Caracterización de comunidades bacterianas y resistencia a antibióticos en humedales construidos

Ricardo Sidrach-Cardona Martínez

Tesis Doctoral

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

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CARACTERIZACIÓN DE COMUNIDADES BACTERIANAS Y RESISTENCIA A ANTIBIÓTICOS EN HUMEDALES CONSTRUIDOS

PhD Thesis

BACTERIAL COMMUNITIES CHARACTERIZATION AND ANTIBIOTIC RESISTANCE IN CONSTRUCTED WETLANDS

Ricardo Sidrach-Cardona Martínez

2015

UNIVERSIDAD DE LEÓN

Instituto de medio Ambiente y Recursos Naturales

Memoria presentada por el Licenciado en Biología Ricardo Sidrach-Cardona Martínez para optar al grado de Doctor en el programa de Doctorado de Ecología Funcional y Aplicada del Departamento de Biodiversidad y Gestión Ambiental de la Universidad de León

Director: Dr Eloy Becares Mantecón

Departamento de Biodiversidad y Gestión Ambiental

Universidad de León

Directora: Dra Olga Sánchez Martínez

Departamento de Genética y Microbiología

Universidad Autónoma de Barcelona

Agradecimientos

A mis directores de Tesis Olga, por su apoyo y ayuda, y Eloy, por darme la primera oportunidad y aguantarme durante todos estos años.

Al Departamento de Genética y Microbiología de la Universidad Autónoma de Barcelona y al Instituto de Ecología Acuática de la Universidad de Girona, en especial a Laura Garrido y Rosalía Trías, por derrochar paciencia enseñándome y acogerme tan bien.

A todos mis coautores, por su colaboración y por hacer posible este trabajo.

A los revisores de los artículos y los editores de las revistas (incluso de las que no aceptaron algún artículo :), por sus valiosos comentarios que ayudaron enormemente a mejorar los manuscritos.

A Héctor y Merce, porque les he dado bastantes quebraderos de cabeza y siempre me han ayudado con su mejor sonrisa.

A todos los compañeros del Departamento de Ecología, y en especial a la subespecie del IMA, Amandito, Irene, Cris, Antonio, Saúl, Patri, Paula, Marias, Natalia, Anna, Irena, Poncho…, De todos he aprendido algo, de algunos mucho, y con todos me lo he pasado en grande.

A mis amigos, los del Trasgu, Tolas, Amandito (bis), Poncho (bis), Lombraña, Pascual, María, Mei, Mey, Osquitar, Ele, y los de otros laos, Ofe, Mária, Fabi, Lopez, Nanin, Natalia…, porque para esos no hacen falta razones.

Y por supuesto, a Tania, a mis padres, y a mi familia, porque siempre están ahí.

GRACIAS!

Tabla de contenido

Abreviaturas y símbolos

	Castellano	Inglés
ANOSIM	Análisis de similitudes	Analysis of similarity
Ct	Ciclo umbral	Cycle threshold
CT	Coliformes totales	
CW	Humedales Construidos	Constructed Wetlands
DBO	Demanda biológica de oxígeno	
DGGE	Electroforesis de gel de gradiente desnaturalizante	Denaturing gradient gel electrophoresis
DNA	Ácido desoxirribonucleico	Deoxyribonucleic acid
DQO	Demanda química de oxígeno	
EDAR	Estación depuradora de aguas residuales	
FM-SF	Flujo superficial-Macrófitos flotantes	Floating macrophytes surface flow
FW-SF	Flujo superficial de lámina de agua libre	Free water surface flow
FW-SSF	Flujo subsuperficial con lámina de agua libre	Free water subsurface flow
MAR	Resistencia múltiple a antibióticos	Multiple antibiotic resistance
MDS	Escalamiento multidimensional	Multidimensional scaling
NH4-N	Nitrógeno amoniacal	
NTK	Nitrógeno total Kjedahl	
PBS	Tampón fosfato salino	Phosphate buffered saline
PCR	Reacción en cadena de la polimerasa	Polymerase chain reaction
PPCPs	Productos farmacéuticos y de higiene personal	Pharmaceuticals and personal care products
rRNA	Ácido ribonucleico ribosomal	Ribosomal ribonucleic acid
rt-PCR	PCR en tiempo real	Real time PCR
SSF	Flujo subsuperficial	Subsurface flow
SST	Sólidos en suspensión totales	
TRH	Tiempo de retención hidráulico	
WWTP	Estación depuradora de aguas residuales	Waste water treatment plant

1 Introducción general

Los humedales construidos (CWs) han sido utilizados durante décadas para el tratamiento de aguas residuales urbanas de pequeñas comunidades así como de diferentes tipos de aguas residuales industriales (Kadlec and Wallace 2009, Vymazal 2009b). En los trabajos realizados en este aspecto se han estudiado infinidad de parámetros, con mayor frecuencia, la eliminación de materia orgánica y nutrientes (Decamp and Warren 2000, Garcia et al. 2008, Molleda et al. 2008), pero también la eliminación de contaminantes prioritarios (PPCP) (Hijosa-Valsero et al. 2011b, Reyes-Contreras et al. 2012), metales pesados (Pedescoll et al. 2015), antibióticos (Hijosa-Valsero et al. 2011a), etc… En la mayoría de los casos, estos estudios son realizados en base a cortos periodos experimentales o de muestreo (hasta 2 años) aunque se supone que los humedales construidos han de mantenerse en funcionamiento durante décadas. Algunos autores han realizado experimentos a más largo plazo (Bulc 2006, Mitsch and Wilson 1996, Vymazal 2009a) pero en estos casos no se utilizaba agua residual urbana real, por lo que el comportamiento de los humedales podría diferir de aquellos trabajando en situaciones reales de funcionamiento. En el primer artículo de esta tesis se estudia el comportamiento de 7 humedales a escala piloto ($1m^2$) a lo largo de un periodo de muestreo de 39 meses, en el que se alimentan con agua residual urbana. Cada uno de los 7 humedales difiere de los demás en alguna de sus características de diseño, como pueden ser la presencia o no de sustrato, la presencia o no de plantas, la especie vegetal usada, o el tipo de flujo y carga.

Así mismo, es sabido que los CWs son los sistemas de depuración que más bacterias fecales eliminan del agua residual (con excepción de los lagunajes) y aunque ha sido relativamente estudiado (Decamp and Warren 2000, Garcia et al. 2008, Molleda et al. 2008) hay ciertos aspectos en esta eliminación que todavía nos son bien conocidos, por ejemplo: a- Cómo afecta la presencia de

plantas en el humedal, b- Cómo influye la especie de planta, b- Como influye el diseño del humedal en dicha eliminación (flujo, sustrato, carga contaminante…). Estos aspectos mencionados son los que pueden influir en la composición de la comunidad microbiana en los diferentes ambientes que se crean. A pesar de la importancia que los microorganismos tienen en estos sistemas (Stottmeister et al. 2003, Kadlec and Wallace, 2009) poco se conoce sobre las especies presentes y su ecología y funcionamiento in situ, especialmente en la rizosfera (Bodelier et al. 2006), aunque se ha demostrado que las plantas de los humedales mejoran la actividad y densidad microbiana en su zona de influencia (Gagnon et al. 2007, Nikolausz et al. 2008) debido al aporte de exudados y de oxígeno, que generan ambientes microaerobios (Armstrong and Armstrong 2001, Lu et al. 2006). De igual manera también son poco conocidas las comunidades que integran la biopelícula de los sustratos (grava), o del líquido intersticial del interior de los humedales y las interacciones que entre todas estas existen. Con el fin de elucidar algunos de estos aspectos, en el capítulo 5 se estudia mediante técnicas moleculares (PCR-DGGE) la composición de la comunidad bacteriana, secuenciando los fragmentos del gen del rRNA 16S e identificando las especies presentes en cada humedal y en cada ambiente (raíz, grava, líquido intersticial), estableciendo las relaciones que existe entre cada uno de ellos y la influencia de la rizosfera sobre la comunidad bacteriana.

La presencia de diferentes ambientes y comunidades así como los tiempos de retención hidráulicos y celulares en los humedales, pueden influir en los tiempos de contacto y en las tasas de intercambio genético entre las bacterias. Estos factores tienen mucha importancia en un tema clave en la salud humana y animal como es el intercambio entre las bacterias de genes de resistencia a antibióticos. La resistencia a antibióticos en los sistemas convencionales de

fangos activados ha sido extensamente estudiada (Fars et al. 2005, Lefkowitz and Duran 2009, Schwartz et al. 2003), no siendo así en el caso de los humedales construidos, en los que no se sabe cómo influye su diseño sobre la eliminación o favorecimiento de estas resistencias, ni de si sus rendimientos en este aspecto son mejores o peores que los sistemas convencionales. Otro asunto relacionado directamente con los vertidos de bacterias resistentes a antibióticos a los medios acuáticos es el impacto que estos tienen sobre los ecosistemas receptores; los patógenos intestinales en estos medios pueden causar enfermedades, agravadas por el hecho de que dichos patógenos sean resistentes a antibióticos (Servais and Passerat, 2009). Además los genes de resistencia pueden ser transmitidos a las bacterias indígenas, contribuyendo a la expansión del problema (Davison 1999), que además es agravado por los vertidos directos de antibióticos o sus metabolitos al medio (Hijosa-Valsero et al. 2011a), creando una presión selectiva sobre las bacterias resistentes y por tanto favoreciendo las resistencias. En estos impactos no solo tienen influencia las plantas de tratamiento de aguas residuales, sino también los vertidos industriales, especialmente aquellos provenientes de industrias de síntesis de antibióticos. En el capítulo 6 se estudian los rendimientos de eliminación de bacterias totales y de bacterias resistentes a antibióticos de cada una de las configuraciones de humedales, comparando los resultados de estos entre sí para determinar los parámetros de diseño más favorables para la eliminación de estas bacterias, así como con una EDAR convencional de fangos activados que depura agua residual urbana. Así mismo, en el capítulo 7 se estudia la prevalencia, por un lado de bacterias fecales resistentes a antibióticos y por otro de genes de resistencia a antibióticos en el río en el que vierten, en menos de 1km, la EDAR convencional y una EDAR industrial perteneciente a una planta de síntesis de antibióticos.

2 Resumen

Objetivos

1. Conocer cómo influye la presencia/ausencia de vegetación en los humedales construidos, así como la especie vegetal, en la eliminación de bacterias.
2. Estudiar la influencia de los factores de diseño (tipo de flujo, tipo de sustrato, carga orgánica) en la eliminación de bacterias y en la composición de la comunidad bacteriana.
3. Comprobar si los diferentes nichos potenciales presentes en los humedales (rizosfera, biopelícula del sustrato o líquido intersticial) albergan comunidades microbianas diferentes.
4. Estudiar la importancia de los humedales construidos en la producción o eliminación de bacterias resistentes a antibióticos, en comparación con sistemas convencionales de fangos activados.
5. Evaluar la influencia de los vertidos domésticos e industriales sobre el resistoma de los cauces receptores.

Material y métodos

1. Zona de Trabajo

Los trabajos realizados en los capítulos 5 y 6 de esta tesis se realizaron en 8 humedales construidos situados en el interior de la estación depuradora de aguas residuales de la ciudad de León y su alfoz (Noroeste de España, 42º33'35.19''N, 5º33'45.35''W) situada a 807 metros sobre el nivel del mar y con un clima mediterráneo (Supramediterraneo) marcado por unos inviernos muy fríos y unos veranos muy secos.

Los humedales fueron alimentados con agua residual proveniente del decantador primario de la EDAR mencionada. Dicha EDAR disponía de un pretatramiento consistente en un desbaste y un desarenador-desengrasador, un decantador primario, un tanque de fangos activado con aireación continua y un decantador secundario. La capacidad de esta EDAR es de 250000 habitantes equivalentes. El vertido final se realiza al rio Bernesga. En este mismo río, 1 km aguas arriba del vertido de la EDAR se encuentra el vertido de una EDAR industrial perteneciente a una fábrica de antibióticos (cefalexina y amoxicilina), también consistente en un sistema de fangos activados.

En el capítulo 7 se estudió el impacto de los vertidos de esas plantas sobre la resistencia a antibióticos de las bacterias del rio receptor, tomándose muestras en diferentes puntos corriente arriba y corriente abajo de dichos vertidos (Figura 2).

2. Diseño de los humedales experimentales

Los humedales construidos consistieron en un contenedor de fibra de vidrio (80 cm de ancho, 130 cm de largo, 50 cm de profundidad) de una superficie aproximada de 1 m^2. Se utilizaron ocho humedales que se diferenciaron en sus parámetros de diseño, resumidos en la Figura 1. Con la excepción de los humedales de flujo vertical, todos los diseños de humedales construidos más empleados fueron utilizados y comparados en los diferentes estudios.

Los humedales se instalaron en Mayo de 2007, fecha en la que se recolectaron las plantas, procedentes de humedales naturales cercanos, y se plantaron en los humedales CW1, CW2, CW3, CW5, CW6 y CW7 con una densidad de 50 plantas/m^2. Los humedales CW1, CW2 y CW3 fueron plantados con *Typha angustifolia* y los humedales CW5, CW6 y CW7 se plantaron con *Phragmites australis*. La cobertura vegetal en los humedales plantados era del 100%, los humedales CW4 y CW8 se dejaron sin plantar.

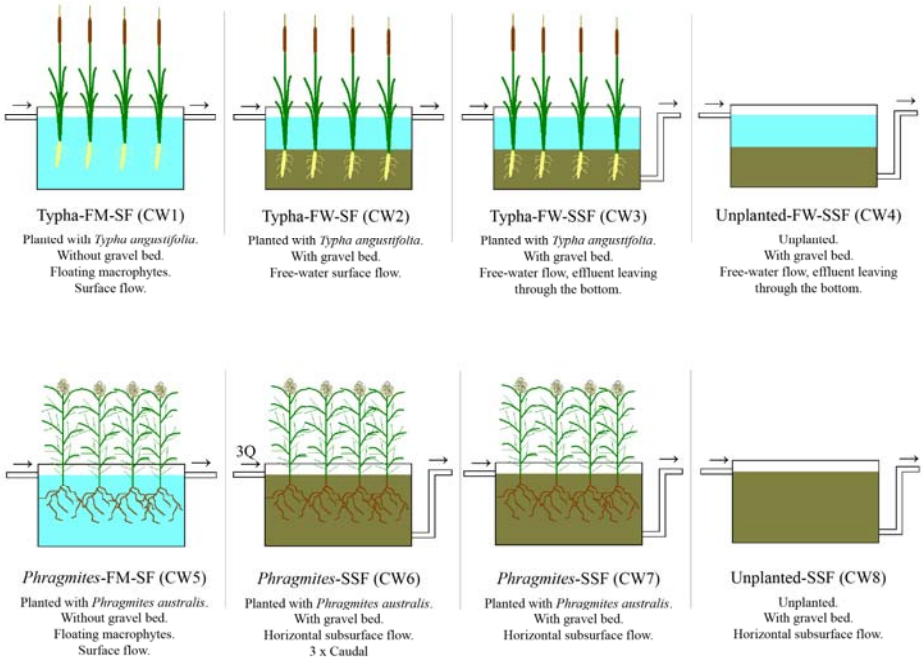

Figura 1.- Representación esquemática de las características del diseño de los humedales construidos. Los sistemas CW1 y CW5 tienen una profundidad de agua de 30 cm y las plantas son sustentadas por unos cilindros de red de 20 cm de longitud y 10 cm de diámetro (4 cm luz). Los sistemas CW2, CW3 y CW4 tienen una capa de agua de 25 cm (Free Water; FW) sobre una capa de 25 cm de grava silícea (d10 = 4mm). Los sistemas CW6, CW7 y CW8 consisten en una sola capa de 45 cm de grava silícea (d10 = 4 mm) a través de la que fluye el agua. La cobertura vegetal es del 100% en los sistemas plantados

El diseño experimental pretendía estudiar diferentes aspectos del funcionamiento de los humedales construidos y comparar diversas condiciones experimentales:

- La comparación entre CW1 y CW5 permite estudiar las diferencias de funcionamiento entre especies diferentes, *Typha* y *Phragmites*, sin que haya un efecto de la grava sobre dicha influencia. El sistema hidropónico en el que se desarrollan las plantas hace que sólo sean las características de las especies (evapotranspiración, biomasa radicular, aireación de la rizosfera, absorción de nutrientes, etc.) las que influyan en la eliminación de nutrientes y bacterias. Por otra parte, estos

sistemas de cultivo hidropónico imitan a sistemas de macrófitos en flotación, lo que permite comparar el funcionamiento de dichos sistemas con otras tecnologías.

- La comparación entre las cubetas CW2 y CW3 permite estudiar el efecto potencial que el tipo de flujo puede tener sobre la calidad del efluente en los diseños de flujo superficial. La cubeta CW2 tiene un flujo estrictamente superficial, es decir el agua entra y sale por la superficie del humedal por lo que sólo la parte superior de la cubeta, la zona de agua libre, será la que tenga una mayor influencia en la eliminación de bacterias y contaminantes. La cubeta CW3 tiene el efluente en la zona profunda de la grava lo que fuerza el flujo de agua a atravesar la grava. En este caso, la zona de grava también influye en el tratamiento, y podría tener un efecto significativo en el funcionamiento del humedal.
- La comparación entre las cubetas CW6 y CW7, de flujo subsuperficial y plantadas con *Phragmites*, permite estudiar el efecto de la carga sobre la composición de la comunidad bacteriana, ya que la cubeta CW6 está doblemente cargada (6-7 gDBO/m2/d) respecto a la cubeta CW7 (3 g DBO/m2/d)
- El efecto de la planta sobre el funcionamiento y la composición bacteriana de los sistemas de flujo superficial se estudia al comparar la cubeta CW3, plantada con *Typha*, y CW4, cubeta no plantada. De la misma forma, la comparación de la cubeta CW7 (plantada) y CW8 (no plantada) permite conocer si la presencia de *Phragmites* tiene un efecto significativo sobre el funcionamiento y comunidad bacteriana de los sistemas de flujo sub-superficial.
- La comparación de las dos cubetas sin plantas, CW4 y CW8 permite conocer el efecto de la grava, o de la presencia de una zona planctónica, sobre el funcionamiento del proceso. Dado que la

vegetación siempre tiene un efecto de sombra, se cubrió parcialmente la cubeta CW4 con una tabla de manera que la intensidad de luz fuese similar a la de una cubeta con vegetación.

- La comparación de las cubetas CW1, CW2, CW3, CW5 y CW6 permite conocer, no solo qué diseño y especie de planta puede ser más eficaz en la eliminación de contaminantes y bacterias, sino también saber hasta qué punto pueden influir dichas variables en la composición de las comunidades bacterianas.

El diseño no tuvo en cuenta todas las posibles combinaciones (ej, enea en flujo sub-superficial, o carrizo en flujo superficial), debido a que muchas de dichas combinaciones han sido ya estudiadas y están claramente establecidas (Kadlec and Knight, 1996). Por ejemplo, la utilización de especies como *Typha* en sistemas de flujo superficial y de *Phragmites* en sistemas de flujo sub-superficial es un aspecto ya establecido en la literatura científica y en la aplicación tecnológica (Kadlec and Wallace, 2009). *Typha* tiene un desarrollo radicular más superficial que *Phragmites* lo que la hace adecuada en sistemas de flujo superficial, mientras que *Phragmites*, por su capacidad para poder profundizar muchos metros en el sedimento (Radoux, 1982) y gran desarrollo radicular, es más adecuado en sistemas de flujo sub-superficial donde la necesidad de zonas aerobias en capas profundas es mucho mayor.

El diseño tampoco tuvo en cuenta la utilización de réplicas para cada una de las condiciones ensayadas. Las limitaciones económicas y de gestión de la planta experimental hubiesen hecho imposible dicho aspecto. En este sentido se utilizó la aproximación experimental que se emplea en la mayoría de estudios sobre este tema, y que se basa en el seguimiento a largo plazo de sistemas que trabajan en paralelo.

La parte aérea de la vegetación se cortó a partir del 2º invierno, dejando la parte sumergida rebrotar en la primavera. Los tiempos de retención hidráulicos medidos experimentalmente difieren bastante en función de la época del año en que nos encontremos y de los calculados teóricamente (ver Tabla 1).

Tabla 1.- Tiempos de retención hidráulicos teóricos, experimentales en julio de 2010 y experimentales en octubre de 2010. Tiempo en días.

	TRH Teórico	TRH Teórico pedescoll	TRH 07-2010	TRH 10-2010	TRH Pedescoll et al. 2012
CW1	50.4	45.7	48.48	12.0	37.4
CW2	79.2	69.0	40.32	10.1	29.6
CW3	122.4	68.0	82.08	23.0	37.8
CW4	146.4	61.5	90.72	15.1	54.0
CW5	69.6	53.6	83.52	27.1	37.4
CW6	19.92	42.2	∞		71.1
CW7	60	22.5	150.72	53.0	52.8
CW8	62.4	34.6	80.16	17.5	41.8

La alimentación de los humedales se realizaba desde un tanque de homogenización (llenado con agua residual proveniente de la EDAR) de 0.5m^3 de manera continua a un caudal de 50 L $día^{-1}$, excepto en el CW6, en el que el caudal de entrada era de 150 L $día^{-1}$ (carga de entrada de 50 mm $día^{-1}$ y carga de DBO5 de 3 $gm^{-2}dia^{-1}$)

3. Variables físico-químicas estudiadas.

Para medir la eficiencia de la depuración a lo largo del periodo experimental se tomaron muestras durante siete campañas de muestreo; verano de 2007, invierno de 2007/2008, verano de 2008, invierno de 2008/2009, verano de 2009, invierno de 2009/2010 y verano de 2010. Durante estas campañas se tomaron muestras del influente y de los efluentes de cada cubeta una vez a la semana, el mismo día a la misma hora en frascos de vidrio ámbar. Una vez en el laboratorio se midieron parámetros de calidad de agua (DQO, DBO_5, SST, NTK,

NH4-N, nitrato y ortofosfato) usando métodos estándar (APHA-AWWA-WPCF 1999).También se midieron in situ temperatura, pH, conductividad, oxígeno disuelto y potencial redox usando equipos de campo.

4. Composición de la comunidad bacteriana

Para identificar la composición de la comunidad microbiana en las cubetas se tomaron muestras de líquido intersticial, de la biopelícula de grava y de las raíces de las plantas, se extrajo el DNA, se amplificó mediante PCR un fragmento de gen del 16S rRNA y se realizó un electroforesis en gel de gradiente desnaturalizante (DGGE), en el que se estudiaron los patrones de bandas de cada muestra y se secuenciaron las bandas más intensas para identificar a qué especie o grupo pertenecían.

4.1. Preparación de las muestras

Las muestras de líquido intersticial, raíces, y biopelicula fueron tomadas en el verano de 2010, cuando las plantas estaban en su máxima actividad, del último tercio de cada cubeta más cercano al efluente de éstas.

El líquido intersticial fue recogido con una jeringa a diferentes profundidades en las cubetas y el agua residual de alimentación fue recogido directamente del tanque de homogeneización, ambos fueron centrifugados a 14000 g durante 30 minutos y el pellet resultante congelado a -20ºC hasta la extracción del DNA.

La biopelícula se extrajo siguiendo el protocolo descrito por Pierzo et al. (1994), esto es, se recogieron 200g de grava, que fue lavada con agua estéril primero y con agua miliQ tamponada después. Las muestras se dividieron en dos submuestras de 100g, que junto con 100 mL de agua miliQ tamponada (en realidad se usó agua de dilución) fueron agitados en un agitador orbital durante

15 minutos a 1000 rpm y posteriormente sonicados durante 4 ciclos de 3 minutos de ultrasonidos y 1 minuto de descanso. El sobrenadante fue centrifugado a 14000 g durante 30 minutos y el pellet fue congelado a -20ºC hasta la extracción del DNA.

En el caso de las raíces, se recogieron alrededor de 25 g (Peso fresco) de rizomas activos, que fueron lavados con agua estéril y posteriormente homogeneizadas con un Ultra-turrax y directamente congeladas a -20ºC (Lu et al. 2006, Yang and Crowley 2000).

El DNA se extrajo usando kits comerciales de extracción, Power Soil kit (MOBIO 12888-50) se usó para el líquido intersticial y la biopelícula, PowerMax Soil (MOBIO 12988-10) se usó para las raíces y UltraClean water DNA kit (MOBIO, 14880-25) se usó para el agua de alimentación. Se comprobó la correcta extracción de DNA con un gel de electroforesis usando DNA mass ladder como estándar.

4.2. PCR-DGGE Fingerprinting

Los fragmentos de DNA extraídos de las muestras fueron amplificados mediante PCR para hacerlos aptos para el análisis DGGE usando el set de primers específicos de bacterias 358f-907rM (Sánchez et al., 2007).

La DGGE fue realizada en una cubeta DCode system (Bio-Rad) según describe Muyzer et al. (1998). El gel de electroforesis contenía un 6% de poliacrilamida con un gradiente de agente desnaturalizante del 30-70% (Urea-Formamida). Después de correr durante 18h a 100 V y 60ºC el gel se tiñó con SybrGold (Molecular probes) y se visualizó con luz UV en un Doc EQ (Bio-Rad). Las bandas más prominentes fueron recortadas del gel, resuspendidas en agua miliQ y reamplificadas para su secuenciación.

