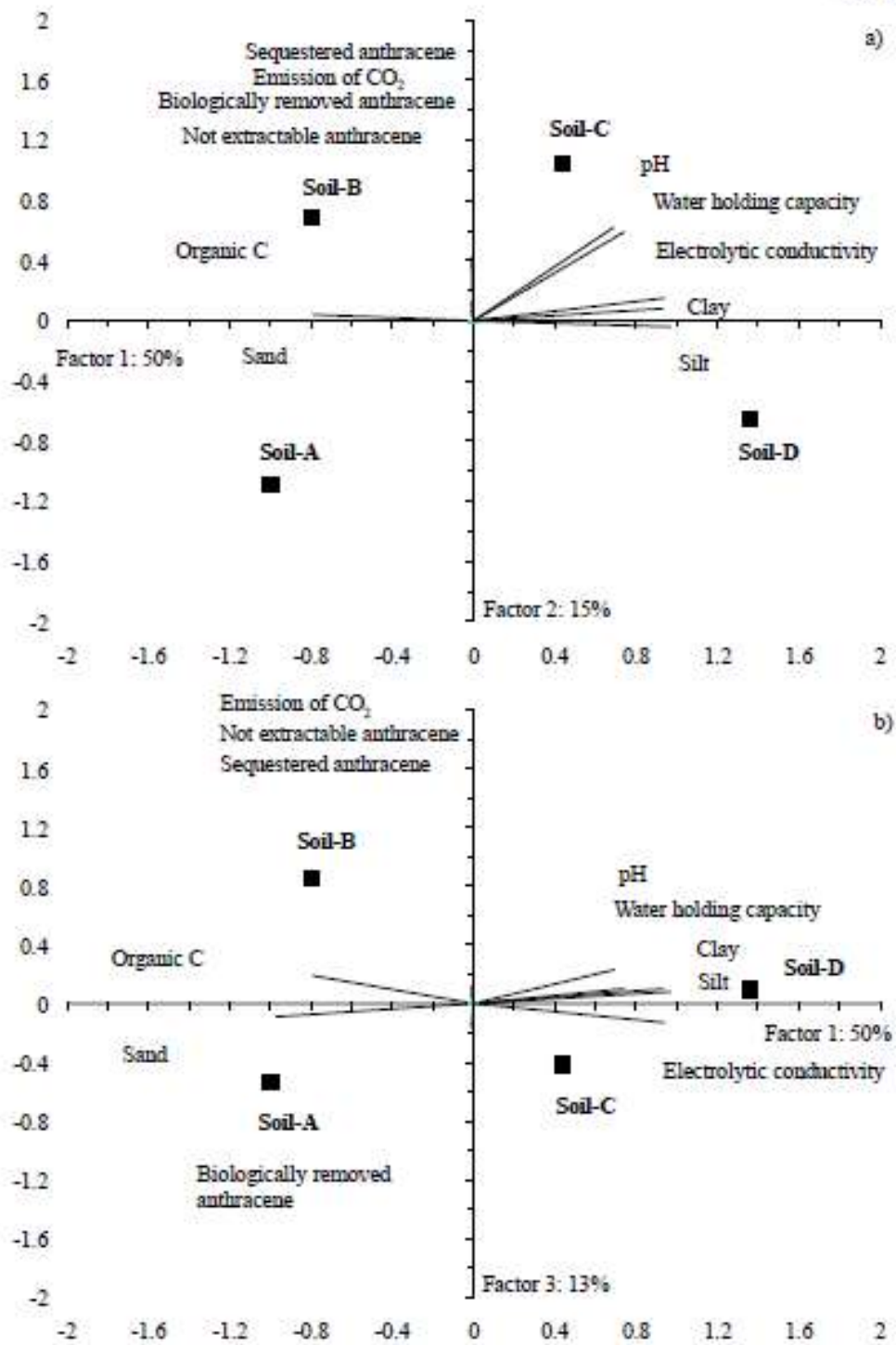
Fig. 4

Table 2. The percentage anthracene extracted from the sterile soil as a percentage of the 550 mg added at day 0 and 56, and the unsterilized soil after 56 days.

| Soil | Extracted with an ehasutive technique | | |
| | Extracted from sterilized soil day 0 | Extracted from sterilized soil at day 56 | Extracted from soil at day 56 |
		(%)	
Soil A	94.8 [a] (5.7) [b]	105.5 (16.6)	72.1 (12.6)
Soil B	97.4 (18.6)	90.2 (15.0)	75.6 (14.1)
Soil C	92.4 (20.1)	101.3 (18.3)	62.4 (8.3)
Soil D	100.5 (6.1)	94.1 (9.7)	71.1 (6.8)
	Extracted with n-butanol		
Soil A	101.0 (9.4)	55.2 (18.0)	50.3 (13.2)
Soil B	103.5 (20.4)	43.2 (16.2)	18.4 (5.5)
Soil C	92.3 (19.6)	57.2 (13.4)	25.7 (11.9)
Soil D	97.0 (30.4)	32.6 (14.9)	18.5 (5.4)
	hydroxypropyl-beta-cyclodextrin		
Soil A	34.1 (11.6)	33.3 (9.5)	27.5 (3.3)
Soil B	73.6 (12.2)	11.1 (4.5)	11.1 (2.5)
Soil C	20.4 (3.6)	24.6 (4.4)	2.0 (4.0)
Soil D	22.9 (7.7)	18.3 (12.2)	13.2 (7.8)