4.3. Secuenciación del gen 16S rRNA

Las bandas extraídas fueron purificadas y secuenciadas por Macrogen (Corea del Sur) con el primer 907rM. Las secuencias obtenidas fueron comparadas con la base de datos de BLAST (Altschul et al. 1997) para obtener una afiliación filogenética. También fueron analizadas por el programa Bellerophon (Huber et al. 2004) pata detectar posibles quimeras.

58 secuencias de genes 16S rRNA se enviaron a la base de datos del EMBL y se registraron con los números de acceso del FM991973 al FM992030.

4.4. Análisis cuantitativos

Las imágenes digitalizadas de las DGGE fueron analizadas por el software Quantity One (Bio Rad), identificándose las bandas que ocupaban la misma posición en diferentes muestras y creándose a partir de esos datos la matriz de presencia-ausencia de bandas.

Esta matriz se usó para calcular índices de similaridad usando el programa PAST (Hammer et al. 2001). Se utilizó el índice Raup-Crick debido a que usa un procedimiento randomizado (Monte Carlo) comparando el número de especies observadas en ambas asociaciones con la distribución de co-ocurrencias de 200 réplicas al azar. Dendrogramas UPGMA (Unweighted-pair group average) resultado de diferentes algoritmos (Jaccard, Dice y Simpson) fueron comparados para comprobar la robustez de los grupos formados. Las diferencias estadísticas entre los grupos fue comprobada mediante análisis no paramétricos de Mann-Withney y Kolmogorov-Smirnov así como con análisis ANOSIM usando PRIMER v5 (Clarke and Gorley 2001).

5. Recuento de bacterias totales

El recuento de bacterias totales se realizó en 4 puntos diferentes en los distintos trabajos: la entrada-salida de los diferentes humedales, la salida de la EDAR de la ciudad de León, la salida de la EDAR de la planta de antibióticos y en diferentes puntos del rio que recibía dichos efluentes, tanto en el agua, como en el sedimento. Para este recuento se usaron diferentes medios selectivos o diferenciales. Para *E. coli* / Coliformes totales se usó Chromocult coliform agar (Merck), para *Enterococcus* se usó SB agar (Slanetz and Barley agar, Merk). Las muestras más o menos diluidas para facilitar el conteo de las colonias fueron filtradas a través de filtros de membrana (S-Pak membrane filters, Millipore) y cultivados durante 24-48h a 37ºC, en todos los casos con 2 réplicas de cada muestra y dilución. En el caso de los sedimentos, para extraer las bacterias se mezclaron 100 g de muestra con PBS (50/50 p/v) y se agitó hasta resuspenderlas (Fernandes Cardoso de Oliveira and Watanabe Pinhata 2008). Al sobrenadante se le aplico posteriormente el mismo protocolo que a las muestras de agua

6. Resistencias a antibióticos

Las resistencias a antibióticos de las bacterias fecales fueron estudiadas en las entradas y salidas de los diferentes humedales, en la salida de la EDAR de la ciudad de León, la salida de la EDAR de la planta de antibióticos y en diferentes puntos del rio antes y después de dichos efluentes, tanto en el agua, como en el sedimento (Figura 2). En el capítulo 6 las resistencias a antibióticos antes y después de las cubetas y su comparación con el vertido de la EDAR de León, fueron estudiadas mediante el método de la dilución de antibiótico en el medio de cultivo (6.1) y mediante el método de la difusión en discos (6.2). En el

capítulo 7 se emplearon estos mismos métodos, además de la detección y cuantificación de genes de resistencia a antibióticos por PCR en tiempo real.

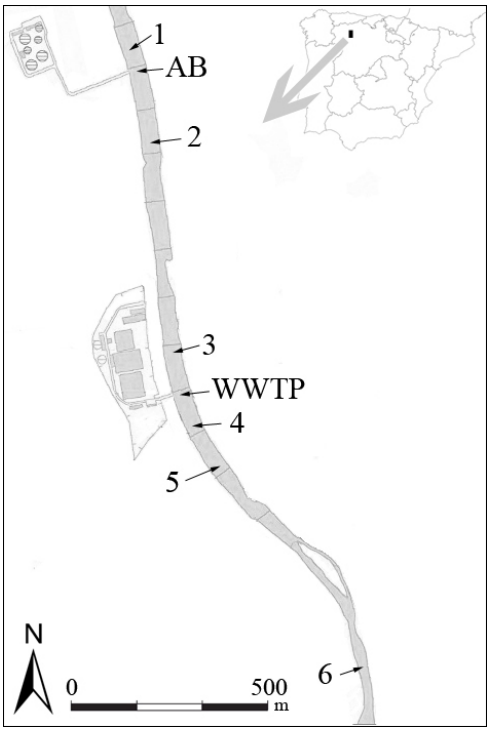

Figura 2.- Puntos de muestreo en el río Bernesga, AB: Vertido de la planta de síntesis de antibióticos, WWTP: Estación depuradora de aguas residuales de León.

6.1. Método de dilución

Este método consiste en diluir directamente el antibiótico en el medio de cultivo para comparar el número de colonias que crecen con el control sin antibiótico añadido (Schwartz et al. 2003, Watkinson et al. 2007b). Este método se usó en 2 artículos variando algunos parámetros en cada uno de ellos.

En el capítulo 6 se estudió la resistencia de *E. coli*, coliformes totales, y *Enterococcus* frente a diferentes concentraciones de Amoxicilina (A), Amoxicilina + Ac. Clavulánico (AC), Azytromicina (AZ) y Doxyciclina (D). Tras preparar una solución stock de cada antibiótico ésta se diluyó directamente en

el medio de cultivo para obtener las siguientes concentraciones finales: 5 y 50 mg L^{-1} de D, AZ, A y AC para *E. coli* y coliformes totales y 1 y 10 mg L^{-1} de D, AZ, A y AC para *Enterococcus.* Para estos ensayos se tomó un litro de muestra de cada punto y se diluyo en solución fisiológica estéril para obtener concentraciones de bacterias fácilmente cuantificables en las placas (entre 10 y 100 colonias), estas soluciones se filtraron por filtros de nitrocelulosa y se aplicaron directamente al medio de cultivo.

En el capítulo 7 se usó el mismo método, pero en este caso se testó la resistencia frente a Cefalexina y Amoxicilina (que son los antibióticos producidos por la planta de síntesis de antibióticos referida en dicho trabajo) a concentraciones de 25 mg L^{-1} y 50 mg L^{-1} respectivamente, también es sedimentos.

6.2. Método de difusión

Este método solo se utilizó en el capítulo 7, siguiendo el protocolo de la NCCLS (2003). En estos casos se obtuvieron colonias aisladas de *E. coli* mediante el cultivo de la muestra en agar Chromocult (medio selectivo y cromogénico) que fueron cultivadas una noche en medio TSB y posteriormente sembradas sobre agar Mueller-Hinton. Sobre esas placas se colocaron los discos de los diferentes antibióticos testados (Beckton Dickinson, BBL™ Sensi-Disc™ Susceptibility Test Discs) (Tabla 2). Las colonias que mostraban comportamientos resistentes o medianamente resistentes (en función del tamaño del halo de inhibición según las instrucciones del fabricante) se clasificaron todas como resistentes.

Tabla 2.- Discos antibióticos usados y su concentración

Antibiótico	Concentración (µg)
Ampicilina	10
Doxiciclina	30
Tetraciclina	30
Estreptomicina	10
Eritromicina	15
Azitromicina	15
Penicilina	10 (U)

6.3. Detección de genes de resistencia

Para detectar genes de resistencia se tomaron 500mL de agua, se filtraron a través de un filtro de nitrocelulosa (0.45µm) para retener las bacterias y éste se congeló a -20ºC. Posteriormente el material en los filtros fue resuspendido en tampón de lisis y el DNA fue extraído usando kits comerciales (DNeasy blood and tissue kit, Quiagen). En el caso de los sedimentos, las muestras se homogenizaron en solución PBS (50/50 p/v) y posteriormente se resuspendieron en tampón de lisis y se extrajo el DNA como en el caso del agua.

La PCR en tiempo real fue realizada para cuantificar los genes bla_{TEM}, $bla_{CTX\text{-}M}$ y bla_{SHV} que confieren resistencias contra antibióticos β-lactamidos, usando métodos previamente descritos (Marti et al. 2013). Todos los resultados fueron normalizados usando el número de copias del gen de la fracción 16S del ribosoma (Maeda et al. 2003). Las curvas patrón de nº de copias fueron generadas representando los valores *Ct* (threshold cycle) de muestras de concentración conocida del gen clonado.

Resultados y discusión

1. Caracterización de la comunidad microbiana.

1.1. Composición de la comunidad bacteriana

Los patrones de bandas de las secuencias amplificadas de rRNA 16S pueden verse en la Figura 3. El número de bandas por carril varió de 10 a 31, con unos valores medios de 20,2 para la biopelícula de la grava, 16,7 para las muestras de raíces y 19,8 para el líquido intersticial. En las diferentes muestras se puede observar diferencias en la posición, la intensidad y el número de bandas, dejando ver diferencias en las comunidades bacterianas de cada ambiente.

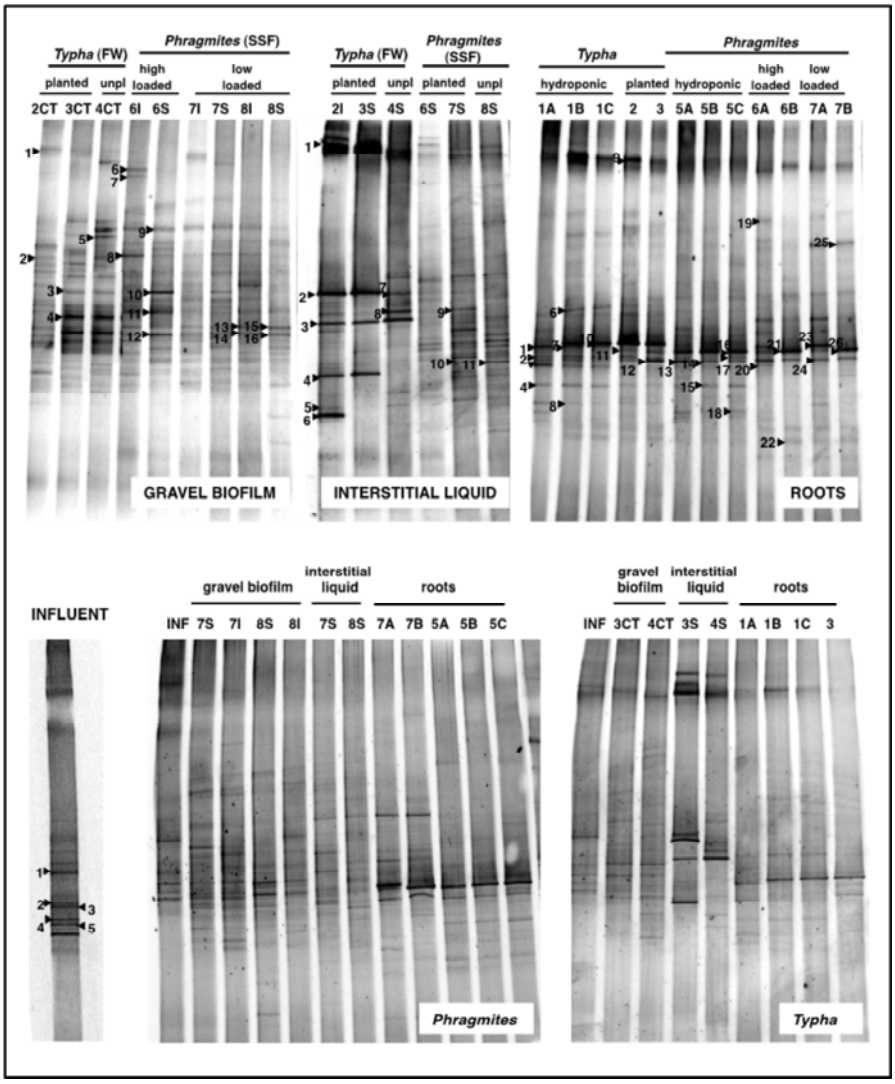

Figura 3.- Patrones de DGGE de las muestras del influente, la biopelicula de la grava, las raíces y el líquido intersticial obtenidos de los diferentes humedales construidos. Los números en la parte superior de los carriles se refieren al número de humedal, la letras A, B, y C a las réplicas dentro del mismo tanque. CT: muestreo central, S: muestreo superficial, I: muestreo profundo, Unpl: sin plantar, SSF: flujo subsuperficial, FW, flujo lámina de agua libre, INF: influente.

1.2. Análisis de la comunidad bacteriana de las cubetas

Un total de 58 bandas fueron extraídas y secuenciadas para obtener su afiliación filogenética. Las coincidencias más cercanas y los porcentajes de similaridad fueron obtenidos mediante una búsqueda BLAST y pueden consultarse en el capítulo 5 (tabla 1)

Bandas asociadas a β-proteobacteria fueron constantes en todos los ambientes, así, algunas bandas de la biopelicula de grava mostraron similitudes mayores del 94% con bacterias no cultivadas de este grupo y se detectaron bandas muy similares a *Acinetobacter johnsonii* (>97%, banda 5I, influente), *Polynucleobacter* sp, bacteria típica de ambientes dulceacuícolas (Hahn 2006) (varias bandas, liquido intersticial) o *Comamonas compostus,* (banda 3R, raíces) esta última con menos porcentaje de similitud. Miembros del grupo Bacteroidetes fueron detectados en todas las muestras de los humedales, *Chryseobacterium joostei* fue encontrada en muestras de biopelícula, mientras *Flavobacterium* sp estaba presente en el líquido intersticial y las raíces de *Phragmites.*

El filo Firmicutes (bacterias gram+ con un bajo porcentaje G+C) se pudo observar en los tres ambientes, aunque las coincidencias eran con especies no cultivadas de este grupo. Por otro lado, secuencias de δ-proteobacteria solo fueron detectadas en muestras de biopelicula de grava y raíces, y algunas de estas secuencias están asociadas a bacterias reductoras de sulfatos, un grupo ubicuo presente en muchos ambientes en los que hay sulfatos, como tratamientos anaerobios de aguas residuales (Ben-Dov et al. 2007, Dar et al. 2005).

Otros grupos sólo fueron detectados únicamente en un ambiente, así Actinobacterias no cultivadas predominan en muestras de líquido intersticial, mientras que grupos como Acidobacteria, Chlorobi o α-proteobacteria fueron

específicos de muestras de raíces. Actinobacteria es un grupo que incluye alguno de los microorganismos más comunes en el suelo, participando en el ciclo del carbono, y en algunos estudios han demostrado su capacidad para degradar contaminantes ambientales en fangos activos (Kim et al. 2007). En el caso de las α-proteobacterias se ha detectado *Rhodobacter blasticus* generalmente aislado en medios contaminados y aguas residuales (Hiraishi et al. 1995, Okubo et al. 2005) y del grupo Chlorobi se han obtenido secuencias similares a bacterias verdes del azufre del genero *Chlorobium;* este grupo de bacterias ha sido tradicionalmente usado para descontaminar aguas residuales y corrientes de gas con presencia de sulfuro (Cork et al. 1983, Kobayashi et al. 1983).

La mayoría de las secuencias identificadas muestran asociaciones bastante cercanas con diferentes especies de bacterias presentes en fangos activos o aguas residuales. Así en el influente aparecen *Acinetobacter* y el anaerobio facultativo *Brachymonas denitrificans,* comunes en fangos activos en los que se produce eliminación de fosfatos (Korstee et al. 1994, Ivanov et al. 2005). Por otro lado *Acidovorax defluvii* fue aislado en una EDAR municipal (Schulze et al. 1999) mientras que *Diaphorobacter* sp es capaz de eliminar nitritos en aguas contaminadas con nitrógeno. En el resto de ambientes la mayoría de las secuencias están asociadas a especies no cultivadas, y por ejemplo entre las proteobacterias las especies cultivadas más cercanas coinciden mayoritariamente con bacterias del ciclo del nitrógeno como *Denitratisoma oestradiolicum* (Fahrbach et al. 2006), o *Dechloromonas denitrificans* (Horn et al. 2005). Por otro lado, entre las muestras del líquido intersticial abundan las secuencias relacionadas con *Polynucleobacter* sp, y aparece por ejemplo *Flavobacterium* sp, que también puede ser encontrada en fangos activos (Park et al. 2007, Shokrollahzadeh et al. 2008, Yu et al. 2007).

1.3. Efecto de la especie de planta y el diseño hidráulico en las comunidades microbianas

Con la matriz de presencias-ausencias obtenida a partir de los geles de DGGE se realizaron dendrogramas y análisis de escalamiento multidimensional (MDS). Los dendrogramas separaron las muestras de acuerdo al diseño y la planta presente en cada humedal (Figura 4). Estos dendrogramas fueron muy consistentes independientemente del algoritmo utilizado, siendo los grupos formados totalmente coincidentes con los obtenidos en el análisis MDS (Figura 5).

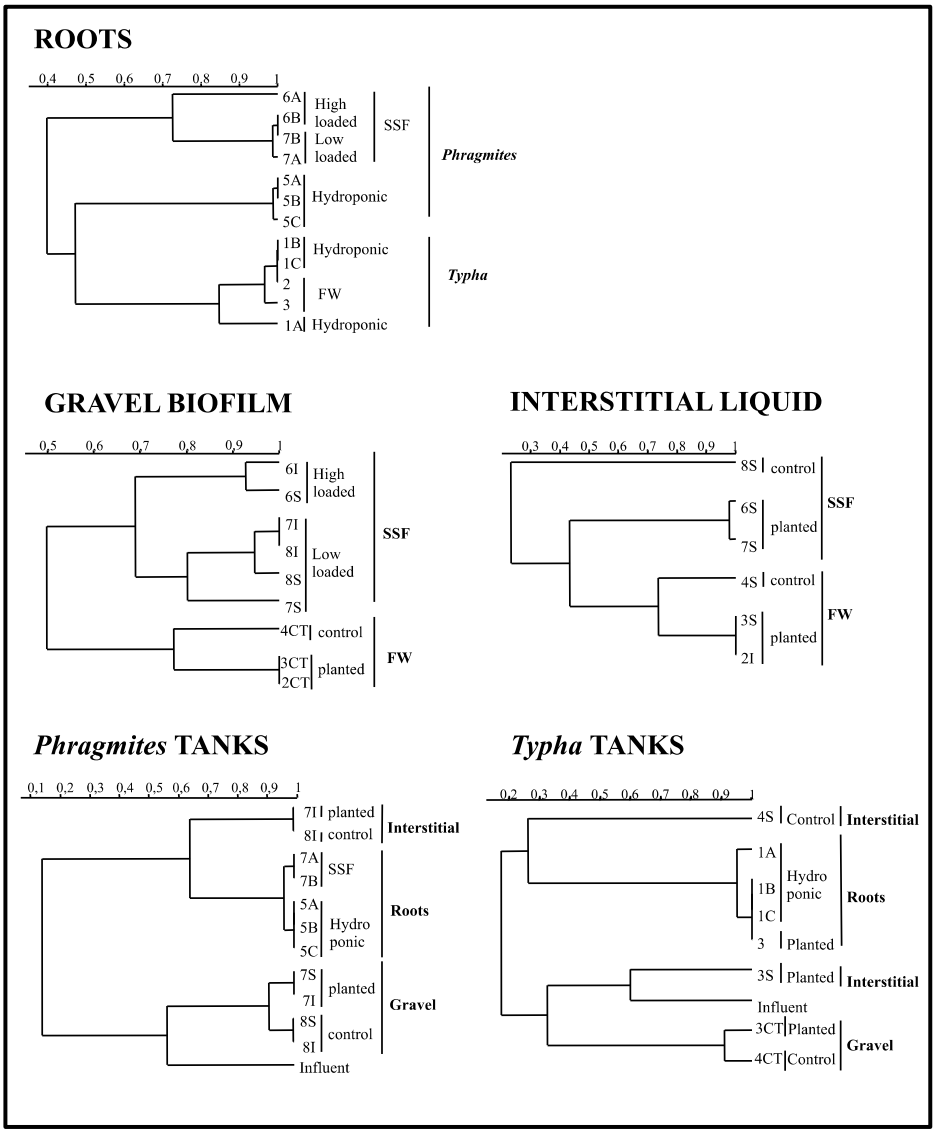

Figura 4.- Dendrogramas de las bandas usando índices de similaridad Raup-Crick. La robustez de las asociaciones se comprobó usando diferentes algoritmos (Jaccard, Dice, y Simpson), obteniendo los mismos grupos. Las letras A, B y C hacen referencia a replicas dentro de la misma cubeta. CT: muestra central, S: muestra superficial, I: muestra profunda. SSF: Flujo subsuoerficial, FW: Lámina de agua libre.

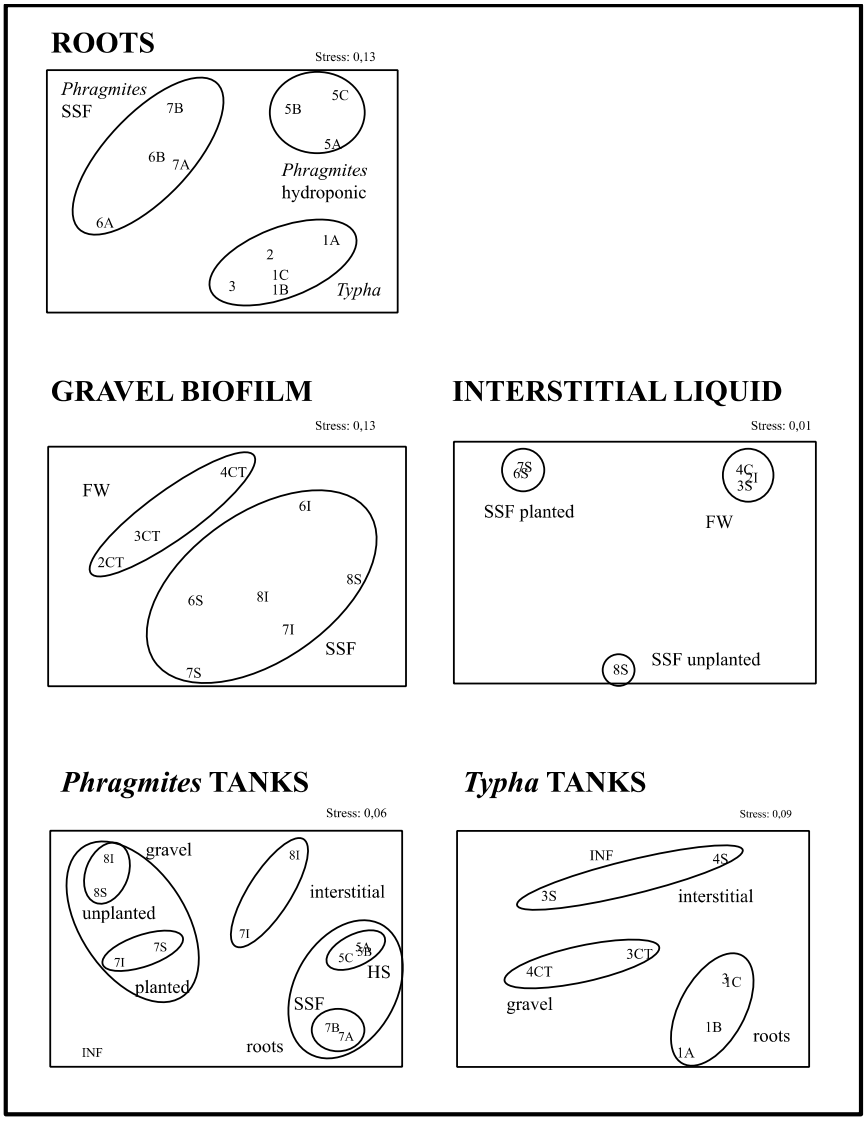

Figura 5.- Análisis de escalado multidimensional (MDS) generados a partir de los patrones de bandas de las DGGEs para cada nicho y para los tanques plantados con cada especie vegetal. Las letras A, B y C hacen referencia a réplicas dentro de la misma cubeta. CT: muestra central, S: muestra superficial, I: muestra profunda. SSF: Flujo subsuoerficial, FW: Lámina de agua libre, HS: Flujo Hidropónico.

Los análisis gráficos y estadísticos diferencian significativamente las comunidades bacterianas entre las raíces de *Typha* y *Phragmites*. Nuestro protocolo no diferencia entre bacterias epifitas y endófitas (McClung et al. 1983, Chelius and Tripplet, 2001) por lo que ambos tipos de bacterias están

incluidas en los resultados. *Typha* y *Phragmites* son claramente diferentes en el desarrollo de las raíces y la producción de oxigeno (Brix 1990, Reddy et al. 1990, Armstrong et al. 1990), siendo plausible pensar que, como ocurre en otras especies de humedales (Lu et al. 2004, 2006), estas diferencias pueden afectar a la composición microbiana de sus rizosferas. En el caso de *Typha angustifolia* la comunidad presente es claramente distintiva dependiendo del ambiente, pero en el caso de *Phragmites australis* se aprecian importantes diferencias entre los humedales con substrato de grava y el hidropónico. Estas diferencias se podrían explicar debido a los cambios en las condiciones ambientales; en nuestro estudio la única diferencia estadísticamente significativa fue el pH que variaba entre el pH 7 del tanque hidropónico y el pH 6.6 de los tanques con substrato de grava.

En cuanto a la influencia del diseño hidráulico en las comunidades, este ha sido probado previamente como una importante variable de control (Gutknecht et al. 2006). En nuestro estudio la influencia del diseño hidráulico ha sido significativa en el caso de los análisis por separado de las raíces de *Phragmites*, el líquido intersticial y la biopelícula de grava (FigurasFigura 4 yFigura 5) cuando se consideran los flujos subsuperficiales y los de lámina de agua.

La principal causa de estas diferencias puede ser el hecho de que el diseño hidráulico afecta a las condiciones redox, siendo los valores mayores en los sistemas con lámina de agua (Lin et al. 2008, Kadlec and Knight 1996). En nuestro experimento los valores redox medios fueron de 234 mV en los humedales con lámina de agua y de 137 mV en los humedales con flujo subsuperficial; así mismo, en los humedales con lámina de agua se puede desarrollar una comunidad plantónica y perifítica que puede también tener influencia en las comunidades bacterianas presentes en los sedimentos y el líquido intersticial (Reddy and DeLaune, 2008).

De todos estos resultados se puede concluir que los humedales construidos albergan una comunidad bacteriana que desempeña un importante papel en la degradación de contaminantes o el ciclo del nitrógeno. Estas poblaciones están influidas por aspectos como la presencia / especie de plantas, o el diseño hidráulico, siendo este último el aspecto más importante en la determinación de la composición de la comunidad. En cuanto a los diferentes ambientes se diferencian claramente, mostrándose un efecto claro de la especie de planta en la composición de la comunidad microbiana asociada a las raíces.

2. Eliminación de bacterias fecales en los humedales

En lo referente a la eliminación de bacterias fecales, todos los humedales construidos funcionan significativamente mejor que la EDAR ($p<0.04$), alcanzando eficiencias de remoción de entre el 90 y el 99% coincidiendo con lo que indican otros autores (Garcia et al. 2008, Karathanasis et al. 2003, Molleda et al. 2008). Los humedales con flujo subsuperficial, y especialmente el nº6 (*Phragmites*, SSF, substrato de grava) se demostró más eficiente que las demás configuraciones en la eliminación de todos los grupos bacterianos a excepción del humedal nº3, que también es mejor que las demás configuraciones en la eliminación de *E.coli.*

Las eficiencias de eliminación son menores en las réplicas sin vegetación, lo que indica cierto grado de efecto de las plantas, especialmente en el caso de *Phragmites,* en línea con lo que demuestran estudios previos (Decamp and Warren 2000, Garcia and Becares 1997, Garcia et al. 2008)(Figura 6).

Estas diferencias en la eliminación de bacterias, especialmente entre los humedales y la EDAR hacen que, aunque los porcentajes de bacterias resistentes sean similares en ambos casos, la diferencia de carga bacteriana

vertida hace que la cantidad de bacterias resistentes que alcanza el medio ambiente sea mucho mayor en el caso de los tratamientos convencionales.

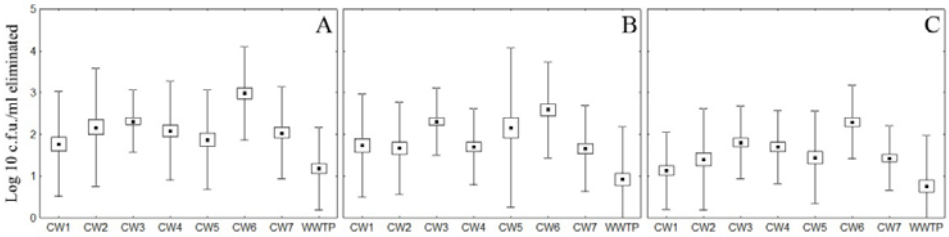

Figura 6.- Log 10 cfu/ml eliminados en cada humedal construido y en la EDAR. A: Coliformes totales, B: *E. coli*, C: *Enterococcus* sp. Media ± error estándar (Box) y desviación estándar (Whisker).

3. Influencia de los humedales construidos en la Resistencia a antibióticos.

En el capítulo 6, se constataron unos porcentajes de resistencia en coliformes totales (CT) y *E. coli* a amoxicilina muy elevados, especialmente en los efluentes de la EDAR y de alguna de las configuraciones de humedales construidos como el 6, el 7 ó el 3, dando valores en algunos casos por encima del 100%, incluso tras añadir ácido clavulánico, lo que indica una resistencia muy importante de este grupo de bacterias a este antibiótico. En cuanto a los *Enterococcus* la resistencia a amoxicilina es mucho menor, no sobrepasando el 40% en ningún caso, siendo también mucho más sensible a la adición de ácido clavulánico y no presentando ninguna colonia resistente en el caso de las concentraciones más altas de antibiótico (Figura 7). Estos resultados son comparables con los encontrados por otros autores (Carroll et al. 2005, Fars et al. 2005, Lefkowitz and Duran 2009).

En el caso de la azitromicina los porcentajes de resistencia en los coliformes totales y *E.coli* son mucho menores y con la concentración alta de este antibiótico no se produce crecimiento en la mayoría de las ocasiones. Cuando se prueba la resistencia a azitromicina de los *Enterococcus* ésta es muy similar en las dos concentraciones de antibiótico, y también bastante similar a la presentada por los CT y la *E. coli* cuando la concentración es baja. En este trabajo también se ensayó la resistencia a doxiciclina, pero no se halló ninguna colonia resistente de ningún grupo bacteriano en ningún humedal construido

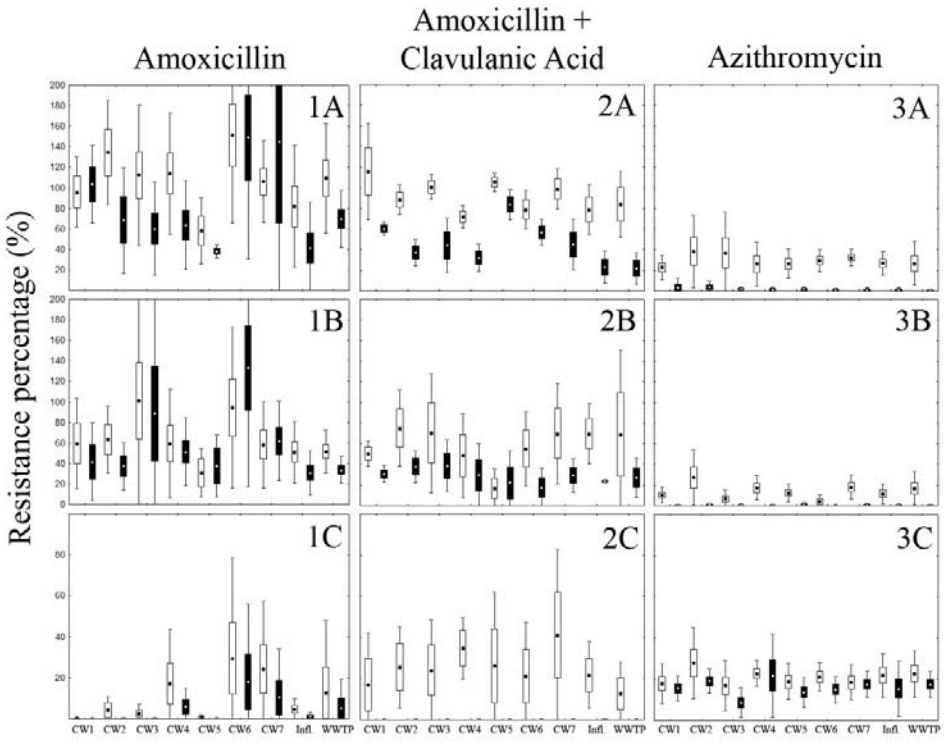

Figura 7.- Porcentaje de resistencia a antibióticos en la entrada (infl) y efluente de cada humedal construido (CW), y en el efluente de la EDAR de León (WWTP). A: Coliformes totales, B: *E. coli*, C: *Enterococcus* sp. Concentración de antibiótico baja (blanco) y alta (negro). media ± error estandar (box) y desviación estandar (whisker)

Para comparar los porcentajes de resistencia entre los diferentes humedales construidos se realizó un análisis no paramétrico Kruskall-Wallis sin encontrar

diferencias significativas, aunque en la mayoría de los casos se produce un incremento de las tasas de resistencia tanto en la EDAR como en los humedales, especialmente en los que tienen flujo subsuperficial, y más concretamente en el nº6 (nº 7 en material y métodos). Esto puede ser debido a la presencia de una matriz sólida en la que la conjugación y transferencia de plásmidos que portan la resistencia es más efectiva (Kalkum et al. 2002, Merlin et al. 2011). También se relacionó el tiempo de retención hidráulico con los porcentajes de resistencia, sin hallar relaciones significativas, esto puede ser debido a que el tiempo mínimo necesario para el intercambio genético es ampliamente superado en todos los humedales.

Estudiando el comportamiento de los diferentes grupos de bacterias independientemente del humedal en que se encuentre, la azitromicina presentó altos grados de efectividad, estando los porcentajes de resistencia entre 0 y 30% en todos los grupos bacterianos. En cuanto a la amoxicilina prácticamente el 100% de las CT fueron resistentes a este antibiótico, incluso añadiendo ácido.clavulánico. *E. coli* y *Enterococcus* fueron menos resistentes a este antibiótico y más sensibles al añadir ácido clavulánico (Figura 8)

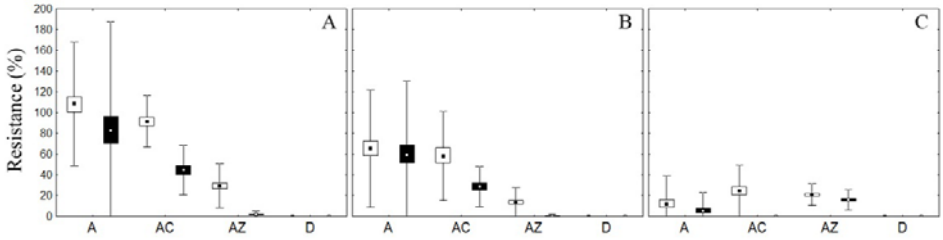

Figura 8.- Porcentaje de resistencia a cada antibiótico. A: Coliformes totales, B: *E. coli*, C: *Enterococcus* sp. Concentración baja de antibiótico (blanco) y alta (negro). Media ± error estándar (Box) y desviación estándar (Whisker).

Los resultados obtenidos corroboran que los humedales construidos son un ambiente idóneo para el intercambio genético y que por tanto cuando en el influente hay un pool de bacterias resistentes, en la mayoría de las

configuraciones estudiadas dichas resistencias se transmiten entre bacterias, de manera similar a lo que ocurre en los sistemas convencionales, aumentando los porcentajes de bacterias resistentes, especialmente en los humedales con una combinación raíces/sustrato más estructurado (3-4, 6-7).

4. Impacto de los efluentes residuales sobre el medio receptor.

4.1. Densidad de bacterias fecales.

En el capítulo 7 se estudia la influencia de los vertidos domésticos e industriales sobre las bacterias fecales presente en el río Bernesga, en un tramo en el que sólo influyen estos impactos. La cantidad de bacterias presentes en el río en el que vierte la EDAR y los humedales se observa que las poblaciones de bacterias se incrementan entre 1 y 3 unidades logarítmicas tras el vertido, siendo la concentración resultante mayor incluso que la del vertido (esto puede ser debido a la rotura de los flóculos en bacterias individuales y a la presencia de crecimiento bacteriano en el río (Figura 9)

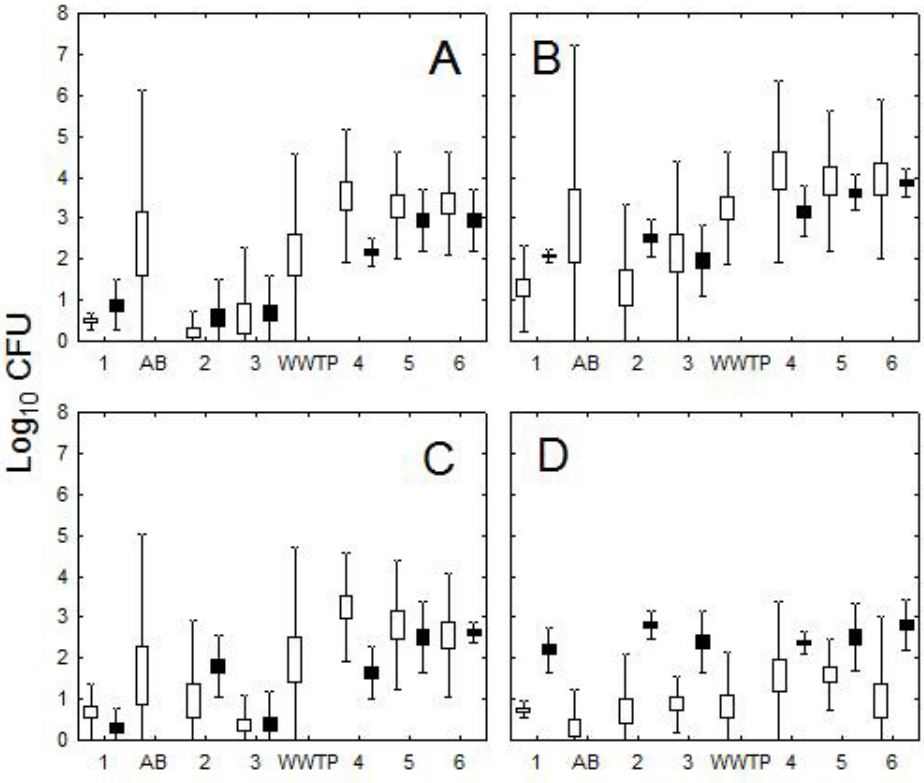

Figura 9.- Abundancias de indicadores fecales en las diferentes muestras de agua (blanco, Log10 CFU ml-1) y sedimento (negro, Log10 CFU gPS-1). A: Coliformes totales, B: *E. coli*, C: *Enterococcus* sp., D: *Clostridium* sp. Media ± error estándar (Box) y desviación estándar (Whisker). Ver Figura 2 para explicación de los puntos de muestreo

Si se tiene en cuenta el caudal de la corriente en los puntos de muestreo, las diferencias se ven más claramente, aumentando de 2 a 4 log10 el número total de bacterias (Figura 10).

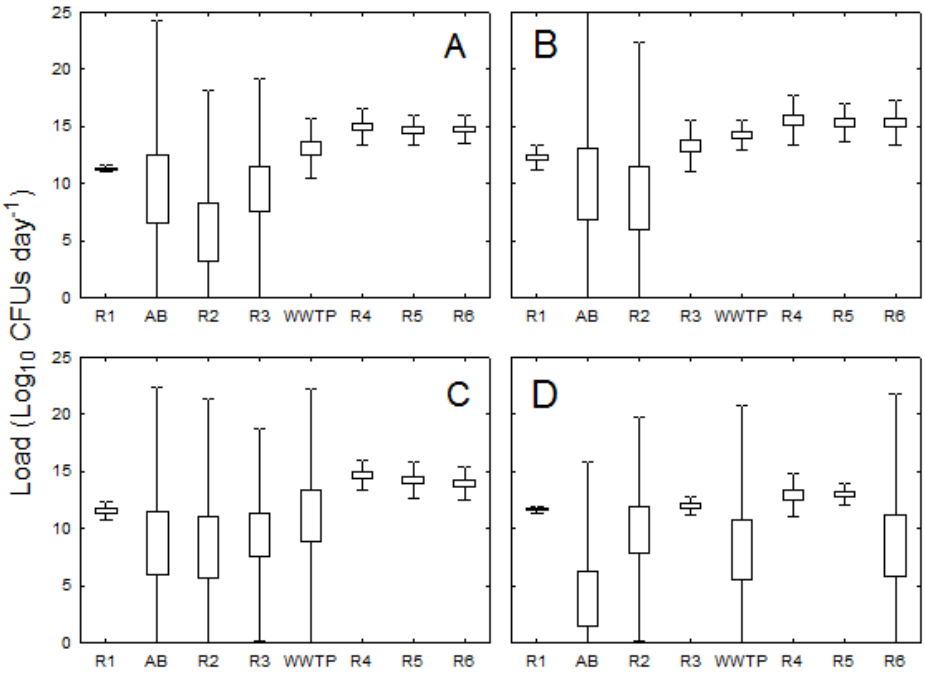

Figura 10.- Carga de indicadores fecales en las diferentes muestras de agua (Log10 CFU ml-1) corregidas en función del caudal medido o calculado en cada punto del rio. A: Coliformes totales, B: *E. coli*, C: *Enterococcus* sp., D: *Clostridium* sp. Media ± error estándar (Box) y desviación estándar (Whisker). Ver Figura 2 para explicación de los puntos de muestreo

En el caso de los sedimentos también se aprecia un incremento en la densidad bacteriana tras los vertidos (Figura 9), con excepción de *Clostridium*, cuya densidad se mantiene bastante constante a lo largo de los puntos de muestreo.

4.2. Efecto de los vertidos sobre la resistencia a antibióticos.

Uno de los aspectos más importantes es el del estudio del impacto que los vertidos de aguas residuales tienen sobre el medio receptor. Las depuradoras de aguas residuales han sido definidas como “puntos calientes” en la producción de bacterias resistentes al medio ambiente. El objetivo del capítulo 7 fue estudiar como influían los vertidos de una industria que producía

antibióticos (cefalosporinas) (AB), y el efecto del vertido de la EDAR de León, sobre el río Bernesga. Las resistencias medidas en el río se pueden agrupar en función del método utilizado para su cuantificación: el método de dilución, comparable al usado en los humedales construidos, el método de difusión (antibiograma), y el estudio del resistoma (genes de resistencia a antibióticos) mediante rt-PCR.

En el caso del método de dilución las muestras tomadas mostraron patrones de resistencia desiguales. Para la cefalexina el grupo bacteriano más resistente eran los *Enterococcus,* alcanzando resistencias de hasta un 60% justo después del vertido de la planta de antibióticos, decreciendo luego a lo largo del rio. En el caso de la amoxicilina tanto los coliformes totales como *E. coli* son los que presentan las mayores tasas de resistencia, también tras el vertido de la planta de AB. En todos los casos, salvo para amoxicilina en *Enterococcus,* existían resistencias a los antibióticos en el rio, antes de recibir el impacto de los vertidos, tanto en agua como en sedimento (Figura 11)

En la mayoría de los casos, los test U de Mann-Whitney realizados no mostraban diferencias entre los puntos de muestreo, si bien estas diferencias sí que existían entre algunos pares de muestras. En el caso de los sedimentos, los patrones fueron muy similares, destacando la alta resistencia a cefalexina de los *Enterococcus* y de los CT a amoxicilina, en ambos casos justo después del vertido de AB, apuntando a que las bacterias resistentes se pueden estar acumulando en los sedimentos tras el vertido de AB.

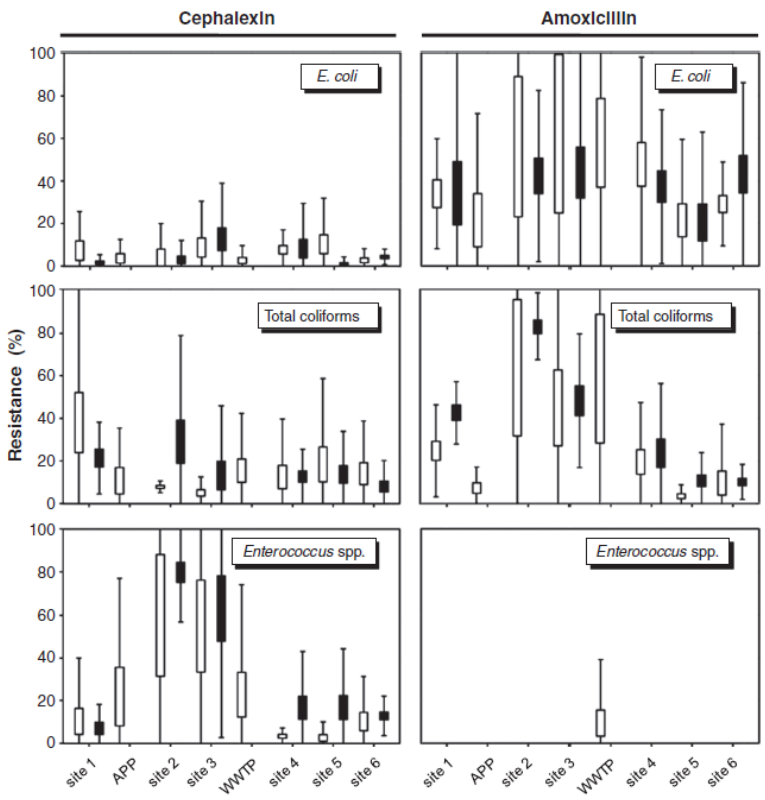

Figura 11.- Porcentajes de indicadores fecales resistentes a 25 µg/ml de cefalexina o a 50 µg/ml de amoxicilina en el rio (blanco) o los sedimentos (negro). Media ± error estándar (Box) y 2 x desviación estándar (Whisker).

En cuanto al método de difusión, se cultivaron un total de 289 colonias de *E. coli*, y se observó que el 100% de las colonias fueron resistentes a la penicilina y prácticamente el 100% resistentes a Eritromicina (Tabla 3). El antibiótico que presentó una menor tasa de resistencia fue la Azitromicina, con valores máximos del 31.6% (similares a los encontrados en los humedales por el método de dilución). En las muestras de agua, de manera general, los porcentajes de resistencia disminuyen justo después del vertido de AB y aumentan después por encima de los valores iniciales para mantenerse luego más o menos constantes a lo largo del río. Los mayores incrementos en los porcentajes de resistencia son para la doxiciclina (del 25% en el punto 1 al 55% en el punto 6) y la tetraciclina (del 35% en el punto 1 al 65% en el 6) (Tabla 3)

Tabla 3.- Patrones de resistencia a antibióticos de colonias de *E. coli* del rio y los vertidos de las plantas de tratamiento.

Antimicrobial	Concentration (µg)	Antimicrobial resistant *E. coli* isolates at each sampling point (%)							
		River 1 (n=20)	AB (n=5)	River 2 (n=6)	River 3 (n=19)	WWTP (n=22)	River 4 (n=29)	River 5 (n=29)	River 6 (n=29)
Ampicillin	10	75.0	40.0	83.3	94.7	81.8	82.8	75.9	65.5
Doxycycline	30	25.0	20.0	0.0	52.6	61.9	58.6	69.0	55.2
Tetracycline	30	35.0	20.0	16.7	63.2	59.1	75.9	58.6	65.5
Streptomycin	10	40.0	100.0	0.0	63.2	52.4	65.5	55.2	34.5
Erythromycin	15	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Azithromycin	15	11.8	0.0	16.7	31.6	9.1	17.2	17.2	20.7
Penicillin	10 (U)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

En los sedimentos los patrones fueron sensiblemente diferentes, comenzaron con porcentajes mayores que en el agua, para luego ir disminuyendo a lo largo del rio, especialmente para tetraciclina y doxiciclina (Tabla 4)

Tabla 4.- Patrones de resistencia a antibióticos de colonias de *E. coli* en el sedimento.

Antimicrobial	Concentration (µg)	Antimicrobial resistant *E. coli* isolates at each sampling point (%)					
		Sediment 1 (n=25)	Sediment 2 (n=7)	Sediment 3 (n=15)	Sediment 4 (n=27)	Sediment 5 (n=27)	Sediment 6 (n=29)
Ampicillin	10	100.0	100.0	100.0	81.5	44.4	82.8
Doxycycline	30	80.0	100.0	46.7	66.7	55.6	55.2
Tetracycline	30	92.0	57.1	66.7	70.4	51.9	48.3
Streptomycin	10	40.0	28.6	46.7	51.9	40.7	34.5
Erythromycin	15	96.0	85.7	100.0	100.0	100.0	100.0
Azithromycin	15	16.0	0.0	7.1	33.3	14.8	3.4
Penicillin	10 (U)	100.0	100.0	100.0	100.0	100.0	100.0

Además de las resistencias a antibióticos individuales, se estudiaron los porcentajes de resistencia a varios antibióticos, si bien todas las colonias son resistentes a al menos dos (por las resistencias a penicilina) el porcentaje de

resistencia a más de 4 antibióticos alcanzó valores medios del 41.7% en agua (Figura 12) y el 50.1% en sedimentos (Figura 13).