[a] mean of five different soil samples ($n = 5$), [b] values between brackets are standard deviations of the mean.

Table 3. Correlation coefficients of soil characteristics with extraction of anthracene with an exhaustive technique, n-butanol or hydroxypropyl-beta-cyclodextrin (HPCD) from sterilized and unsterilized soil after 0 or 56 days.

Technique	Soil	Incubation time (day)	pH	Organic C	WHC [a]	Sand	Clay	Silt	EC [b]	CO_2 [c]
Exhaustive	Sterilized	0	0.060	-0.049	-0.004	-0.040	-0.001	0.061	0.025	-0.206
		56	-0.240	0.039	-0.202	0.185	-0.192	-0.175	-0.077	-0.003
	Unsterilized	56	-0.157	0.143	-0.394	0.196	-0.200	-0.188	-0.286	-0.182
n-butanol	Sterilized	0	-0.108	0.111	-0.174	0.255	-0.166	-0.300	-0.082	-0.078
		56	-0.233	0.063	-0.103	0.461	-0.408	-0.474 *	-0.146	0.152
	Unsterilized	56	-0.774***	0.094	-0.699 ***	0.475 *	-0.511 *	-0.440	-0.399	-0.453 *
HPCD	Sterilized	0	-0.004	0.776 ***	-0.210	0.385	-0.395	-0.369	-0.681 **	0.168
		56	-0.504 *	-0.238	-0.440	0.274	-0.345	-0.227	-0.068	-0.275
	Unsterilized	56	-0.778 ***	0.368	-0.799 ***	0.365	-0.318	-0.379	-0.454	-0.673 **

[a] WHC: Water holding capacity, [b] EC: electrolytic conductivity, [c] CO_2: CO_2 emission rate, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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Table 5. Rotated loadings on the principal components.

	Principal components		
	PC1	PC2	PC3
Eigenvalues	6.19	2.13	1.03
Proportions	0.56	0.19	0.09
Measurements	Rotated loading on retained components [a]		
pH	53 *	82 *	7
Organic C	-79 *	-6	16
Water holding capacity	60 *	73 *	-23
Sand	-96 *	-18	-2
Clay	91 *	21	3
Loam	95 *	16	1
Electrolytic conductivity	89 *	26	-19
Emission of CO_2	-27	87 *	-16
Anthra extracted with the exhaustive technique d56	-13	-5	93 *
Anthra extracted with the n-butanol d56	-28	-69 *	-21
Anthra extracted with the HPCD d56	-24	-85 *	11

[a] Parameters with significant loadings on the within column principal component, [b] Only principal components with Eigenvalues > 1 and that explain >10% of the total variance were retained.

CONCLUSIONES

- Los tratamientos PAH y ACETONA incrementaron la producción de CO_2.
- Los microorganismos del suelo del exlago de Texcoco son capaces de remover el 56% del antraceno en los primeros 14 días de la incubación aerobia de 56 días.
- El control abiótico refleja que no hubo ningún efecto fisicoquímico sobre el contenido de antraceno en suelos del ex lago de Texcoco.
- Las condiciones fisicoquímicas del suelo del exlago de Texcoco: pH, capacidad de retención de agua, y la actividad microbiana afectaron la medición del antraceno con las técnicas no exhaustivas n-butanol y HPCD.
- El mezclado, la incubación del suelo y el antraceno afectaron la comunidad bacteriana del exlago de Texcoco.
- La adición de antraceno incrementó el porcentaje de actinobacterias comparado con el suelo control o el tratamiento acetona mientras que disminuyó el porcentaje de proteobacterias después de 14 días.

PERSPECTIVAS

Los resultados obtenidos en esta investigación tienen gran utilidad para determinar las fluctuaciones de las comunidades bacterianas cuando están en presencia de un contaminante, sin embargo, deben enriquecerse mediante la implementación de experimentos donde se incorporen a los suelos, hidrocarburos marcados con ^{13}C, que permitan identificar los microorganismos metabólicamente activos en la degradación de los contaminantes. Otra alternativa sería implementar medios donde la única fuente de carbono sea un PAHs, para así poder cultivar bacterias del exlago de Texcoco que degraden el contaminante.

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