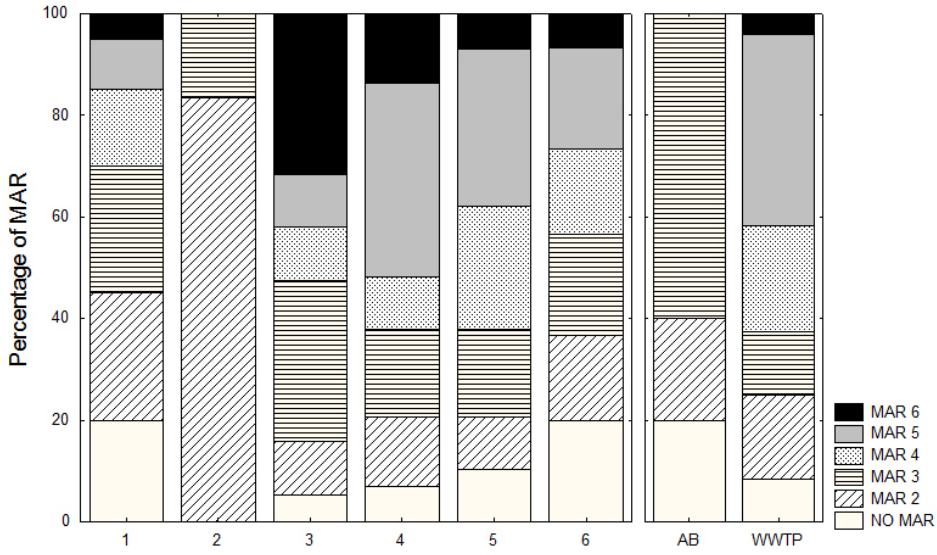

Figura 12.- Porcentaje de colonias de *E. coli* resistentes a múltiples antibióticos en el agua de cada punto de muestreo y los efluentes de las plantas de tratamiento.

Fijándonos en los vertidos de las 2 plantas de tratamientos, se puede observar cómo en el de la planta de antibióticos (columna AB, Figura 12) hay resistencia a 4 antibióticos, mientras que en el vertido de la EDAR (columna WWTP), hay colonias resistentes a todos los antibióticos (8) y el porcentaje de las que son resistentes a más de 4 antibióticos es del 62.5%. Esto puede ser debido a que en la EDAR las bacterias están en contacto con una amplia gama de antibióticos frente a los que pueden generar resistencias (Hijosa-Valsero et al. 2011a), en contraposición al vertido de AB, en el que sólo están en contacto con los producidos en la planta.

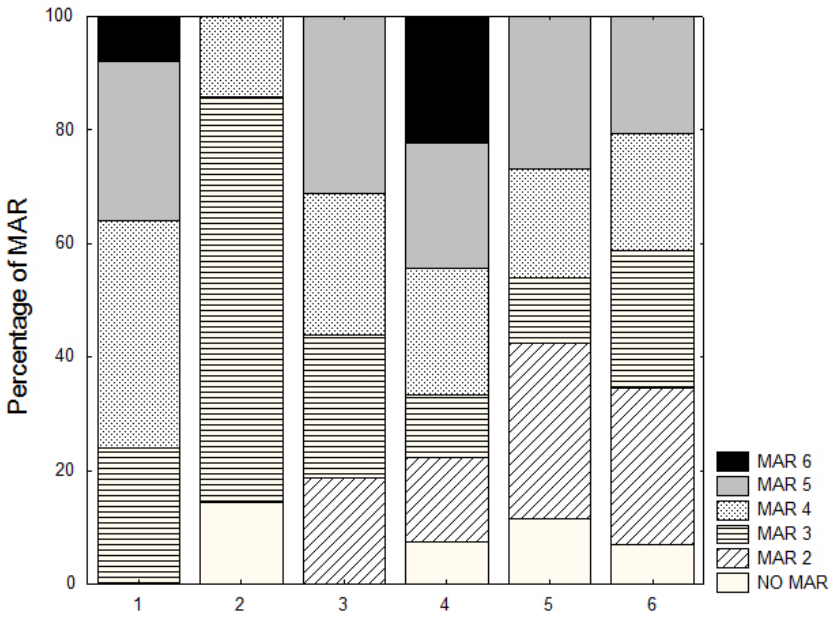

Figura 13.- Porcentaje de colonias de *E. coli* resistentes a múltiples antibióticos en el sedimento de cada punto de muestreo.

La cuantificación mediante rtPCR de genes de resistencia, que fue utilizado en el capítulo 7, mostró que todos los genes medidos estaban presentes en algún punto de río o de los sedimentos y que las abundancias de estos son mayores en las muestras tomadas en el vertido de AB (para luego descender en los puntos inmediatamente corriente abajo) y también bastante altas en las muestras del vertido de la EDAR, aunque en este caso se mantienen estables aguas abajo, apareciendo incluso el gen bla_{SHV} en los sedimentos en los que hasta ese vertido no se encontraban presentes (Figura 14).

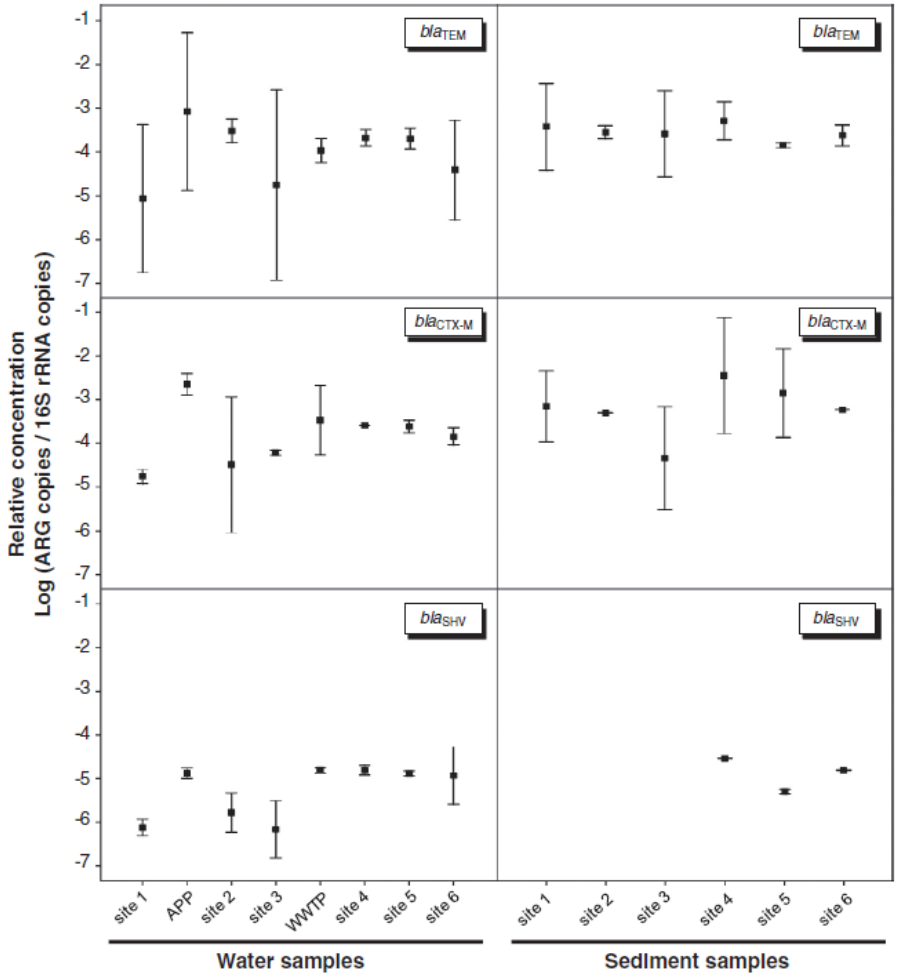

Figura 14.- Concentración relativa de ARGs (Genes de resistencia a antibióticos) en muestras de agua y sedimentos a lo largo del rio y en los vertidos de AB y la EDAR de León. Media ± Error Estándar

Tras comparar los tres métodos de cuantificación de las resistencias en los diferentes ambientes se puede ver cómo la presión selectiva por parte de las actividades humanas ha favorecido aquellas bacterias que han incorporado mecanismos de resistencia (Alonso et al. 2001) y cómo la descarga de poblaciones resistentes a medios acuáticos produce también el intercambio genético con poblaciones previas no resistentes (Davison 1999)

Aunque la mortalidad de estas bacterias es muy alta en medios extra enterales, la alta densidad que presentan (McFeters 1990) y ciertos ambientes más favorables para su supervivencia, pueden hacer que sobrevivan durante largos periodos (Davies-Colley et al. 1999). Esta mayor longevidad es especialmente perceptible en humedales o sedimentos, cómo han demostrado otros autores (Alm et al. 2003, Fernandes Cardoso de Oliveira and Watanabe Pinhata 2008, Howell et al. 1996), que además presentan una alta correlación en la densidad bacteriana con el agua que las rodea (Alm et al. 2003, Fernandes Cardoso de Oliveira and Watanabe Pinhata 2008, Junco et al. 2001), indicando la presencia de un flujo continuo de microorganismos entre los dos medios, y por tanto de su material genético, factores que explicarían la alta tasa de resistencia a antibióticos en los sedimentos después del vertido de AB, de la EDAR o en los diferentes ambientes de los humedales construidos.

Así mismo, se puede observar cómo la presencia de resistencias a varios antibióticos está extendida en el ambiente, y cómo la EDAR vierte porcentajes elevados de bacterias altamente MAR, que incrementan también las bacterias altamente MAR en el rio, aunque estos porcentajes tienden a estabilizarse aguas abajo de los vertidos cuando la presión selectiva disminuye.

Lo que parece claro es que los vertidos tienen un impacto en la corriente receptora en cuanto a las tasas de resistencia de las bacterias y sobre todo en cuanto a la presencia de genes de resistencia en el medio ambiente.

3 Conclusiones

Las conclusiones obtenidas en estos trabajos, en función de los objetivos planteados se pueden resumir de la siguiente manera:

1. La presencia o ausencia de plantas tiene un efecto dispar en la función de eliminación de bacterias, si bien los humedales con plantas eliminaron más bacterias que sus réplicas sin plantas en todos los casos, este efecto sólo es estadísticamente significativo en el caso de Phragmites. Si comparamos las dos especies de plantas presentes, Phragmites siempre es más efectivo eliminando bacterias que los humedales "equivalentes" con Typha, si bien estas diferencias no son significativas.

2. En cuanto a la influencia de los parámetros de diseño, las cubetas con substrato de grava, sea cual sea su flujo, eliminan más bacterias que las hidropónicas. En cuanto a las cubetas con grava, las que tienen flujo subsuperficial eliminan más bacterias que las que tienen flujo estrictamente superficial. El diseño más eficaz eliminando bacterias es el del humedal de flujo subsuperficial y presencia de *Phragmites*. Si comparamos los rendimientos de eliminación de bacterias de los humedales con los de la EDAR convencional, los de los humedales son significativamente mayores, redundando en una carga mucho menor de bacterias al medio receptor.

3. Los análisis de la riqueza de bandas de la DGGE muestra que los diferentes ambientes estudiados (raíz, grava y líquido intersticial) se separan claramente en cuanto a la composición de su comunidad bacteriana, detectándose especies de bacterias exclusivas para cada nicho. Así mismo analizando cada ambiente por separado se comprueba que las comunidades en las raíces de Typha y Phragmites

son diferentes, demostrando el efecto de la planta. Estas diferencias se aprecian también en el caso del líquido intersticial y la biopelícula de la grava, estando en este caso la composición de la comunidad principalmente influenciada por el tipo de flujo (o la presencia de lámina de agua libre)

4. No hay diferencias en los rendimientos de eliminación porcentual de bacterias resistencias a antibióticos entre los humedales y la EDAR convencional, en ambos sistemas no se ha producido una eliminación sino un aumento en los porcentajes de bacterias resistentes entre la entrada y la salida. La menor concentración de bacterias en los efluentes de los humedales supone un aporte mucho menor de bacterias resistentes al medio receptor en comparación con las EDAR convencionales.

5. Los vertidos de las plantas depuradoras, tanto de la EDAR como de la industria de antibióticos, tienen un efecto directo en el aumento de la resistencia a antibióticos en los puntos de muestreo cercanos del cauce receptor, efecto que se va diluyendo aguas abajo. Así mismo se detectó una elevado porcentaje de bacterias fecales multirresistentes a varios antibióticos, así como la presencia y aumento de prevalencia de genes de resistencia en la comunidad bacteriana del rio. Estos resultados confirman a las plantas de tratamiento de aguas residuales como importantes vías para la propagación de la resistencias a antibióticos.

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Molecular Characterization of Microbial Communities in Constructed Wetlands: The effect of Plant Species, Organic Matter and Hydraulic Design[1]

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1 Este capítulo corresponde con:
R. Sidrach-Cardona, Sánchez, O., Garrido, L., Mas, J., Bécares, E. Molecular characterization of microbial communities in constructed wetlands: The effect of plant species, organic matter and hydraulic desing. En Barret, L.M. Wastewater Treatment: Processes, Management Strategies and Environmental/Health Impacts, Nova Science Publishers Inc, 2015, p. 45-67.

In: Wastewater Treatment
Editor: Lydia M. Barrett

ISBN: 978-1-63482-467-5

Chapter 2

MOLECULAR CHARACTERIZATION OF MICROBIAL COMMUNITIES IN CONSTRUCTED WETLANDS: THE EFFECT OF PLANT SPECIES, ORGANIC MATTER AND HYDRAULIC DESIGN

Ricardo Sidrach-Cardona[1*], Olga Sánchez[2*†], Laura Garrido[2], Jordi Mas[2] and Eloy Bécares[1]

[1]Department of Ecology, Faculty of Biology, University of León, León, Spain
[2]Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Spain

ABSTRACT

Constructed wetlands are becoming an interesting alternative for wastewater reuse since high concentrations of contaminants and pathogenic microorganisms can be removed with these natural treatment systems. In this study, experimental constructed wetlands treating diluted wastewater were used to study the effect of plant species (*Typha angustifolia* or *Phragmites australis*), hydraulic design (free-water flow or sub-subsuperficial flow) and organic loading (3 or 9 $gBOD_5/m^2/d$) on the microbial composition of the rhizoplane, gravel biofilm and interstitial water. The analysis of DGGE band patterns showed statistically significant differences in community assemblages between plant species. Hydraulic configuration, and plant presence in a lesser extend, were more important than organic load in shaping microbial communities in the studied wetlands. Distinctive communities were found for roots, gravel biofilm and interstitial water inside the same mesocosm, being differences among these communities higher for *Phragmites* than *Typha* planted tanks. Plants had an effect on all the microbial communities studied into the mesocosms, proving that their influence affect interstitial water and gravel-associated bacteria far beyond their roots. Environmental conditions,

* These authors contributed equally to this work.
† Corresponding author: Email: olga.sanchez@uab.es.

mainly redox, are suggested as main driving forces in organizing microbial assemblages in the studied wetlands.

INTRODUCTION

Natural wastewater treatment systems such as constructed wetlands constitute a relevant option to conventional methods due to their efficiency, low stablisment costs and reduced operation and management requirement. In these systems, microbial processes are crucial since many reactions are microbiologically mediated (Stottmeister et al., 2003; Kadlec & Wallace, 2009). Thus, the wetland rhizosphere is characterized by the presence of oxic-anoxic interfaces in which obligatory aerobic to strictly anaerobic microbes operate in close proximity, thereby facilitating elemental cycling (Schlesinger, 2004). Despite the impact of microbes in wetland ecosystems, little is still known about the species present and their ecology and functioning *in situ*, especially in the rhizosphere (Bodelier et al., 2006). However, former limitations of cultivation dependent techniques on the study of wetland microbiology have been overcome by the development of molecular techniques like FISH (Criado & Bécares, 2005) and specially fingerprinting methods (Gutknecht et al., 2006; Truu et al., 2009; Adrados et al., 2014, Morató et al., 2014). Thus, it has been proved that wetland plants enhance microbial density and activity in their rhizosphere (Gagnon et al., 2007; Nikolausz *et al.,* 2008), mainly due to the fact that plants provide carbon compounds through root exudates and a microaerobic environment via root oxygen release (Armstrong & Armstrong, 2001; Lu et al., 2006). Microbial densities and activities can also differ depending on the presence of plants or on the species present in the wetland (Angeloni et al., 2006; Gagnon et al., 2007; Li et al., 2008; Ruiz- Rueda et al., 2008; Wang et al., 2008). A few studies found higher functional microbial diversity or higher microbial activity in rhizosphere sediments compared to non-rhizosphere (bulk) sediments (Tam et al., 2001; Vacca et al., 2005) indicating some kind of connection between plants and their adjacent microbial communities. On the other hand, other studies (Ahn et al., 2007; Baptista et al., 2008) pointed to an absence of a vegetation effect on microbial community. The influence of plants on their surrounding environment, clearly evidenced in terrestrial plants (see e.g. Hawkes et al., 2007) seems to be much less clear in aquatic ecosystems, probably due to a potentially more homogeneous environmental conditions in wetlands in comparison with soil.

Polluted areas and constructed wetlands have been the focus of the majority of microbially-based research on plants-bacteria coupling (Gutknecht et al., 2006). Constructed wetlands have been extensively developed in the last decades for the treatment of point and diffuse pollution and their widespread use and research has also improved the knowledge on the functioning of, not only constructed, but also natural wetlands (Reddy and DeLaune, 2008). An important part of the treatment in these systems is attributable to the presence and activity of plants and to the interactions between plant and bacteria. Nevertheless, until recently the microbial ecology of constructed wetlands has remained relatively uncharacterized (Truu et al., 2009) and there is still little understanding of microbial community structure in constructed wetlands and how it is influenced by plants or other potential factors like hydraulic fluxes or organic matter. In this study an experimental constructed wetland was built with the objective to study, a) the effect of plants on their surrounding rhizosphere by comparing planted and unplanted tanks b) the differences

between *Typha angustifolia* and *Phragmites australis* concerning their microbial root-associated communities and, c) the effect of hydraulic design (superficial vs. sub-superficial) and organic loading on the main microbial habitats of planted tanks.

MATERIALS AND METHODS

Experimental Pilot Plant

The constructed wetland pilot plant was located in León (north-west of Spain) and received primary settled wastewater from the urban wastewater treatment plant as influent (mean influent BOD_5 of 105 mg O_2/L). Further details on the pilot plant and wastewater characteristics are described elsewhere (Hijosa-Valsero et al., 2012; Pedescoll et al., 2013). Briefly, eight mesocosm tanks 1 m^2 surface 0.5 depth were used for the experiment and divided in three sets (Figure 1).

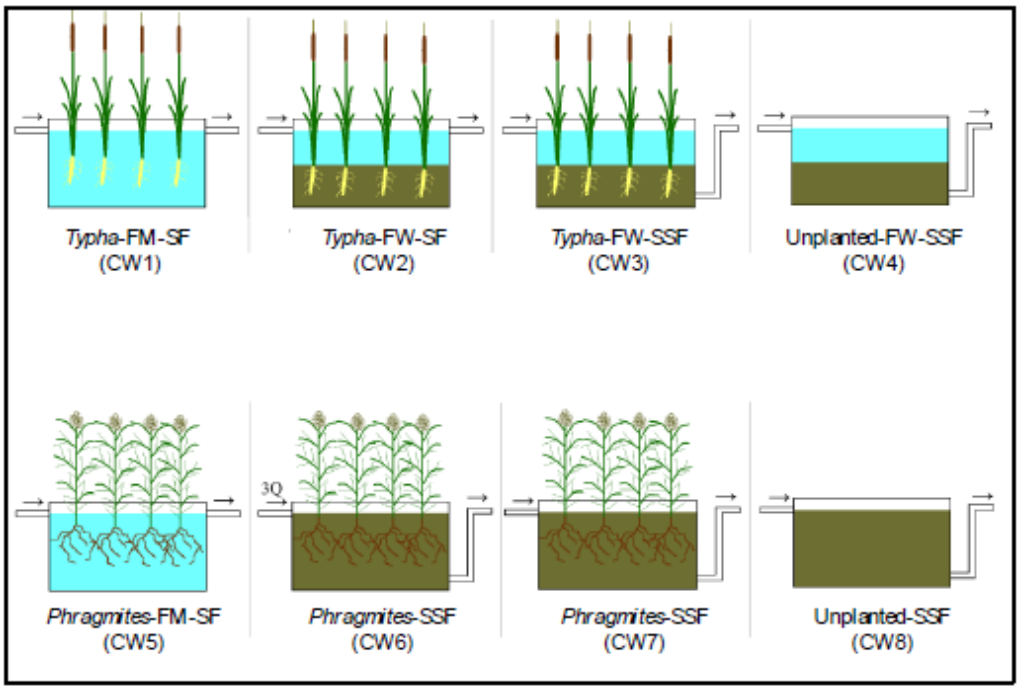

Figure 1. Schematic design characteristics of the CWs. FM: floating macrophytes, FW: free-water layer, SF: surface flow; SSF: sub-surface flow. Systems CW1 and CW5 had a water layer of 0.25 m and plant growth was supported by 25 cm length and 15 cm diameter garden-net cylinders (2 cm pore size). Systems CW2, CW3 and CW4 had a 0.25 m of free-water (FW) layer over a 0.25 m layer of silicious gravel. Systems CW6, CW7 and CW8 consisted of a 0.5 m silicious gravel layer with sub-surface flow (SSF).

A set of three tanks (tanks numbers 2, 3 and 4) were free-water flow systems (FW), half-filled with gravel (0.7-15 mm diameter) up to 0.25 m and water up to 0.5 m. Two of the tanks (tanks 2 and 3) were planted with *Typha angustifolia* and the other (tank 4) was unplanted and considered as control for plant effect comparisons. Influent and effluent were superficial in

tank 2 (i.e. effluent from the surface at the end of the tank), whereas effluent was sub-superficial, (i.e. effluent from the bottom at the end of the tank) in tanks 3 and 4. Comparison of tanks 2 and 3 was therefore used to test flow effect (superficial *vs.* sub-superficial) and comparison of tanks 3 and 4 to test differences between *Typha* planted and unplanted tanks. Another set of three tanks (tanks numbers 6 to 8) were conventional sub-surface flow systems (SSF). Tanks were filled up (0.5 m depth) with siliceous gravel, water level being 4 cms below gravel surface. Two tanks (tanks 6 and 7) were planted with *Phragmites australis* and the other tank (tank 8) was left unplanted and considered as control. Tank 6 was loaded with 9 $gBOD_5/m^2/d$ whereas the rest of the tanks used in the experiment were loaded with 3 $gBOD_5/m^2/d$. Comparison of tanks 6 and 7 allowed to check for loading effects while comparison of tank 7 and 8 for *Phragmites* effect. The last set of tanks (tanks 1 and 5) were filled up with 0.25 m water without any gravel inside (hydroponic tanks). Tank 1 was planted with *Typha* and plant 5 with *Phragmites* to compare bacterial community differences among plant species. Plant support in the hydroponic tanks followed previous experiences (Soto et al., 1999), using rolled strips (15 cm diameter, 25 cm length) of garden plastic nets 2 cms pore size to allow both, root support and development. Rolled strips were tided-up to PVC-tube frames to give more stability to the structure when plants reached full development. All tanks received the same wastewater as influent with a load of 3 g $BOD_5/m^2/d$, with exception of the aforementioned tank 6 which received three times more. Detailed information on wastewater characteristics and efficiencies are presented elsewhere (Hijosa-Valsero et al., 2012).

Collection of Samples

Samples were taken in late summer, when plants were in their maximum development (100% coverage). Biofilm samples from the gravel and plant roots, and from the interstitial liquid of each of the tanks were simultaneously taken in two zones in the last third of the tanks, close to the final effluent. Bacteria attached to the gravel were dislodged using protocols described by Pierzo et al. (1994). About two hundred grams of gravel was firstly washed with sterile tap water to remove all particles loosely attached to the gravel and later with buffered miliQ water. Sample was divided in tubes with 100 g gravel each and 100 ml buffered miliQ. Tubes were shaken in an orbital shaker for 15 min at 1000 rpm and sonicated later at 3 min interval 1 min rest for four times following previous experiences. Supernatant was centrifuged in 45 ml tubes at 14.000 g for 30 min in a refrigerated (-4°C) centrifuge. Pellet was preserved at -20°C until DNA extraction.

Bacterial biofilm from plant roots was extracted using protocols described in Lu et al. (2006), Yang & Crowley (2000) and Ibekwe et al. (2003). About 25g fresh weight of small active roots were taken form the plants rhizomes. Roots were first washed with sterile tap water and buffered miliQ water and later homogenized with an Ultra-turrax. Homogenized samples were kept in 30 ml Falcon tubes at -20°C until DNA extraction. Interstitial water from the gravel bed was sampled with a 100 mL syringe at 15 cm. depth from the gravel surface. Samples were centrifuged at 14.000 g for 30 min in a refrigerated (-4°C) centrifuge and kept at -20°C until extraction.

Several replicates from each tank and environment were taken but due to DGGE limitations not all replicates were included in the same gel.

DNA Extraction

Different kits were utilized for DNA extraction. The DNA Power Soil kit from MOBIO (12888-50) was used for DNA extraction of interstitial liquid and gravel biofilm, while the PowerMax soil kit from MOBIO (12988-10) was utilized for roots. DNA from the influent was extracted with the UltraClean water DNA kit (MOBIO, 14880-25). DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder as a standard (Invitrogen).

PCR-DGGE Fingerprinting

Fragments of the 16S rRNA gene suitable for DGGE analysis were obtained by using the bacterial specific primer set 358f-907rM (Sánchez et al., 2007). Polimerase chain reaction (PCR) was carried out with a Biometra thermal cycler using the following program: initial denaturation at 94°C for 5 min; 10 touchdown cycles of denaturation (at 94°C for 1 min), annealing (at 63.5-53.5°C for 1 min, decreasing 1°C each cycle), and extension (at 72°C for 3 min); 20 standard cycles (annealing at 53.5°C, 1 min) and a final extension at 72°C for 5 min. PCR mixtures contained the template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U Taq DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) at a final concentration of 600 $\mu g \cdot ml^{-1}$ was added to minimize the inhibitory effect of humic substances (Kreader, 1996). The volume of reactions was 50 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen). The DGGE was run in a DCode system (Bio-Rad) as described by Muyzer et al. (1998). A 6% polyacrylamide gel with a gradient of 30-70% DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). Seven hundred ng of PCR product were loaded for each sample and the gel was run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Gel Doc EQ (Bio-Rad). Prominent bands were excised from the gels, resuspended in milli-q water overnight and reamplified for its sequencing.

rRNA Sequencing

Purification of PCR products from DGGE bands and sequencing reactions were performed by Macrogen (South Korea) with primer 907rM. They utilized the Big Dye Terminator version 3.1 sequencing kit and reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type. Sequences were subjected to a BLAST search (Altschul et al., 1997) to obtain an indication of the phylogenetic affiliation, and to the Bellerophon program (Huber et al., 2004) to determine potential chimeric artefacts.

Accession Numbers

Fifty-eight 16S rRNA gene sequences were sent to the EMBL database (http://www.Ebi.ac.uk/embl) and received the following accession numbers: from FM991973 to FM992030.

Quantitative Analyses

Digitalized DGGE images were analyzed with Quantity One software (Bio-Rad). Bands occupying the same position in the different lanes of the gels were identified. A matrix was constructed for all lanes, taking into account the presence or absence of the individual bands. This matrix was used to calculate similarity index for presence-absence data using the Past program (Hammer et al., 2001). Raup-Crick index was utilized as this method uses a randomization (Monte Carlo) procedure, comparing the observed number of species occurring in both associations with the distribution of co-occurrences from 200 random replicates. Dendrograms using unweighted-pair group average (UPGMA) given by other three different algorithms (Jaccard, Dice and Simpson) were also compared in order to assess the robustness of the groupings. Statistical differences between groups were checked with non-parametrics Man-Withney and Kolmogorov-Smirnov test, and with the ANOSIM permutation/randomization test using PRIMER v5 (Clarke & Gorley, 2001). The R statistic produced by this test fluctuates from 0 (no differences) to 1 (perfect separation) with $R>0.75$ indicating well separated groups, $R>0.5$ overlapping but clearly different, or barely separated at all when $R<0.25$. Non-metric multidimensional scaling MDS was also used to check for 2D grouping of sampling points. Raup Crick similarity matrixes from the PAST program (Hammer et al., 2001) were also used as data input for both, ANOSIM and MDS tests. As groups from MDS were mostly the same than those from UPGMA dendrograms only the MDS graphs will be presented.

RESULTS

Bacterial Community Composition

Analysis of the bacterial community composition by PCR-DGGE was performed on influent, gravel biofilm, interstitial liquid and root samples collected in summer 2007 for the different experimental wetlands. DGGE was performed independently for each of the environments (roots, gravel and interstitial samples), and also independently for the different hydraulic designs studied: hydroponic, subsurface flow (SSF) and free-water flow (FW). Banding patterns for the 16S rRNA DGGE-PCR amplicons are presented in Figure 2. The number of bands per lane varied from 10 to 31 (mean values of 20.2, 16.7 and 19.8 for gravel biofilm, roots and interstitial liquid respectively). Some differences could be observed in band position, intensity, and number of bands present in the different samples for each environment, demonstrating that different bacterial communities developed.

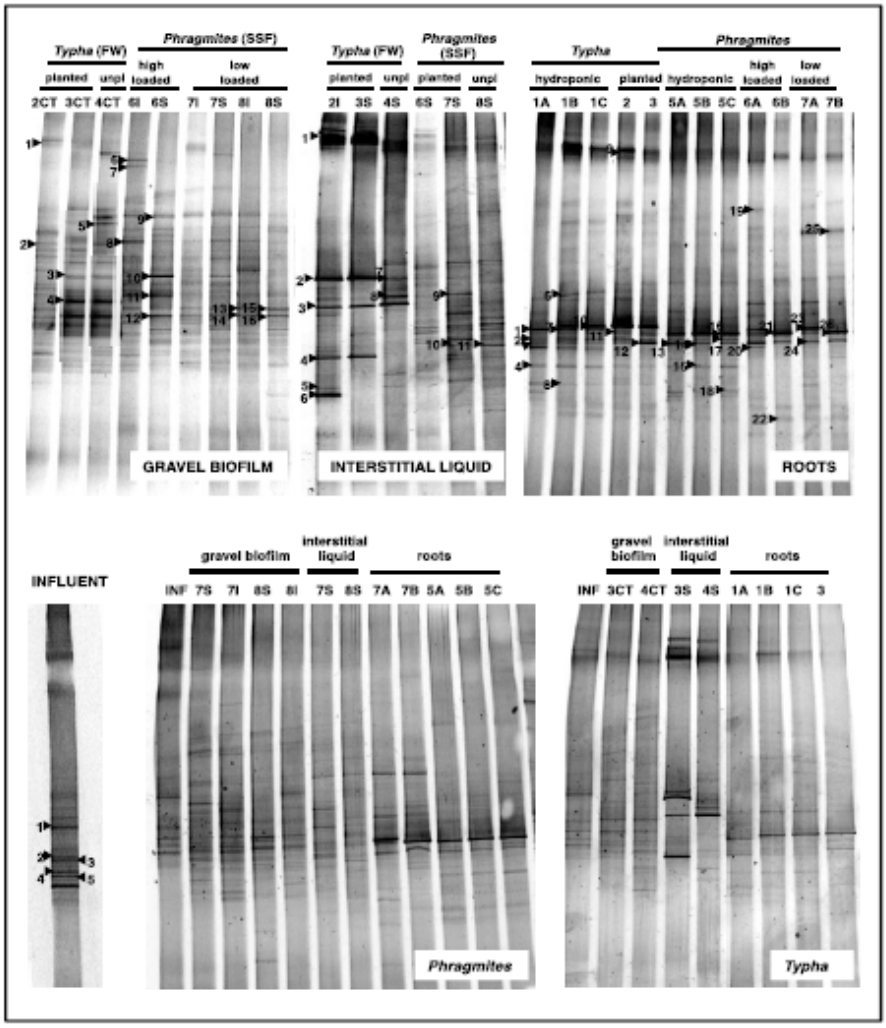

Figure 2. DGGE fingerprints from samples of influent, gravel biofilm, interstitial liquid and roots obtained from the different CW tanks. Numbers on the top of the lanes refer to the experimental tank; letters A, B and C refer to replicates inside the same tank (when available); CT: central sampling, S: surface sampling, I: lower part sampling, unpl: unplanted, SSF: sub-surface flow, FW: free water flow, INF: influent.

Dendrograms and multidimensional scaling (MDS) were applied to discriminate microbial assemblages between the different microenvironments. The dendrograms based on the DGGE banding pattern from roots, gravel biofilm and interstitial water (Figure 3) separated the samples according to the different wetland design and plant species. Dendrograms were very consistent independently of the algorithm used, being groups fully coincident with those observed in the MDS (Figure 4).

Samples from roots split in three main clusters or MDS associations according to the different types of constructed wetlands, and on the base of plant type (Figures 3 and 4). MDS clearly differentiated 3 groups: Samples from *Typha* planted tanks (samples 1A, 1B, 1C, and root samples from tanks 2 and 3), samples from the hydroponic *Phragmites* tank (samples 5A, 5B and 5C), and samples from the gravel (SSF) *Phragmites* planted tanks (samples 6A,

6B, 7A and 7B). DGGE gel patterns from *Typha* and *Phragmites* roots proved to be statistically different when using one-way ANOSIM test (R = 0.582, P = 0.001). Two-way ANOSIM test also showed differences for plant type (R= 0.864, P=0.007) and also for the type of tank, being the hydroponic tank planted with *Phragmites* (tank 5) statistically different from the gravel planted ones (tanks 6 and 7) (R= 0.663, P=0.009).

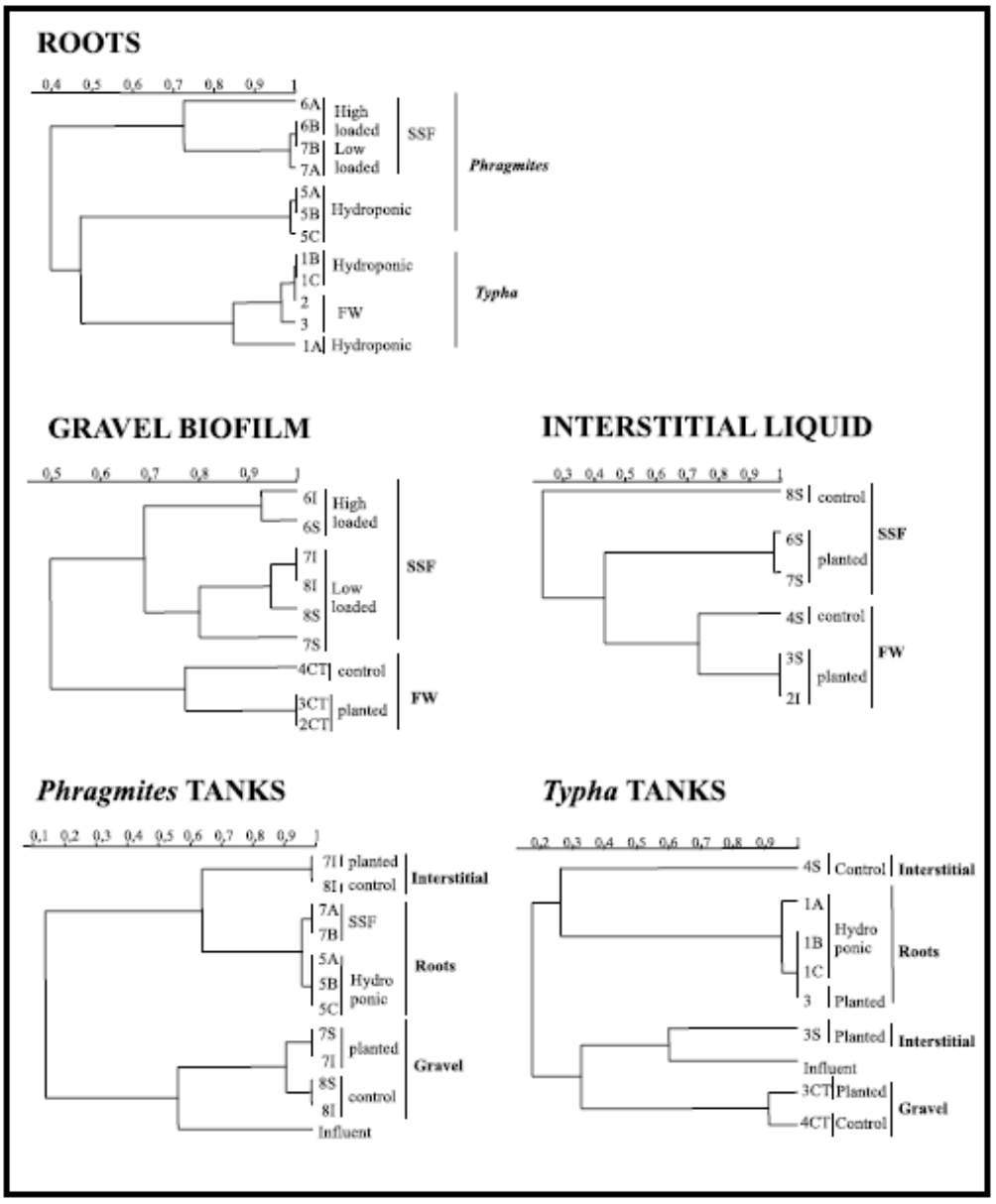

Figure 3. Dendrograms of the bands using similarity Raup-Crick index. Robustness of the associations were confirmed by using three different algorythms (Jaccard, Dice and Simpson), obtaining the same groups. Dendrograms were carried out individually for root associated bacteria, gravel biofilm and interstitial water, as well as for *Phragmites* and *Typha* tanks. Letters A, B and C refer to replicates inside the same tank (when available); CT: central sampling, S: surface sampling, I: lower part sampling, unpl: unplanted, SSF: sub-surface flow, FW: free water flow.

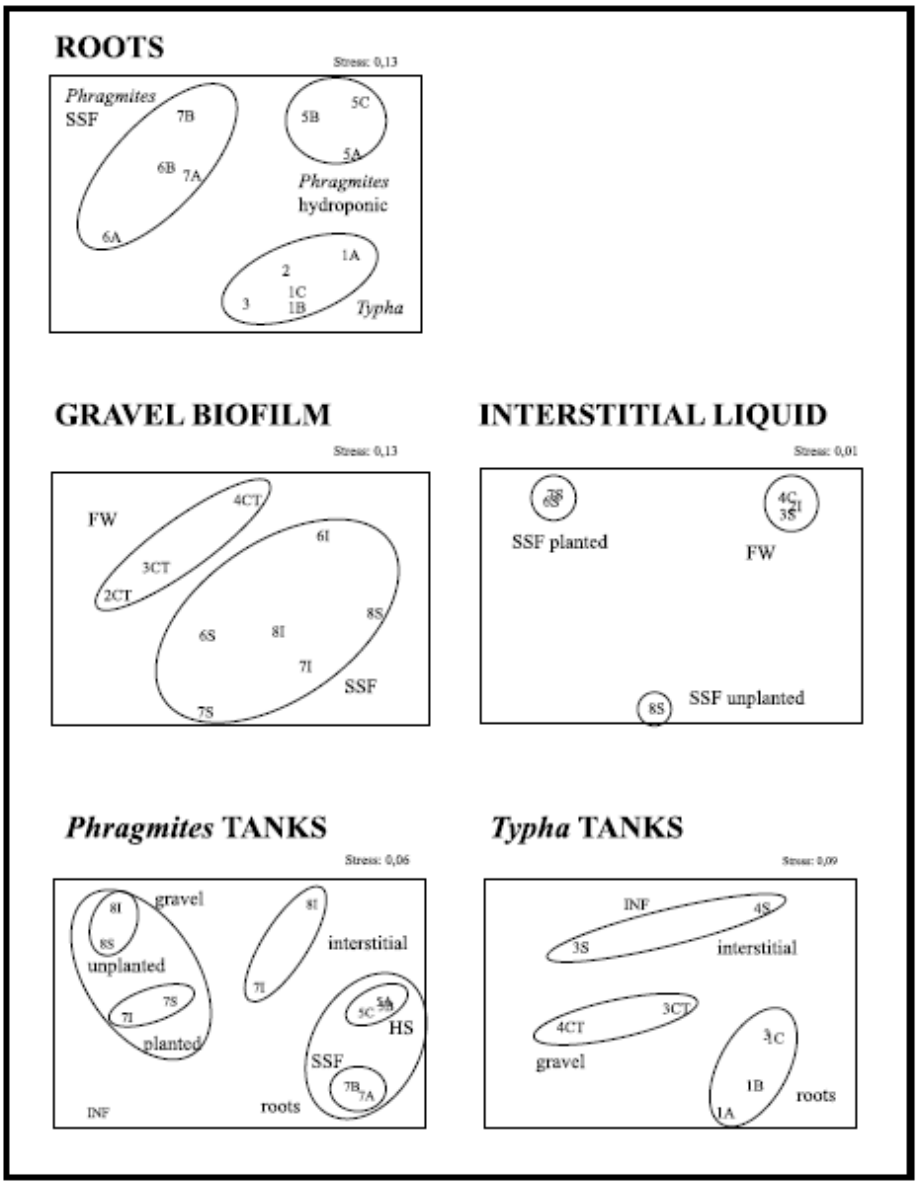

Figure 4. Multidimensional scaling (MDS) analyses generated from the DGGE profiles of the different samples analyzed for each environment (gravel biofilm, interstitial liquid and roots), as well as for each planted tanks (*Phragmites* and *Typha* planted tanks). Type of flow, sub-surface (SSF) and free-water (FW) of planted or unplanted tanks is indicated. Sample number refers to tank type. Letters A, B and C refer to replicates inside the same tank (when available); CT: central sampling, S: surface sampling, I: lower part sampling, INF: influent to the tanks. Circles refers to main groups in the dendrograms (Figure 3).

Bacterial assemblages growing on the roots of *Typha* are therefore statistically different from those growing on *Phragmites* roots. With regard to the hydraulic design of the tank, *Typha* roots harboured the same community composition independently on the type of tank as no differences were found between hydroponic (tank 1) and FW tanks (tanks 2 and 3) (Figure 4). On the other hand, bacterial community from *Phragmites* roots growing on the hydroponic tank was statistically different from that growing on SSF gravel tanks, being no statistical

differences between high organically loaded (tank 6) and low loaded (tank 7) conditions (K-S test, p=0.73). Microbial community composition on *Phragmites* roots was therefore statistically different depending on the support media (hydroponic or gravel-based) whereas no differences were found for *Typha*.

Within the gravel biofilm (Figure 4), samples split into two main groups depending on the type of constructed wetland (SSF or FW) being these two groups statistically different using ANOSIM (R= 0.519, p=0.029). Statistical differences between plant types (*Typha* vs. *Phragmites*) (R= 0.64, p=0.067), planted vs. un-planted (control) tanks (R=0.143, p= 0.4), or high vs. low loaded tanks (R=0.25, p=0.66) did not show significant differences using ANOSIM. The type of flow (superficial in tank 2 vs. sub-superficial in tank 3) did not influence bacterial communities present on the gravel of FW tanks. Presence (FW) or absence (SSF) of free water surface is therefore statistically more important than other experimental conditions when comparing bacterial communities growing on gravel biofilm.

The community composition of interstitial liquid was strongly separated (MDS stress: 0,01) according to wetland design (SSF or FW) or plant presence (Figure 4). MDS clearly separated the unplanted SSF tank (tank 8) from the *Phragmites* SSF planted tanks (tanks 6 and 7) and from the FW tanks (tanks 2, 3 and 4) being differences statistically significant (R=1, p= 0.017). Pairwise test showed significant differences between plant species (*Typha* vs. *Phragmites* tanks, R= 1, p=0.01), and between planted vs. control SSF tanks (R=1, P=0.033). These results suggest that interstitial communities were significantly influenced by the type of hydraulic design (SSF or FW), and by the presence of plants in the case of sub-superficial (SSF) systems. Following the pattern previously observed in the gravel biofilm, no differences were found between loading conditions (tank 6 vs. 7), neither between *Typha* planted and unplanted tank (tanks 2 or 3 vs. tank 4) nor between surface (tank 2) vs. sub-surface flow (tank 3) *Typha* planted tanks with regards to bacterial communities in the interstitial water.

DGGE gels with samples belonging either to the *Typha* planted tanks (tanks 1, 3 and its control tank 4) or to the *Phragmites* planted tanks (tanks 5, 7, and its control tank 8), combining the three different environments, and the influent, in the same gel were also run (Figure 2). Both, the dendrograms and MDS obtained from these fingerprints showed that samples separated on the base of the microenvironment (gravel biofilm, interstitial water and roots) (Figure 4). One-way ANOSIM for *Phragmites* planted tanks including unplanted tank 8 showed significant statistical differences among the three environments (gravel, roots and interstitial) (R= 0.954, P=0,001). Pairwise tests showed significant differences for all the three combinations, gravel vs. interstitial (R= 0.821, p=0.05), gravel vs. roots (R=1, p=0.008), and interstitial vs. roots (R=1, p= 0.04). No differences were found between planted and unplanted gravel tanks (samples 7 vs. 8) neither between roots from the hydroponic and gravel planted tanks (samples 5 vs. 7). Concerning FW tanks (gravel tanks 2 and 3 planted with *Typha* including control tank 4) (Figure 4), differences among the three environments were significant for the global ANOSIM test (R=0.85, p=0.005), being pairwise test almost significant for gravel (samples 4 and 3) vs. roots (samples 1 and 3) (R=1, p=0.06), interstitial (samples 3 and 4) vs. roots (R=0.857, p=0.06) and clearly not significant for gravel vs. interstitial (R=0.25, p=0.33). In general, bacterial communities from the three environments (roots, interstitial and gravel biofilms) were statistically different inside the same tank, being differences more evident for *Phragmites* than *Typha* planted tanks.

Analysis of Predominant Bacterial Species by PCR-DGGE

A total of 58 band positions were excised and sequenced in order to determine their phylogenetic affiliation. The closest matches (and percentages of similarity) for the sequences retrieved were determined by a BLAST search (Table 1). The number of bases used to calculate each similarity value is also shown in Table 1 as an indication of the quality of the sequence. Five bands could be retrieved from the DGGE corresponding to the influent. They belonged preferentially to different Betaproteobacteria, while one band (band 5I) affiliated with a high similarity (>97%) to *Acinetobacter johnsonii*, a Gammaproteobacterium. Bands affiliated to Betaproteobacteria constituted a constant in the different environments studied in this work. Thus, some DGGE bands corresponding to different gravel biofilm samples showed similarities higher than 94.4% to uncultured members of this phylogenetic group, while some DGGE bands of samples corresponding to interstitial liquid, significantly affiliated to the freshwater widespread *Polynucleobacter* sp., as well as to uncultured Betaproteobacteria. One DGGE band obtained from root samples (band 3R) was affiliated to *Comamonas compostus*, although at a low similarity value, and the rest of Betaproteobacteria bands from this environment matched well with non-cultured members of this group. Members of uncultured Bacteroidetes were present in all samples of constructed wetlands. Besides, sequences of *Chryseobacterium joostei* were found in gravel biofilm samples (bands 1L and 14L), whereas sequences of *Flavobacterium* sp. were present in interstitial liquid and roots of *Phragmites* (band 13R).

Firmicutes (Gram+ bacteria with low G+C content) could also be observed in the three environments studied from artificial wetlands, although closest matches corresponded to uncultured members of this group. On the other hand, sequences from Deltaproteobacteria were only present in samples from gravel biofilm and roots, and some taxonomic groups were found exclusively in one of the environments. Thus, uncultured Actinobacteria predominated in some samples of interstitial liquid, while groups such as Acidobacteria, Chlorobi or Alphaproteobacteria were specific of roots samples. Sequence identity of most of the bands in Table 1 showed that they were closely associated with different species or bacteria from sludge or wastewater environments.

Table 1. Phylogenetic affiliation of sequences obtained from DGGE bands, with closest uncultured and cultured matches. Number of bases used to calculate the sequence similarity is shown in parentheses in the third column

Band	Closest match	Similarity (%) (n° bases)	Taxonomic group	Acc n° (Gen Bank)	Cultured closest match (% similarity)
INFLUENT					
WETLE-1I	*Acinetobacter johnsonii*	97.6 (532)	γ-proteobacteria	DQ870719	
WETLE-2I	*Diaphorobacter oryzae*	98.9 (527)	ß-proteobacteria	EU342381	
WETLE-3I	*Acidovorax defluvii*	99.1 (528)	ß-proteobacteria	AM943035	
WETLE-4I	Uncultured ß-proteobacterium	98.9 (537)	ß-proteobacteria	AM940952	*Acidovorax temperans* (98.7)
WETLE-5I	*Brachymonas denitrificans*	99.3 (540)	ß-proteobacteria	EU434449	

Table 1. (Continued)

Band	Closest match	Similarity (%) (n° bases)	Taxonomic group	Acc n° (Gen Bank)	Cultured closest match (% similarity)
GRAVEL BIOFILM					
WETLE-1B	Uncultured diatom chloroplast	99.4 (521)	Bacillariophyta	AY168726	*Phaeodactylum tricornutum* (97.9)
WETLE-2B	Uncultured bacterium	97.6 (523)	Bacteroidetes	AB237701	*Flexibacter canadensis* (89.9)
WETLE-3B	Uncultured ß-proteobacterium	95.6 (517)	ß-proteobacteria	AY947965	*Azovibrio* sp. (93.3)
WETLE-4B	Uncultured bacterium	96.2 (525)	δ-proteobacteria	EU443000	*Desulfobacula toluolica* (94.3)
WETLE-5B	Uncultured bacterium	97.6 (401)	Bacteroidetes	AB237701	*Flexibacter canadensis* (87.8)
WETLE-6B	*Chryseobacterium joostei*	99.4 (522)	Bacteroidetes	EF204455	
WETLE-7B	*Chryseobacterium joostei*	99.4 (522)	Bacteroidetes	EF204455	
WETLE-8B	Uncultured Bacteroidetes	99.6 (534)	Bacteroidetes	EF111172	*Alistipes onderdonkii* (85.3)
WETLE-9B	Uncultured bacterium	98.9 (534)	Firmicutes	AY532555	*Erysipelothrix rhusiopathiae* (92.4)
WETLE-10B	Uncultured bacterium	94.2 (506)	Bacteroidetes	AM086159	*Prolixibacter bellariovorans* (90.1)
WETLE-11B	Uncultured bacterium	99.3 (542)	δ-proteobacteria	EU234252	*Desulfobacter postgatei* (98.9)
WETLE-12B	Uncultured bacterium	99.3 (541)	ß-proteobacteria	AJ318917	*Dechloromonas denitrificans* (96.9)
WETLE-13B	Uncultured bacterium	94.4 (388)	ß-proteobacteria	EU529730	*Rhodocyclus tenuis* (93.4)
WETLE-14B	Uncultured bacterium	97.1 (529)	ß-proteobacteria	AM909879	*Siderooxidans paludicola* (94.5)
WETLE-15B	Uncultured *Gallionella*	97.2 (529)	ß-proteobacteria	AB252929	*Denitratisoma oestradiolicum* (94.5)
WETLE-16B	Uncultured bacterium	98.9 (539)	ß-proteobacteria	AB355063	*Denitratisoma oestradiolicum* (95.6)
INTERSTITIAL LIQUID					
WETLE-1L	*Flavobacterium* sp.	99.8 (533)	Bacteroidetes	AB426577	*Flavobacterium succinicans* (98.7)
WETLE-2L	Uncultured bacterium	98.9 (539)	ß-proteobacteria	EU234274	*Azovibrio restrictus* (94.9)
WETLE-3L	*Polynucleo-bacter* sp.	99.6 (533)	ß-proteobacteria	AB426572	*Polynucleobacter necessarius* (98.7)
WETLE-4L	Uncultured actinobacterium	100 (526)	Actinobacteria	AY948008	*Salinibacterium aquaticus* (98.1)
WETLE-5L	Uncultured bacterium	98.1 (517)	Actinobacteria	AJ863316	*Leifsonia xyli* (97.9)
WETLE-6L	Uncultured bacterium	98.5 (517)	Actinobacteria	AJ863316	*Leifsonia xyli* (98.3)
WETLE-7L	*Polynucleo-bacter* sp.	99.4 (541)	ß-proteobacteria	AB426572	*Polynucleobacter necessarius* (98.5)
WETLE-8L	*Polynucleo-bacter* sp.	100 (542)	ß-proteobacteria	AB426572	*Polynucleobacter necessarius* (99.1)
WETLE-9L	Uncultured bacterium	98.8 (513)	Firmicutes	AY754834	*Clostridium botulinum* (90.6)

Band	Closest match	Similarity (%) (n° bases)	Taxonomic group	Acc n° (Gen Bank)	Cultured closest match (% similarity)
WETLE-10L	*Haslea salstonica* chloroplast	93.0 (494)	Bacillariophyta	AF514854	
WETLE-11L	Uncultured bacterium	89.7 (490)	ß-proteobacteria	EU133809	*Rubrivirax gelatinosus* (88.8)
ROOTS					
WETLE-1R	Uncultured bacterium	98.9 (538)	Acidobacteria	EU499471	*Geobacter pickeringii* (83.2)
WETLE-2R	*Lolium perenne* chloroplast	99.8 (522)	Spermatophyta	AM777385	
WETLE-3R	*Comamonas compostus*	90.1 (491)	ß-proteobacteria	EF015884	
WETLE-4R	Uncultured bacterium	96.9 (402)	Acidobacteria	EU499471	*Desulfomonile limimaris* (84.9)
WETLE-5R	*Lolium perenne* chloroplast	100 (519)	Spermatophyta	AM777385	
WETLE-6R	Uncultured bacterium	93.5 (507)	ß-proteobacteria	FJ037637	Candidatus *Nitrotoga arctica* (91.3)
WETLE-7R	ß-proteo-bacterium	91.9 (487)	ß-proteobacteria	AY297807	*Rhodocyclus* sp. (91.1)
WETLE-8R	Uncultured bacterium	95.2 (511)	Bacteroidetes	DQ093919	*Bacteroides* sp. (91.8)
WETLE-9R	Uncultured bacterium	98.7 (512)	Firmicutes	AY754834	*Clostridium botulinum* (90.0)
WETLE-10R	Uncultured bacterium	89.3 (469)	ß-proteobacteria	AY785239	*Rhodocyclus tenuis* (86.9)
WETLE-11R	Uncultured Rhodocyclaceae	95.1 (504)	ß-proteobacteria	EU266786	*Dechloromonas hortensis* (94.3)
WETLE-12R	Uncultured bacterium	99.4 (540)	ß-proteobacteria	EF667706	*Dechloromonas hortensis* (98.7)
WETLE-13R	*Flavo-bacterium* sp.	99.8 (533)	Bacteroidetes	AB426577	*Flavobacterium succinicans* (98.7)
WETLE-14R	Uncultured bacterium	98.3 (509)	α-proteobacteria	EU284319	*Rhodobacter blasticus* (97.5)
WETLE-15R	Uncultured bacterium	99.4 (515)	α-proteobacteria	EU284319	*Rhodobacter blasticus* (97.5)
WETLE-16R	Uncutlured Acidobacteria	98.2 (534)	Acidobacteria	DQ676412	*Holophaga fetida* (83.1)
WETLE-17R	Uncultured bacterium	98.2 (532)	Acidobacteria	EU499471	*Desulfofrigus* sp. (82.9)
WETLE-18R	Uncultured bacterium	98.5 (533)	Acidobacteria	EU499471	*Desulfofrigus* sp. (82.7)
WETLE-19R	Uncultured bacterium	95.2 (499)	α-proteobacteria	AF502221	*Rhizobium giardinii* (90.8)
WETLE-20R	Uncultured soil bacterium	95.2 (519)	ß-proteobacteria	DQ297980	*Methylibium* sp. (94.3)
WETLE-21R	*Lolium perenne* chloroplast	100 (519)	Spermatophyta	AM777385	
WETLE-22R	*Lolium perenne* chloroplast	99.2 (516)	Spermatophyta	AM777385	
WETLE-23R	Uncultured *Geobacter* sp.	99.3 (542)	δ-proteobacteria	AM159357	*Pelobacter propionicus* (98.7)
WETLE-24R	Uncultured ß-proteobacterium	99.1 (540)	ß-proteobacteria	AB265946	*Denitratisoma oestradiolicum* (95.4)
WETLE-25R	*Chlorobium* sp.	98.3 (524)	Chlorobi	AB210277	

DISCUSSION

Bacterial Assemblage Diversity as Revealed by DGGE

Excision of prominent DGGE bands and subsequent sequencing allowed characterization of predominant microorganisms from the influent as well as from gravel biofim, interstitial water and roots. Most of these sequences belonged to uncultured microorganisms, while others matched well with cultured bacteria. In general, they were closely associated with different species or bacteria from sludge or wastewater environments. Thus, within the Beta and Gammaproteobacteria predominating in the influent, the genus *Acinetobacter* (a Gammaproteobacterium) and the facultative anaerobe *Brachymonas denitrificans* (a Betaproteobacterium) occur in a wide variety of activated sludges in which enhanced biological phosphate removal is observed (Korstee et al., 1994; Ivanov et al., 2005). On the other hand, *Acidovorax defluvii* (a Betaproteobacterium) was isolated from activated sludge samples from a municipal wastewater treatment plant (Schulze et al., 1999). Furthermore, *Diaphorobacter* sp. (Beta) is able to perform partial nitrification followed by further aerobic removal of nitrite in high-nitrogen-containing wastewaters. Overall, ammonia-oxidizing bacteria seem to play an important role in the nitrogen cycle in natural and constructed wetlands.

Also, sequences of Betaproteobacteria, Bacteroidetes and Firmicutes could be retrieved from the three environments studied in the different wetlands. Within the Betaproteobacteria, most sequences had uncultured closest matches; in these cases, cultured closest matches corresponded mostly to microorganisms related to the nitrogen cycle. For example, several sequences from gravel biofilm and roots were associated to *Denitratisoma oestradiolicum*, a denitrifying bacterium isolated from activated sludge of a wastewater treatment plant (Fahrbach et al., 2006), or to *Dechloromonas denitrificans*, a N_2O-producing facultative aerobe (Horn et al., 2005). On the other hand, samples of interstitial liquid abounded in sequences closely related to *Polymucleobacter* sp., a typical freshwater microorganism detected in acidic, neutral and alkaline habitats located in different climatic zones (Hahn, 2006). A number of sequences belonged to the phylum Bacteroidetes. For instance, *Flavobacterium* sp. is also a typical genus which can be found in activated sludge (Park et al., 2007; Shokrollahzadeh et al., 2008; Yu et al., 2007); it was retrieved in samples of interstitial water and roots.

Other sequences, however, belonged to microorganisms present only in specific environments. Thus, sequences affiliated to uncultured Deltaproteo-bacteria could be detected in gravel biofilm and roots, but were not retrieved in prominent bands of interstitial liquid. Some of these sequences were associated to sulphate-reducing bacteria, a ubiquitous group of microorganisms that can be found in many natural and engineered environments where sulphate is present, such as anaerobic wastewater treatment plants (Ben-Dov et al., 2007; Dar et al., 2005).

Other groups were just detected in one particular environment. Thus, sequences affiliated to Actinobacteria could only be retrieved in interstitial water samples, while samples belonging to Acidobacteria, Alphaproteo-bacteria and Chlorobi were found in roots. Actinobacteria include some of the most common soil life microorganisms, performing an important role in decomposition of organic materials, and thereby playing a vital part in

organic matter turnover and carbon cycle. Some studies have reported their ability to degrade environmental pollutants in activated sludge from wastewater treatment (Kim et al., 2007). Acidobacteria was another taxonomic group only observed in root samples. Sequences belonging to this widespread distributed phylum have been found traditionally in soil, aquatic environments and wastewater treatment plants (Ludwig et al., 1997; Juretschko et al., 2002). On the other hand, Alphaproteobacteria related to the phoheterotrophic purple nonsulfur bacterium *Rhodobacter blasticus* have also been detected in roots; this microoganism has been isolated mainly from wastewater and polluted environments such as photosynthetic sludge processes (Hiraishi et al., 1995; Okubo et al., 2005). In general, the genus *Rhodobacter* includes species with an extensive range of metabolic capabilities which allow them to survive in a number of varied habitats, thus appearing in all types of aquatic environments. In fact, the group Alphaproteobacteria has also been found as attached bacteria from different species of plants in aquatic environments (Crump & Koch, 2008). Sequences belonging to *Chlorobium* sp., a green sulphur bacterium, were also detected only in roots. The ability of this microorganism to use sulphide has been exploited in the past for the treatment of sulphide-containing wastewaters and gas streams (Cork et al., 1983; Kobayashi et al., 1983). Thus, many of the species recovered from DGGE bands in this work have the potential to act as degraders of environmental pollutants in natural and constructed wetlands.

Differences between Plant Species Microbial Communities

Graphical and statistical analysis showed significant differences between *Typha* and *Phragmites* root-associated microorganisms. Our protocol did not differentiate between endophytic and rhizoplane bacteria (McClung et al., 1983; Chelius & Tripplet, 2001), therefore both root habitats were pooled in the analysis. Plants species in soils clearly influence the microbial communities of their roots, each plant species harbouring a distinctive microbial assemblage (see e.g. Hawkes et al., 2007; Ehrenfeld et al., 2005, for revision). Nevertheless, a small body of research has demonstrated this aspect in the roots of wetland plants as mostly are focused on the rhizosphere and not on the proper roots or their rhizoplanes. Abundances of Fe-oxidizing bacteria were different among five wetland species studied by Emerson et al. (1999) which isolated different strains in the rhizoplane of different plant species. Bergholz et al. (2001) compared the physiological diversity of root-asociated bacteria of *Spartina patens* with results from a previous work studying *S. alterniflora* and *Juncus roemerianus* rhizoplains (Bagwell et al., 1998). Substantial differences among roots communities were explained due to bacterial oxygen preference and therefore to differences in oxygen production by the plant species. The same reasoning was applied by Briones et al. (2003) to explain differences in band patters among different rice cultivars. A more evident work on species differences was carried out by Chelius & Lepo (1999), who made RFLP analysis from the rhizosphere of *Spartina alterniflora* and *Sesbania macrocarpa*, revealing differences in the community structure of N_2-fixing bacteria. Concerning constructed wetlands, Ruiz-Rueda et al. (2008) also found different nitrogen-related microbial communities when comparing *Typha* and *Phragmites* species. In our work, *Typha angustifolia* roots harboured a significantly distinctive community than *Phragmites australis*, with substantial variations between hydroponically and gravel grown plants in this last species.

Differences in bacterial assemblages between hydroponically and gravel-filled *Phragmites* tanks could be due to the environmental differences in these tanks. Wirsel et al. (2001) found that endophytic fungi biodiversity in *Phragmites* roots changed depending on the type of soil media. In our case, the study of main environmental conditions (redox, pH, or dissolved oxygen) showed that pH was the only variable with statistical differences (ANOVA, F= 116, $p<0,001$) between hydroponic tank 5 (pH=7) and gravel planted tanks 6 and 7 (both with a pH= 6.6). Organic loading, another important factor potentially influencing root development and oxygen release due to the toxic effect of low redox conditions (see e.g. Armstrong & Armstrong, 2005) did not affect differences among redox conditions in the experimental tanks probably due to the low loadings applied (redox values of 162mV and 166 mV for high and low loaded tanks, respectively). Growth substrata (i.e. hydroponic or gravel) seemed therefore to have more influence on bacterial assemblages of *Phragmites* roots than the organic load assayed.

The Effect of Plants and Hydraulic Design on Microbial Communities

Hydrology has consistently been proved to be an important control variable in wetland communities (Gutknecht et al., 2006). In our work the influence of hydraulic design on bacterial communities was significant in *Phragmites* roots, interstitial water and gravel biofilm associated communities when considered separately (Figures 3 and 4), as bands from SSF and FW systems (or hydroponic for *Phragmites* roots) kept significantly different in all environments. These results agree with Vacca et al. (2005), who found that hydraulic design (horizontal or vertical) produced differences in band patterns in bulk soil of constructed wetlands. Hydraulic patterns were also driving forces shaping bacterial communities in a 0.1 ha constructed wetland (Popko et al., 2006). Hydraulic design mainly affects redox conditions in water due to a better exchange of oxygen in FW systems than SSF systems, FW having therefore higher redox than SSF systems (Lin et al., 2008; Kadlec & Knight, 1996). The presence of a water layer in FW wetlands also allows the development of a planktonic and periphytic community in these wetlands which could also influence the microbial community of their sediments and their interstitial water (Reddy & DeLaune, 2008). Studies have revealed that the redox state of the rhizosphere has a significant effect on the intensity of oxygen release through the roots of helophytes (Wiessner et al., 2002; Armstrong & Armstrong, 2001). In our experiment mean redox conditions were clearly different between FW and SSF tanks (mean values of 234mV and 137 mV, respectively), with significantly lower values for the unplanted gravel tank 8 (80 mV) than the *Phragmites* planted (tanks 6 and 7, 164 mV mean) (ANOVA, F=23.5, $p<0,001$). In accordance with the previously mentioned results on bacterial communities in the roots, higher organic loading in SSF tank 6 (9 g $BOD_5/m^2/d$) did not make effect on microbial communities in comparison with low-loaded tank 7 (3 g $BOD_5/m^2/d$). This could be due to the still low loading assayed as 9 g $BOD_5/m^2/d$ is below the recommended design value in some wetland manuals (e.g. Reed, 1990).

Also, no difference was found between gravel and interstitial communities of tank 2 (FW superficial flow) and tank 3 (FW sub-superficial flow). This is in accordance with the absence of differences in redox conditions between tank 2 (120 ±75 mV) and tank 3 (180±71mV). The effect of redox conditions in shaping the rhizosphere microbial communities has been

observed in both, natural and constructed wetlands (Dong et al., 2006; Nikolausz et al., 2008; Wang et al., 2008; Ahn et al., 2009). *Typha* and *Phragmites* are clearly different in root development and oxygen production (Brix, 1990; Reddy et al., 1990; Armstrong et al., 1990), being plausible to think, as it has already shown with other wetland species (Lu et al., 2004; 2006), that these differences could affect the microbial composition of their rhizospheres.

Separate analysis of DGGE band patterns for SSF and FW tanks significantly differenced three community groups: roots, interstitial and gravel communities (Figure 4, *Phragmites* and *Typha* tanks). Differences were, in accordance with previously mentioned results, more significant for SSF tanks than for FW tanks. Microbial communities are therefore different for the different habitats under the same hydraulic design, having plant species a secondary influence on these differences. Differences between planted and non-planted control tanks were only evident for *Phragmites* interstitial water communities but such clear results were not found for *Typha*. These results agree with Vacca et al. (2005) who found differences in band patterns between planted and non planted *Phragmites* tanks and also between the type of soil used for growing. Lower differences among studied habitats for *Typha* tanks could be explained as consequence of a higher influence of this species on their surrounding rhizosphere and to the "planktonic" effect of the free-water environment developed on top of the tanks. In accordance to this hypothesis, redox, dissolved oxygen and pH were significantly higher for *Typha* than *Phragmites* planted tanks (mean redox values of 208±42 mV and 163±49 mV, 1.3±0.1 and 0.46±0.2 mgO_2/L of dissolved oxygen, 7±0.1 and 6.6±0.2 pH units, for *Typha* and *Phragmites* tanks, respectively).

Comparison of our results with previous works on the same topic is problematic because experiments in constructed wetlands treating wastewater are highly variable in operational conditions (e.g. organic loading, redox values, wastewater characteristics, plant species, plant density). Moreover, constructed wetlands experiments are usually designed to test their efficiency for wastewater treatment, and not specifically to test the effect of plants on wastewater treatment. A detailed look on these papers suggest that either high organic loading, low plant density or inadequate redox conditions for root development could have influenced the absence of plant effects in constructed wetlands (Baptista et al., 2008; Ahn et al., 2007; DeJournett et al., 2007; Gorra et al., 2007), whereas in other works opposed conditions could suggest plant effects on their microbial communities (Wang et al., 2008; Li et al., 2008; Ruiz-Rueda et al., 2008; Ibekwe et al., 2007; Vacca et al., 2005; see also Stottmeister et al., 2003). This also fits with Ravit et al. (2006) suggestions that plant effects on associated microbial communities are less evident in human-disturbed (organically loaded) natural wetlands. Plant effects on their associated bacterial communities could be therefore more evident as physico-chemical conditions in constructed wetlands approach those in natural wetlands. In our work, plants fully covered all tanks and ground biomass reached high values (mean values of 10 and 4 kg DW/m^2 for *Typha* and *Phragmites* planted tanks, respectively) which maximize oxygen release into the rhizosphere (Wiessner et al., 2002). Moreover, organic load was kept much lower than recommended in constructed wetlands manuals (EPA, 2000). Under these circumstances, we were able to find significant differences between plant species with regard to the microbial community structure of their roots.

CONCLUSION

Constructed wetlands harbour microbial communities which play an important role in processes such as degradation of contaminants or nutrient cycling, therefore controlling wastewater treatment efficiency. These populations could be influenced by aspects like the type of plant species, organic matter load or hydraulic design. In this chapter, experimental constructed wetlands were used to study the effect of those factors in microbial communities of rhizoplane, gravel and interstitial water. Hydraulic design of the wetland (superficial or sub-superficial flow) seemed to be more important than plant presence in shaping microbial assemblages of their rhizosphere for *Typha* but not for *Phragmites* planted tanks. Microbial communities from roots, gravel biofilm and interstitial water were clearly different inside the same planted tank being these differences more important for *Phragmites* than *Typha* planted mesocosms. This paper gives therefore evidences to support the influence of wetland plant species on their root-attached microbial communities and their effect on their surrounding habitats, and also how this last influence is also driven by the hydraulic patterns of the wetland.

ACKNOWLEDGMENTS

The authors would like to thank Juan Carlos Sánchez, María Hijosa-Valsero, Mercedes López-Vázquez and Héctor Astiarraga for their valuable help in field and laboratory work. This work was funded by the projects MICROWET (CTM2005-06457-C05-03/TECNO), CONSOLIDER-TRAGUA (CSD2006-00044) and PET2008-0165-02.

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Fecal indicator bacteria resistance to antibiotics in experimental constructed wetlands[2]

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[2] Este capítulo corresponde con:
Sidrach-Cardona, R., Bécares, E., (2013). Fecal indicator bacteria resistance to antibiotics in experimental constructed wetlands, Ecological Engineering, Volume 50, Pages 107-111, 0925-8574 (JCR Impact factor: 2.958)

Ecological Engineering 50 (2013) 107–111

Contents lists available at SciVerse ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Fecal indicator bacteria resistance to antibiotics in experimental constructed wetlands

Ricardo Sidrach-Cardona[a,*], Eloy Bécares[b]

[a] Instituto de Medio Ambiente, Universidad de León, C/La Serna n° 58, 24007 León, Spain
[b] Facultad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain

ARTICLE INFO

Article history:
Received 10 September 2011
Received in revised form
26 December 2011
Accepted 5 January 2012
Available online 24 February 2012

Keywords:
Antibiotic resistant bacteria
Fecal indicators
Urban wastewater
Constructed wetlands
Activated sludge
Wastewater treatment

ABSTRACT

Antibiotic resistant bacteria (ARB) to amoxicillin, amoxicillin + clavulanic acid, azithromycin and doxycycline were tested in the effluent water of seven different pilot scale constructed wetlands (CW) and in an urban wastewater treatment plant (WWTP) treating the same influent. It was also analysed the ability of the CW to remove bacteria from the influent. CW differed from each other in flow type (free water surface flow, subsurface flow or hydroponics), plant species (*Typha angustifolia*, *Phragmites australis* or unplanted) and effluent position (upper part or bottom of the tank). Three groups of bacteria were tested, total Coliforms, *Escherichia coli*, and *Enterococcus*, and cultivated after membrane filtering in media with different antibiotic concentrations. No significant differences were found in the proportions of ARB among the CW, or between these and the WWTP. However, hydraulic design and plant presence were found to be extremely important in reducing total number of bacteria, which is related to the total number of resistant bacteria. Higher bacterial removal in CW (especially in those with subsuperficial flow) than in activated sludge means much lower ARB loadings to the environment by CW.

1. Introduction

Although antibiotics have a direct impact on the microorganisms of the receiving environment and in many cases are continuously emitted due to their widespread use, they have received comparatively little attention as pollutants (Constanzo et al., 2005). Indiscriminate use of these substances has resulted in an increase in antibiotic resistant bacteria (ARB) (Levy, 2002). This problem is so alarming that many countries monitor this resistance, mainly in hospitals, although usually focusing on pathogenic bacteria, while aquatic ecosystems receive much less attention (Servais and Passerat, 2009). The presence of enteric pathogens in aquatic environments can cause disease, risk of which is greater when bacteria are AR (Servais and Passerat, 2009). Fecal bacteria can also transmit the resistance to indigenous bacteria through lateral transfer, thus contributing to the spread of ARB (Davison, 1999). This is coupled with the direct discharge of antibiotics or their metabolites to the environment (Hijosa-Valsero et al., 2011), increasing selective pressure on the bacteria, and thus favouring resistance.

ARB have already been isolated from wastewaters, and high rates of resistance have been reported in wastewater treatment systems (da Costa et al., 2006; Reinthaler et al., 2003). While the removal efficiency of fecal bacteria has been widely studied in constructed wetlands for wastewater treatment (Decamp and Warren, 2000; Garcia et al., 2008; Molleda et al., 2008), to our knowledge, rate of resistance to antibiotics has not yet been tested in CW. Although a wide body of research exists in activated sludge (Fars et al., 2005; Lefkowitz and Duran, 2009; Schwartz et al., 2003) hydraulic and cellular retention times clearly differ between these and CW, impacting contact time and genetic exchange rates and therefore potential spread of antibiotic resistance genes (da Costa et al., 2006).

The present study aims to describe the abundance and elimination of antibiotic resistant fecal bacteria in different configurations of constructed wetlands receiving urban wastewater, and to compare them with conventional WWTP, evaluating the different configurations to optimise the removal of the resistance in fecal bacteria.

2. Methods

2.1. Description of constructed wetlands

The experiment was carried out between April 2008 and March 2009, samples being taken in winter and summer. In May 2007, seven pilot-scale constructed wetlands were arranged in the

* Corresponding author. Tel.: +34 987291000x3135.
E-mail addresses: rsidm@unileon.es (R. Sidrach-Cardona), ebecm@unileon.es (E. Bécares).

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doi:10.1016/j.ecoleng.2012.01.001

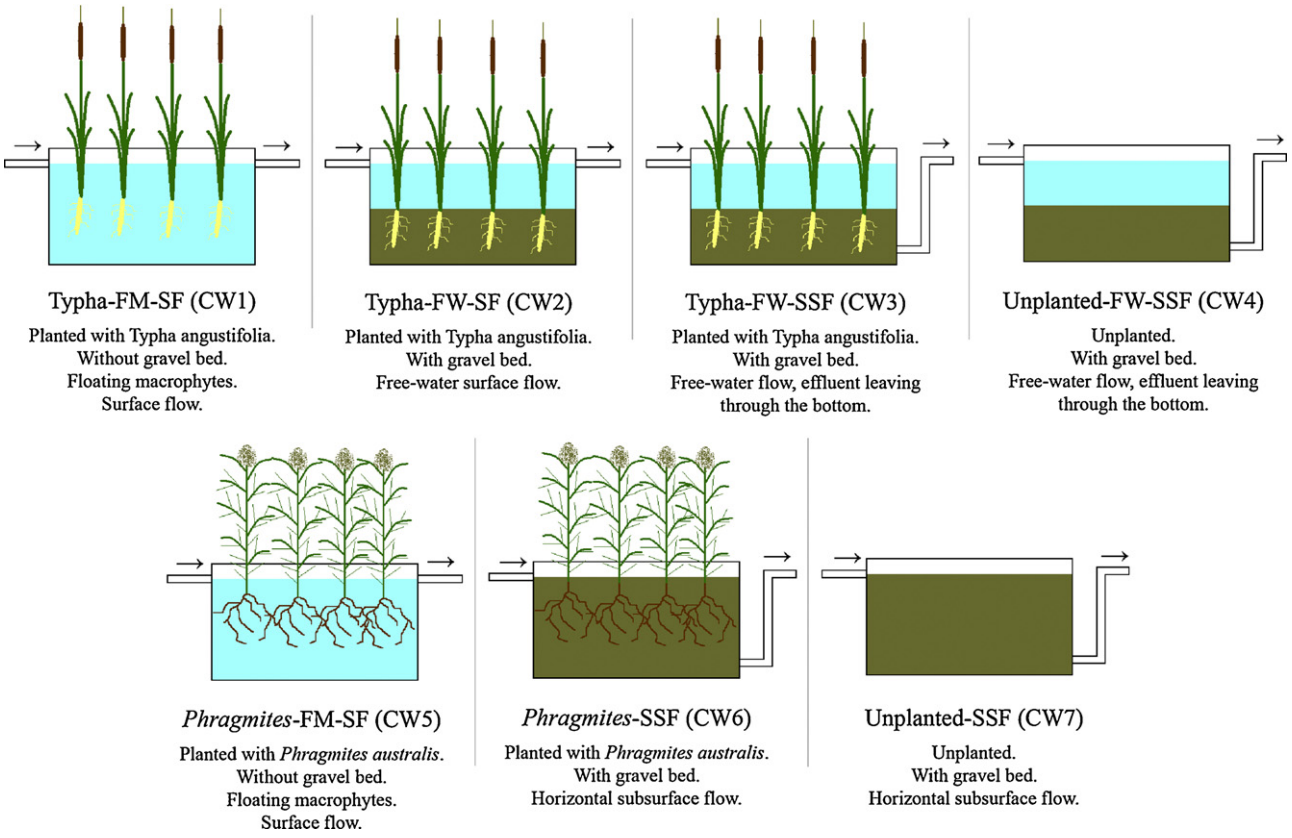

Fig. 1. Schematic design characteristics of the CWs. Systems CW1 and CW5 had a water depth of 30 cm and plant growth was supported by 20 cm long and 10 cm diameter garden-net cylinders (4 cm pore size). Systems CW2, CW3 and CW4 had a 25 cm layer of free-water (FW) over a 25 cm layer of siliceous gravel (d_{10} = 4 mm). Systems CW6 and CW7 consisted of a 45 cm siliceous gravel (d_{10} = 4 mm) layer, through which a 40 cm water layer flowed. Vegetation coverage was 100% in the planted systems.

open air inside the facilities at the León Wastewater Treatment Plant (WWTP), in the northwest of Spain. All constructed wetlands consisted of a fibreglass tank (80 cm width, 130 cm length, 50 cm height) with a surface area of 1 m^2. The constructed wetlands differed from each other in their design parameters (see supplementary material), summarised in Fig. 1 (Hijosa-Valsero et al., 2011). The experiment basically consisted of a simultaneous comparison of several wetlands with different plant and flow conditions, based on the most commonly used designs. In order to ascertain whether plant species impact treatment, two plant species *Typha angustifolia* and *Phragmites australis* were compared when growing in hydroponic conditions (i.e. without a gravel substrate) (tanks FM-SF). A conventional horizontal sub-surface flow system (SSF tank) planted with *Phragmites* was compared to two variations of the free-water (FW) surface flow design, both planted with *Typha*. One of the FW tanks was a surface flow system (FW-SF tank) with the effluent flowing from the surface of the water layer, which means that the gravel of the tank will not affect the treatment. The other tank used for comparison was a free-water sub-surface flow (FW-SSF tank) with the effluent flowing from the bottom of the tank in order to evaluate both the effect of the free-water layer and the potential effect of the gravel biofilm on treatment. In this case, the goal was to compare this FW-SSF tank with the previous FW-SF system and with the conventional SSF planted with *Phragmites*. Finally, to test for the effect of plants, unplanted tanks were included as controls for both the FW-SSF planted with *Typha* and the SSF tank planted with *Phragmites* (Fig. 1).

The aerial part of the plant was harvested at the end of the season during the experimental period although the roots remained inside the beds and continued growing until the experiment concluded. The experimental hydraulic retention time (HRT) values in summer of tanks CW1, CW2, CW3, CW4, CW5, CW6 and CW7 were 48.67, 40.40, 82.08, 90.83, 83.59, 150.72 and 80.13 h, respectively.

The León WWTP consists of a primary treatment (screening, sand removal, fat removal and primary clarifier) and a secondary treatment (plug-flow activated sludge with nitrification/denitrification and secondary clarifier). The plant was designed to treat the wastewater of 330,000 equivalent inhabitants with an inflow of 123,000 $m^3\ d^{-1}$, an HRT of about 6 h and a SRT of about 7 days in summer. Urban wastewater from the primary clarifier of León WWTP was piped to a 0.5 m^3 homogenisation tank. All the constructed wetlands were fed with this homogenised urban wastewater at a continuous flow rate of 50 $L\ d^{-1}$.

2.2. Sampling procedure

One litre samples from the CW influent and effluents and from the effluent of the León WWTP were taken over a year and a half into 3 periods (summer–winter–summer) in which it was sampling 2 times a week. A total of 31 samples were collected.

2.3. Antibiotic resistance

Four antibiotics were tested for each bacteria group; amoxicillin (A), azithromycin (AZ), amoxicillin + clavulanic acid (AC), and doxycycline (D).

Amoxicillin was prepared by diluting amoxicillin trihydrate (Fluka 31586) in water, and amoxicillin + clavulanic acid was prepared by mixing amoxicillin trihydrate (Fluka 31586) with potassium clavulanate (Sigma P3494) in a 4:1 rate diluted in water. Azithromycin was prepared by diluting it in ethanol and then in

water. Doxycycline was prepared by diluting doxycycline hyclate (Sigma D9891).

1000 mg L^{-1} solutions of each antibiotic were diluted directly in the culture media to obtain 5, 50 (A, AZ, AC for *Escherichia coli* and total coliforms), 1 and 10 mg L^{-1} (D and A, AZ, AC for *Enterococcus*) culture media without any antibiotic diluted in them being used as control (Schwartz et al., 2003). Strains of *E. coli* CET 516 were used as natural resistance control following the criteria established by the NCCLS (2004).

2.4. Sample processing and culture media

Samples from the CW influent and effluents and from the effluent of the León WWTP were diluted with physiological NaCl Solution (0.4%) and membrane filtered (S-Pak Membrane Filters, Millipore HAWG04756). Two replicates of each sample were made and the filters were applied on the culture media and incubated for 24 h (*E. coli* and total Coliforms) and 48 h (*Enterococcus*) (APHA, 1999).

Chromocult coliform agar (Merck 1.10426.0500) was used for *E. coli* and total Coliforms (TC), and SB agar (Membrane-filter *Enterococcus* selective agar acc. to Slanetz and Barley) (Merck 1.05262.0500) for *Enterococcus*.

2.5. Statistical analysis

Average bacteria counts were calculated from duplicate samples obtained from each CW. For statistical analyses, non-parametric Kruskall–Wallis ANOVA tests were carried out using statistical software (Statistica v8.0, Statsoft Inc.).

3. Results and discussion

3.1. Antibiotic resistance

Previous analyses evidenced no winter vs. summer differences in resistance percentages, both sampling periods being therefore considered together for the remainder of the study.

Both TC and *E. coli* had very high rates of resistance to amoxicillin (Fig. 2; 1A and 1B), showing percentages over 100%, although in most cases higher resistance was observed when the antibiotic concentration was greater, and when clavulanic acid was added (Fig. 2; 2A and 2B). As regards *Enterococcus,* resistance to amoxicillin was much lower and did not exceed 40% in the worst case (Fig. 2; 1C), in addition to which it was much more sensitive to the addition of clavulanic acid, exhibiting no growth when combined with the high concentration of amoxicillin (10 mg L^{-1}) (Fig. 2; 2C). These results are comparable to those found by other authors in wastewaters (Carroll et al., 2005; Fars et al., 2005; Lefkowitz and Duran, 2009).For azithromycin, the resistance rates in TC and *E. coli* are far lower than in the case of amoxicillin, and with high concentration (10 mg L^{-1}) no growth is observed in most cases (Fig. 2; 3A and 3B). *Enterococcus* resistance is very similar at both concentrations of azithromycin tested, and is also similar to the resistance rate of TC and *E. coli* to the low concentration of the antibiotic (Fig. 2; 3C).Doxycycline was also tested in the experiment, although no

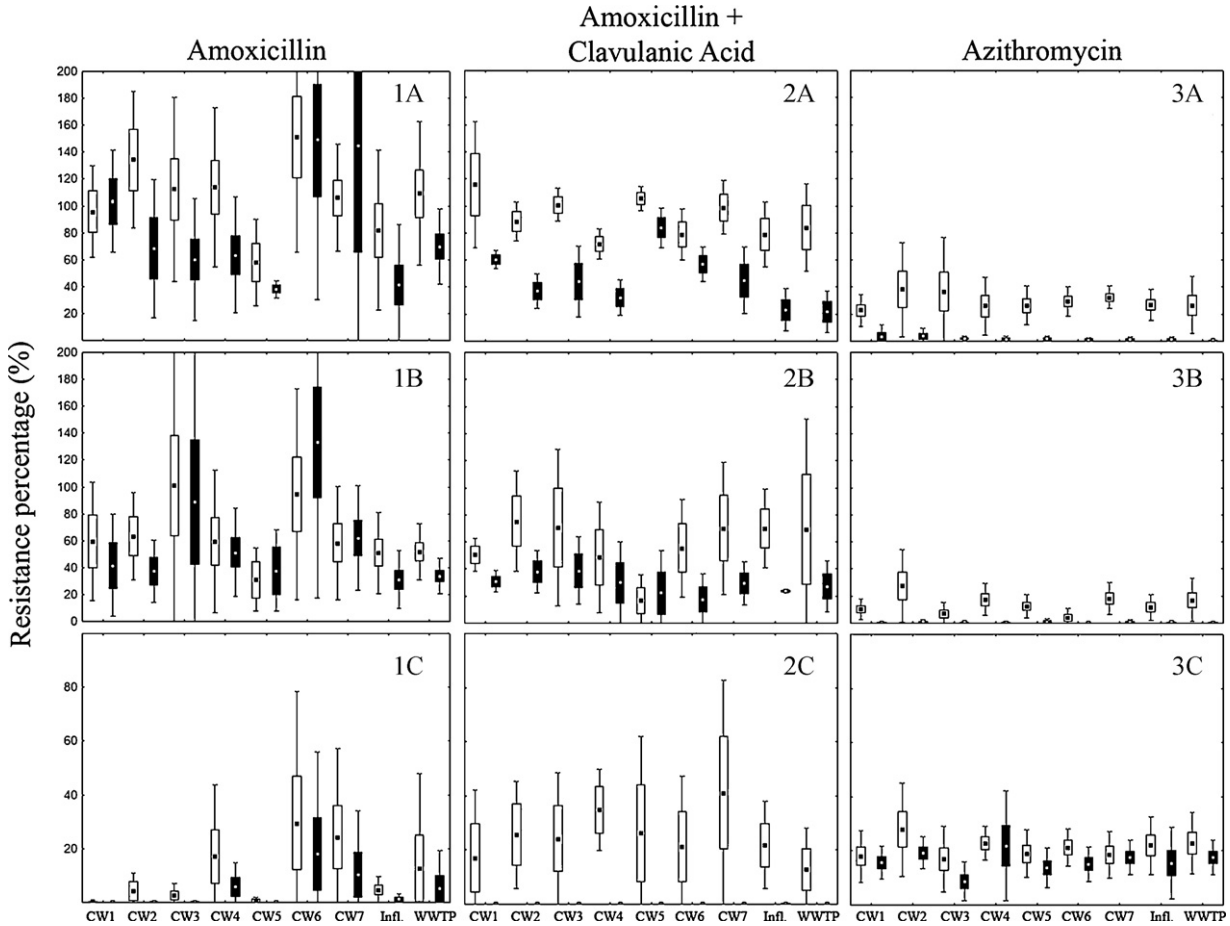

Fig. 2. Antibiotic resistance percentage in each constructed wetland (CW) in the wastewater treatment plant (WWTP), and in the common influent (Infl.). (A) Total coliforms, (B) *E. coli*, (C) *Enterococcus* sp. Low antibiotic concentration (white) and high antibiotic concentration (black). Mean ± standard error (Box) and standard deviation (Whisker).

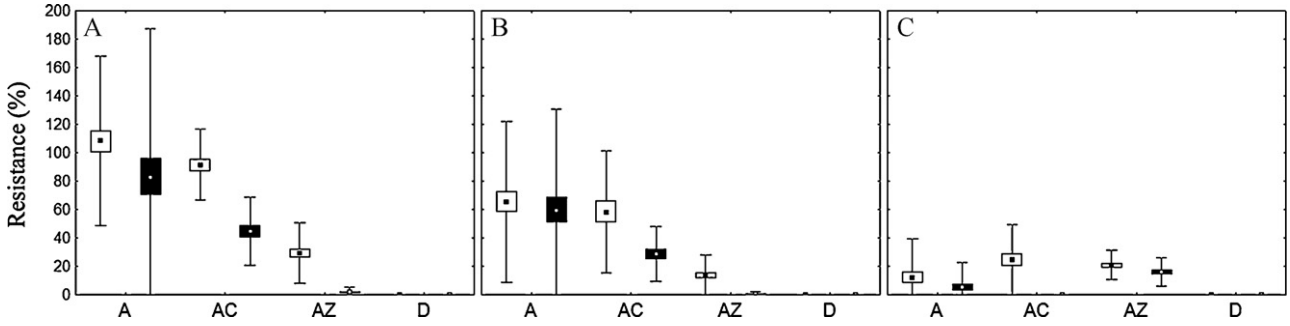

Fig. 3. Resistance percentage to each antibiotic. (A) Total coliforms, (B) *E. coli*, (C) *Enterococcus* sp. Low antibiotic concentration (white) and high antibiotic concentration (black). Mean ± standard error (Box) and standard deviation (Whisker).

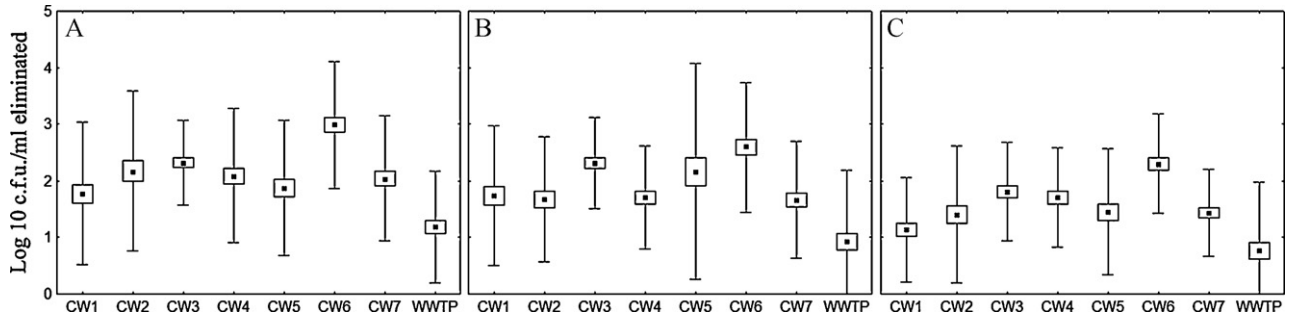

Fig. 4. Log 10 cfu/mL eliminated in each constructed wetland and in the WWTP. (A) Total coliforms, (B) *E. coli*, (C) *Enterococcus* sp. Mean ± standard error (Box) and standard deviation (Whisker).

resistance was found among the different bacteria groups in all the CW.

Non-parametric Kruskall–Wallis ANOVA tests were carried out to compare resistance among the different configurations of constructed wetlands, no significant differences being found, although in most cases resistance rates increased in all the CW from the influent to the effluent, as well as through the WWTP. The CWs with the highest increases in resistance rates were those with sub-surface flow, particularly CW6. This may be due to the transfer of plasmids encoding resistance to antibiotics being more effective in spatially organised communities because of the relative stability offered by the biofilm to the mating partners in the conjugation (Kalkum et al., 2002; Merlin et al., 2011) which occurs more easily in CW with flow through the solid matrix (CW6, CW7, CW3 and CW4). With regard to HRT it was found no statistically significant relationship with the resistance, this may be because the minimum time needed for the genetic exchange to take place is widely exceeded in all the CW.

Bacterial response to antibiotic resistance was strongly dependent on the antibiotic used. Considering all the CW, doxycycline presented no resistant bacteria in this study and azithromycin evidenced high rates of effectiveness, resistance percentages ranging between 0 and 30%. With regard to amoxicillin, almost all total Coliforms were resistant to this antibiotic, even after adding clavulanic acid (which is supposed to inhibit beta-lactamase). *E. coli* and particularly *Enterococcus* were, nevertheless, less resistant to both amoxicillin and its combination with clavulanic acid (Fig. 3).

3.2. Bacteria removal

As regards bacteria removal, all CW configurations worked significantly better than the WWTP ($p < 0.04$), reaching removals between 90 and 99%, values comparable to previous works (Garcia et al., 2008; Karathanasis et al., 2003; Molleda et al., 2008). Sub-surface flow CW, and particularly CW6 (*Phragmites*, SSF, gravel substrate), proved significantly better at removing all bacteria groups ($p < 0.034$ for TC, $p < 0.035$ for *E. coli*, and $p < 0.023$ for *Enterococcus*) than all other configurations except CW3, which was also significantly better than the other settings for the removal of *E. coli* ($p < 0.04$). All of these differences mean an average elimination in the CWs of 1.9 logarithm units [(2.2 log units in TC, 1.9 in *E. coli*, and 1.6 in *Enterococcus*) (in the case of CW6, the differences increase up to 2.6 log units in *E. coli*, 2.9 in TC, 2.3 log units in *Enterococcus*)], and 0.95 logarithm units in WWTP (1.2 log units in TC, 0.9 in *E. coli*, and 0.76 in *Enterococcus*) (see Fig. 4). Higher removal rates in CW6 may be due to a predatory effect. Chrysophyta algae bloom was detected during the study period, some authors having reported that certain genera of the group graze on bacteria, causing major losses in their abundance (Bird and Kalff, 1986; Unrein et al., 2010). Removal efficiency is lower in unplanted CW4 and CW7 than in their planted replicates, which means some plant effect (greater in the case of *Phragmites*) in the reduction of bacteria numbers, in line with previous studies (Decamp and Warren, 2000; Garcia and Becares, 1997; Garcia et al., 2008). The effect of HRT was also compared and no statistically differences were found, although it can be appreciate that CW with greater HRT have slightly higher capacity to remove bacteria (average slope = 0.0105). These differences in bacteria elimination, particularly between wetlands and the WWTP, means that, although the resistant bacterial load is similar in both cases, the amount of resistant bacteria that reaches the environment is greater in the case of conventional water treatments.

4. Conclusions

Antibiotic resistant bacteria were widely present in the studied wastewater, abundance rates being similar between conventional WWTP and the various CW configurations. Nevertheless, CWs

eliminate significantly more bacteria than WWTP, design parameters influencing their performance, those with sub-surface flow proving better than hydroponic, and planted better than unplanted. For this reason, although the percentage of antibiotic resistant bacteria in the effluents are similar between CW and WTTP, the abundance of bacteria in CW effluents were much lower than in the conventional activated sludge system which means much lower ARB loadings in the receiving environments.

Acknowledgments

This study was funded by the Spanish Ministry of Science and Innovation through project CTM2008-06676-C05-03. The authors wish to thank Diego Junquera for his help in field sampling and laboratory work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ecoleng.2012.01.001.

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Prevalence of antibiotic-resistant fecal bacteria in a river impacted by both an antibiotic production plant and urban treated discharges[3]

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[3] Este capítulo corresponde con:
R. Sidrach-Cardona, Hijosa-Valsero, M., Marti, E., Balcázar, J.L., Becares, E. (2014). Prevalence of antibiotic-resistant fecal bacteria in a river impacted by both an antibiotic production plant and urban treated discharges, Science of The Total Environment, Volumes 488–489, Pages 220-227. (JCR Impact factor: 4.099)

Science of the Total Environment 488–489 (2014) 220–227

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Prevalence of antibiotic-resistant fecal bacteria in a river impacted by both an antibiotic production plant and urban treated discharges

Ricardo Sidrach-Cardona [a], María Hijosa-Valsero [b], Elisabet Marti [c], José Luis Balcázar [c], Eloy Becares [a,b,*]

[a] Instituto de Medio Ambiente, Universidad de León, c/La Serna 58, 24007 León, Spain
[b] Facultad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain
[c] Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, c/Emili Grahit 101, 17003 Girona, Spain

HIGHLIGHTS

- Domestic effluents increased fecal bacteria concentration in the river.
- Antibiotic production effluents increased the prevalence of antibiotic resistance.
- Multirresistant *E. coli* increased in the river after both industrial and domestic effluents.

ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form 21 April 2014
Accepted 23 April 2014
Available online xxxx

Editor: D. Barcelo

Keywords:
Antibiotic resistance
Fecal indicators
Urban wastewater treatment plant
Antibiotic production plant
River
Sediments

ABSTRACT

In this study, the abundance and spatial dynamics of antibiotic-resistant fecal bacteria (*Escherichia coli*, total coliforms and *Enterococcus* spp.) were determined in water and sediment samples from a river impacted by both antibiotic production plant (APP) and urban wastewater treatment plant (WWTP) discharges. Agar dilution and disk diffusion methods were also used for antimicrobial susceptibility testing. Two antimicrobial agents, cephalexin (25 μg/ml) and amoxicillin (50 μg/ml), were evaluated using the agar dilution method for *E. coli*, total coliforms (TC) and *Enterococcus* spp., whereas the degree of sensitivity or resistance of *E. coli* isolates to penicillin (10 U), ampicillin (10 μg), doxycycline (30 μg), tetracycline (30 μg), erythromycin (15 μg), azithromycin (15 μg) and streptomycin (10 μg) was performed using the disk diffusion method. Real-time PCR assays were used to determine the prevalence of three antibiotic-resistance genes (ARGs). The agar dilution method showed that most *E. coli* isolates and TC were resistant to amoxicillin, especially after receiving the APP discharges. Antibiotic resistances to amoxicillin and cephalexin were higher after the APP discharge point than after the WWTP effluent. The disk diffusion method revealed that 100% of bacterial isolates were resistant to penicillin and erythromycin. Multidrug-resistant bacteria were detected and showed a higher proportion at the WWTP discharge point than those in the APP. Highly multidrug-resistant bacteria (resistance to more than 4 antibiotics) were also detected, reaching mean values of 41.6% in water samples and 50.1% in sediments. The relative abundance of the bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} genes was higher in samples from the treatment plants than in those collected upstream from the discharges, especially for water samples collected at the APP discharge point. These results clearly demonstrate that both the APP and the WWTP contribute to the emergence and spread of antibiotic resistance in the environment.

1. Introduction

Following the introduction of antibiotics into medical practice in the second half of the twentieth century, the over- and misuse of these compounds in human and veterinary medicine, animal husbandry, agriculture, aquaculture and food technology have resulted in an increase in antibiotic resistance and multidrug-resistant bacteria (Barbosa and Levy, 2000; Baquero et al., 2008).

Several studies suggest that antibiotic resistance detected in clinical settings is closely associated with mechanisms found in environmental bacteria. In fact, aquatic ecosystems may provide an ideal setting for the acquisition and spread of antibiotic resistance, because they are constantly exposed to anthropogenic environmental changes such as pollution from urban, agricultural, and industrial sources (Koczura et al., 2012, 2013; Korzeniewska et al., 2013; Marti et al., 2013).

Fecal bacteria may reach aquatic ecosystems not only by release of wastewater, but also through surface runoff and soil leaching (Servais

* Corresponding author at: Instituto de Medio Ambiente, Universidad de León, c/La Serna 58, 24007 León, Spain.
E-mail address: ebecm@unileon.es (E. Becares).

http://dx.doi.org/10.1016/j.scitotenv.2014.04.100
0048-9697/

and Passerat, 2009). The presence of fecal bacteria, including pathogens, in these ecosystems may be harmful to public health with a greater risk when bacteria are resistant to antibiotics. Antibiotic-resistant fecal bacteria can also transfer the resistance to autochthonous bacteria via horizontal gene transfer, which is responsible for the development of antibiotic-resistant bacteria (Davison, 1999). This together with the direct discharge of antibiotics or their metabolites (Hijosa-Valsero et al., 2011) increases selective pressure on bacteria, favoring the emergence and spread of antibiotic resistance.

Previous studies have demonstrated that antibiotic-resistant fecal bacteria may be found in different aquatic ecosystems including rivers (Boon and Cattanach, 1999; Ash et al., 2002; Watkinson et al., 2007a; Tao et al., 2010), estuaries (Parveen et al., 1997), lakes (Edge and Hill, 2005) and coastal areas (Kimiran-Erdem et al., 2007; de Oliveira and Pinhata, 2008). Several studies have also reported high rates of antibiotic-resistant bacteria in wastewater environments (Reinthaler et al., 2003; da Costa et al., 2006; Korzeniewska et al., 2013; Harris et al., 2014). However, the effect of industrial discharges, especially those from antibiotic manufacturing facilities, has only been partly explored. These discharges may be an important reservoir of antibiotic-resistant bacteria and antibiotic resistance genes (ARGs) due to the constant interaction between bacterial populations and antibiotic residues. Given this, the presence of both urban and industrial discharges, separated from each other by less than 1 km makes the study area particularly interesting for studying the effect of anthropogenic activities on the emergence and prevalence of antibiotic resistance.

The aims of this study were therefore to describe the abundance of antibiotic-resistant fecal bacteria and ARGs in water and sediment samples from a river receiving both urban and antibiotic production plant discharges and to study the variation rates of antibiotic resistance and number of bacteria along the river.

2. Materials and methods

2.1. Study site

Water and sediment samples were collected in the Bernesga River downstream from the city of León (Northwest Spain). The urban wastewater treatment plant (WWTP), a conventional activated sludge system treating waste from a population of 250,000 inhabitants including a hospital, is located 1 km downstream of a cephalexin and amoxicillin production plant (APP) with its own activated sludge treatment facility. Six samples were taken from different sites along the river, as well as two samples from the effluents of both treatment plants (Fig. 1) on three different days from August 20th to September 10th, 2010. There are no more discharges in the study area, the nearest one being 10 km upstream from sampling site 1 due to the presence of a small village.

2.2. Sample collection

Water samples were taken with a core along the whole width and depth of the river at each of the sampling sites and pooled to obtain a representative sample at that point. Sediments were also collected with a core, but, because of river morphology, could only be collected in the river areas where they accumulated.

2.3. Flow measurements

To calculate the river flow, current speed and depth were measured every 5 m in transects across the river. A model 2030R flowmeter (General Oceanics Inc.; Miami, FL, USA) was used to measure the current speed. River flow was measured at sampling sites 1 and 6, whose values were used to estimate the flow at the other points.

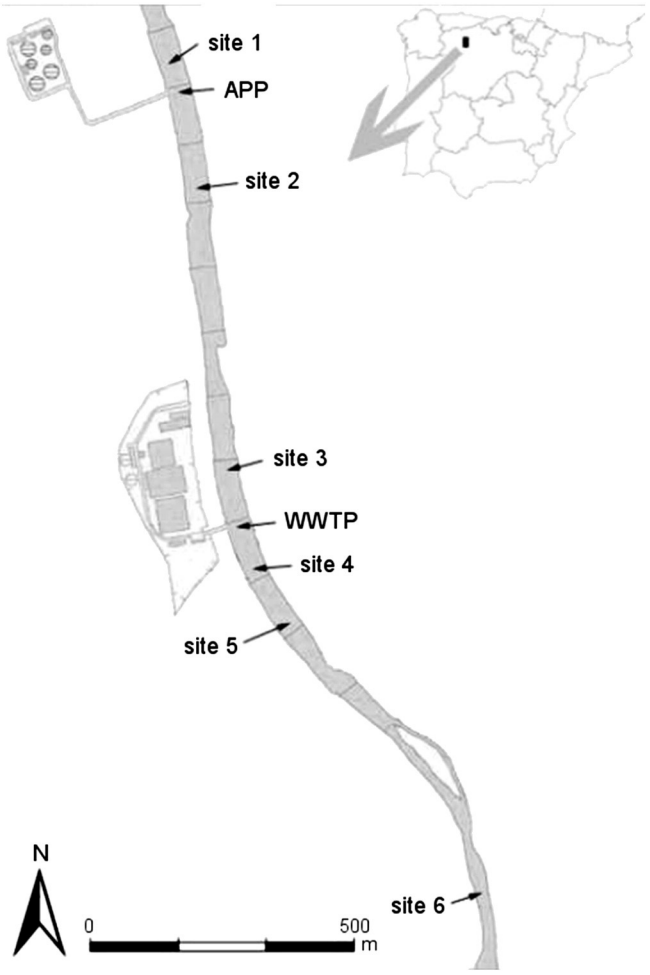

Fig. 1. Location of sampling sites in the Bernesga river. APP: antibiotic production plant; WWTP: urban wastewater treatment plant.

2.4. Enumeration and isolation of bacterial indicators

For the enumeration of *Escherichia coli* and total coliforms (TC), water samples were ten-fold diluted in sterile saline solution (0.4% NaCl) and filtered through a 0.45 μm membrane (Millipore; Darmstadt, Germany). The filters, in duplicate, were then placed on the culture media and incubated at 37 °C for 24 h (*E. coli* and TC) or 48 h (*Enterococcus* spp.) (APHA, 1999). Sediment samples were weighed (100 g each), added to a sterile phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) solution (ratio 50:50 w/v) and shaken to wash out and extract the bacteria; the supernatant was processed in the same way as the water samples (de Oliveira and Pinhata, 2008). For enumeration of sulfite-reducing *Clostridium*, 2 ml of water or sediment supernatant was mixed with the culture medium and incubated in an anaerobic atmosphere for 24 h (APHA, 1999).

Chromocult coliform agar (Merck; Darmstadt, Germany) was used for the isolation of *E. coli* and TC, membrane-filter *Enterococcus* selective (SB) agar (Merck) was used for *Enterococcus* spp., and sulfite polymyxin sulfadiazine (SPS) agar was used for sulfite-reducing *Clostridium* (Merck).

2.5. Antimicrobial susceptibility testing

Two methods were used to test the antimicrobial susceptibility, antibiotic dilution method and disk diffusion method. *E. coli* strain CECT 516 was used as a control following the criteria established by

the National Committee for Clinical Laboratory Standards (NCCLS, 2004).

2.5.1. Antibiotic dilution method

Antibiotics were added to the nutrient medium to evaluate the percentage of antibiotic-resistant bacteria by comparison with plating assays without antibiotics (Schwartz et al., 2003; Watkinson et al., 2007b). Two antibiotics, cephalexin and amoxicillin, which are produced by the APP, were tested using this method for each bacterial group (except *Clostridium*). Despite *Enterococcus* having been defined as intrinsically resistant to cephalosporins in human diseases (Murray, 1990), previous papers (e.g. Sidrach-Cardona and Becares, 2013) have proved their sensitivity to cephalexin in water samples. Stock solutions were prepared in sterile water, which were diluted directly in the culture media to get a final concentration of 25 μg/ml for cephalexin and 50 μg/ml for amoxicillin (Sidrach-Cardona and Becares, 2013).

2.5.2. Disk diffusion method

The agar disk diffusion method was carried out following NCCLS criteria (NCCLS, 2003). *E. coli* isolates, which were obtained on Chromocult agar plates, were used to prepare bacterial suspensions in trypticase soy broth (Conda-Pronadisa; Madrid, Spain) and cultured overnight. These suspensions were spread on Mueller–Hinton agar plates (Conda-Pronadisa) with a sterile handle, and antimicrobial disks (Becton, Dickinson and Co.; Franklin Lakes, NJ, USA) were then dispensed on the plates. After an incubation period of 24 h at 37 °C, the resistance of *E. coli* isolates was tested against 7 antimicrobials; two β-lactams [penicillin (10 U), ampicillin (10 μg)], two tetracyclines [doxycycline (30 μg), tetracycline (30 μg)], two macrolides [erythromycin (15 μg), azithromycin (15 μg)] and one aminoglycoside [streptomycin (10 μg)]. All isolates showing "resistant" or "intermediate resistant" patterns were classified as "resistant", whereas all other isolates were classified as "sensitive" (Reinthaler et al., 2003; Constanzo et al., 2005; Servais and Passerat, 2009).

2.6. Detection and quantification of ARGs

Water samples (500 ml) were collected at each sampling site and filtered through a 0.45 μm pore-size membrane (Millipore) in order to retain bacterial cells. The membranes were then resuspended in lysis buffer (20 mM Tris–Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100; and 20 mg/ml lysozyme), and genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen; Valencia, CA, USA), in accordance with the manufacturer's instructions. Sediment samples were also collected, weighed and homogenized in PBS solution (ratio 50:50 w/v). The homogenates were then resuspended in lysis buffer and genomic DNA was extracted as mentioned above.

Real-time PCR assays were used to quantify the bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} genes, which confer resistance on β-lactam antibiotics. All real-time PCR assays were performed on an Mx3005P system (Agilent Technologies; Santa Clara, CA, USA) using SYBR Green detection chemistry, as previously described (Marti et al., 2013). The following primers were used: for the bla_{TEM} gene, forward (5′-GCK GCC AAC TTA CTT CTG ACA ACG-3′) and reverse (5′-CTT TAT CCG CCT CCA TCC AGT CTA-3′); for the $bla_{CTX\text{-}M}$ gene, forward (5′-CTA TGG CAC CAC CAA CGA TA-3′) and reverse (5′-ACG GCT TTC TGC CTT AGG TT-3′); for the bla_{SHV} gene, forward (5′-CGC TTT CCC ATG ATG AGC ACC TTT-3′) and reverse (5′-TCC TGC TGG CGA TAG TGG ATC TTT-3′). All data were normalized using the 16S ribosomal RNA (rRNA) gene copy numbers (forward 5′-GTG STG CAY GGY TGT CGT CA-3′ and reverse 5′-ACG TCR TCC MCA CCT TCC TC-3′), as previously described (Maeda et al., 2003). Standard curves were generated by plotting *Ct* values against the known quantities of the cloned target gene.

2.7. Statistical analysis

Average bacterial counts were calculated from duplicate samples in three dilutions obtained at each sampling site. Due to the absence of normality, both non-parametric Friedman Test and Mann–Whitney U tests were used for data analysis using Statistica software, version 8.0 (StatSoft Inc.; Tulsa, OK, USA).

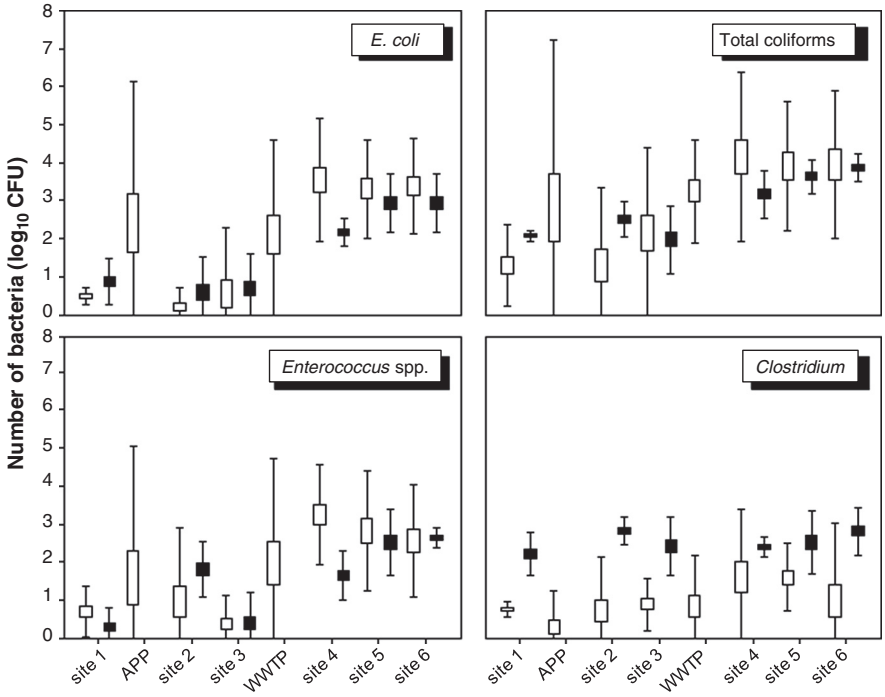

Fig. 2. Abundances of fecal indicators in the different water (white) (log_{10} CFUs ml^{-1}) and sediment (black) (log_{10} CFUs gDW^{-1}) samples. Box: mean ± standard error, whisker: mean ± standard deviation.

3. Results

3.1. Enumeration of fecal indicator bacteria

Mean values of fecal bacteria in water ranged over 4 logs, and showed an increase (2–3 logs) in colony-forming units (CFUs) in samples collected downstream of the treatment plants, especially after receiving the WWTP discharges (Fig. 2). The number of bacteria was low at sampling sites 1, 2 and 3; however, this concentration increased substantially at sampling sites 4, 5 and 6, which are located downstream of the WWTP discharge point. These values were even higher that those detected in the WWTP effluents. All differences between sampling sites became evident when the number of bacteria was related to the river and treatment plant flows, the trend being to increase with the rate of the bacterial loads (from 10^{11} CFUs/day at sampling site 1 to 10^{15} CFUs/day at sampling site 6 for *E. coli*, from 10^{12} to 10^{15} CFUs/day for TC, from 10^{12} to 10^{14} CFUs/day for *Enterococcus* spp., and from 10^{12} to 10^{13} CFUs/day for *Clostridium*). Flows were measured at site 1 (1.2 $m^3\ s^{-1}$) and site 6 (3 $m^3\ s^{-1}$), including the APP and WWTP discharges (0.6 and 1.2 $m^3\ s^{-1}$, respectively), thus it was possible to deduce the flow at all of the sampling sites (1.8 $m^3\ s^{-1}$ at sites 2 and 3 and 3 $m^3\ s^{-1}$ at sites 4 and 5).

In the sediment samples, the range of fecal bacteria concentration extended over 4 logs, and also showed a tendency to increase at those sites located downstream of the WWTP discharge point (Fig. 2). Abundance of *Clostridium* was very constant in sediment samples collected along the river, with no apparent influence of the treatment plant discharges (Fig. 2).

3.2. Antimicrobial susceptibility testing

3.2.1. Antibiotic dilution method

Collected water samples showed variable antibiotic resistance patterns. In the case of cephalexin, *Enterococcus* spp. was the group with the highest resistance, which reached 60% immediately after receiving the APP discharges, but it decreased along the river (Fig. 3). For amoxicillin, *E. coli* and TC had high resistance rates, especially after receiving the APP discharges (Fig. 3). No amoxicillin-resistant *Enterococcus* isolates were detected in water samples collected in the river or at the APP discharge point, whereas the rate of resistant isolates was about 10% at the WWTP discharge point (Fig. 3). In all cases (except for amoxicillin in *Enterococcus*) there were resistant isolates in the environment before receiving the treatment plant discharges.

Mann–Whitney U tests for *E. coli* resistance showed no significant differences in resistance rates among sampling sites for both cephalexin and amoxicillin in water samples. In the case of TC resistance to amoxicillin, there were significant differences ($p \leq 0.05$) between sites 2 and 3 and sites 5 and 6, but no differences were found for cephalexin in water samples with the exception of site 1 which differed from sites 2 and 3 ($p \leq 0.05$). In the case of *Enterococcus*, there were significant differences between site 3 and site 4 ($p = 0.04$), between site 3 and site 5 ($p = 0.04$), between APP and site 4 ($p = 0.01$), and between APP and site 5 ($p = 0.03$) for cephalexin (Fig. 3). The effect of the APP effluent on bacterial resistance in the river water was generally higher than that of the domestic WWTP effluent.

In the case of sediment samples, antibiotic resistance patterns were similar to those detected in water samples. *Enterococcus* and TC showed

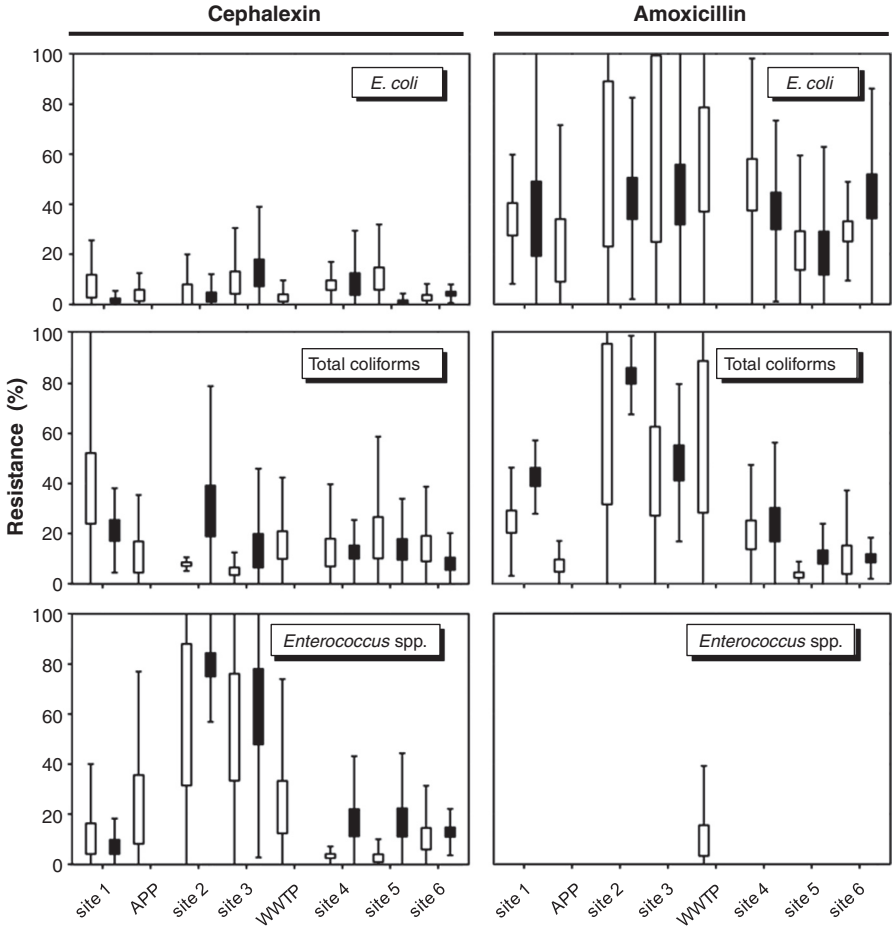

Fig. 3. Percentages of resistant fecal indicator to 25 μg/ml of cephalexin or 50 μg/ml of amoxicillin in water (white) and sediment (black) samples. Box: mean ± standard error, whisker: mean ± standard deviation.

Table 1
Antibiotic resistance patterns in *E. coli* isolates from water samples collected at each sampling site. APP: effluent from the antibiotic production plant; WWTP: effluent from the urban wastewater treatment plant.

Antibiotic	Concentration (μg)	Antibiotic-resistant *E. coli* isolates at each sampling site (%)							
		Site 1 ($n = 20$)	APP ($n = 5$)	Site 2 ($n = 6$)	Site 3 ($n = 19$)	WWTP ($n = 22$)	Site 4 ($n = 29$)	Site 5 ($n = 29$)	Site 6 ($n = 29$)
Ampicillin	10	75.0	40.0	83.3	94.7	81.8	82.8	75.9	65.5
Doxycycline	30	25.0	20.0	0.0	52.6	61.9	58.6	69.0	55.2
Tetracycline	30	35.0	20.0	16.7	63.2	59.1	75.9	58.6	65.5
Streptomycin	10	40.0	100.0	0.0	63.2	52.4	65.5	55.2	34.5
Erythromycin	15	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Azithromycin	15	11.8	0.0	16.7	31.6	9.1	17.2	17.2	20.7
Penicillin	10 (U)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

high resistance to cephalexin and reached their maximum level in the sediments after receiving the APP discharges (Fig. 3). Amoxicillin resistance was not observed in *Enterococcus* found in river sediments. *E. coli* and TC had a high resistance level to amoxicillin and their resistance rates remained almost constant along the river for *E. coli* (Fig. 3). A significant increase in amoxicillin resistance was observed in TC after receiving the APP discharges; however, a moderate decrease was observed along the river, even after receiving the WWTP effluent. Sampling sites 2 and 3 had higher rates of resistance to cephalexin in *Enterococcus* ($p < 0.01$) and amoxicillin in TC ($p < 0.01$) than those from other sites. These results may confirm that antibiotic-resistant bacteria are being accumulated in the sediments after receiving the APP discharges, and that the resistance caused by the APP effluent in the river sediment was higher than the resistance caused by the WWTP effluent.

3.2.2. Disk diffusion method

A total of 289 *E. coli* isolates from 14 sampling sites, including discharges, river water and sediments were tested using the disk diffusion method. It was observed that 100% of bacterial isolates were resistant to at least 2 antibiotics: penicillin and erythromycin. A low rate of resistance was observed against azithromycin, with maximum values of 31.6% in water samples. The highest rates of resistance were observed against doxycycline (from 25.0 to 55.2%), tetracycline (from 35.0 to 65.5%) and azithromycin (from 11.8 to 20.7%) along the river (Table 1). Additionally, patterns were quite different in sediment samples, because initial percentages of resistance were higher than those detected in water samples, which gradually decreased along the river (Table 2), especially against tetracycline (from 92.0 to 48.3%) and doxycycline (from 80.0 to 55.2%).

In addition to the percentage of resistant *E. coli* isolates to individual antibiotics at different sites on the river, there were also potential trends in the number of resistant bacteria, which could be grouped in multidrug-resistant bacteria (resistance to 4 or less antibiotics) and highly multidrug-resistant bacteria (resistance to more than 4). In this study, most isolates had multidrug resistance because of the high resistance rates to penicillin and erythromycin and, in the case of high multidrug resistance, mean values were 41.6% in water samples (Fig. 4) and 50.1% in sediments (Fig. 5). There were no isolates highly resistant to multidrugs in water samples collected at the APP discharge point (Fig. 4). In contrast, their concentration was 62% of the total isolates collected at the WWTP discharge point (Fig. 4).

3.3. Quantification of ARGs

Three ARGs, including bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV}, and the 16S rRNA gene were quantified by real-time PCR in the water and sediment samples. Relative concentrations of ARGs (normalized to the 16S rRNA gene copy number) at the sampling sites are shown in Fig. 6. All ARGs were detected in the samples, except the bla_{SHV} gene in some sediment samples. Relative abundances of the three ARGs were higher in water samples collected at the APP discharge point than those found at the first sampling site; however, the relative abundance of these genes decreased at the sites after receiving those discharges. Likewise, constant values of ARGs were observed in water samples collected downstream of the WWTP discharge. As regards sediment samples, differences were not observed in the relative concentration of the bla_{TEM} gene; however, the concentration of the $bla_{CTX\text{-}M}$ gene was higher in samples collected after receiving the WWTP discharges than that found at the other sampling sites, and the bla_{SHV} gene was detected only in sediment samples collected after receiving those discharges.

4. Discussion

Although antibiotic resistance is a natural phenomenon developed by bacteria as a defense mechanism or competitive strategy (Martinez, 2009), the selective pressure to which they are exposed, such as anthropogenic activities, favors the emergence and spread of antibiotic resistance (Alonso et al., 2001; Marti et al., 2014). The discharge of antibiotic-resistant bacteria into the aquatic environment may also contribute to the spread of antibiotic resistance among non-resistant bacterial communities through horizontal gene transfer processes (Davison, 1999).

Previous studies suggest that the mortality of fecal indicator bacteria is very high in extra-enteral habitats; however, their great abundance (Geldreich, 1990) and some environmental conditions may make them viable over a long period (Davies-Colley et al., 1999). This is especially relevant in sediments, which may act as a reservoir of bacteria (Howell et al., 1996; Alm et al., 2003; de Oliveira and Pinhata, 2008), due to the availability of nutrients (Davies et al., 1995), the extra

Table 2
Antibiotic resistance patterns in *E. coli* isolates from sediment samples collected at each sampling site.

Antibiotic	Concentration (μg)	Antibiotic-resistant *E. coli* isolates at each sampling site (%)					
		Site 1 ($n = 25$)	Site 2 ($n = 7$)	Site 3 ($n = 15$)	Site 4 ($n = 27$)	Site 5 ($n = 27$)	Site 6 ($n = 29$)
Ampicillin	10	100.0	100.0	100.0	81.5	44.4	82.8
Doxycycline	30	80.0	100.0	46.7	66.7	55.6	55.2
Tetracycline	30	92.0	57.1	66.7	70.4	51.9	48.3
Streptomycin	10	40.0	28.6	46.7	51.9	40.7	34.5
Erythromycin	15	96.0	85.7	100.0	100.0	100.0	100.0
Azithromycin	15	16.0	0.0	7.1	33.3	14.8	3.4
Penicillin	10 (U)	100.0	100.0	100.0	100.0	100.0	100.0

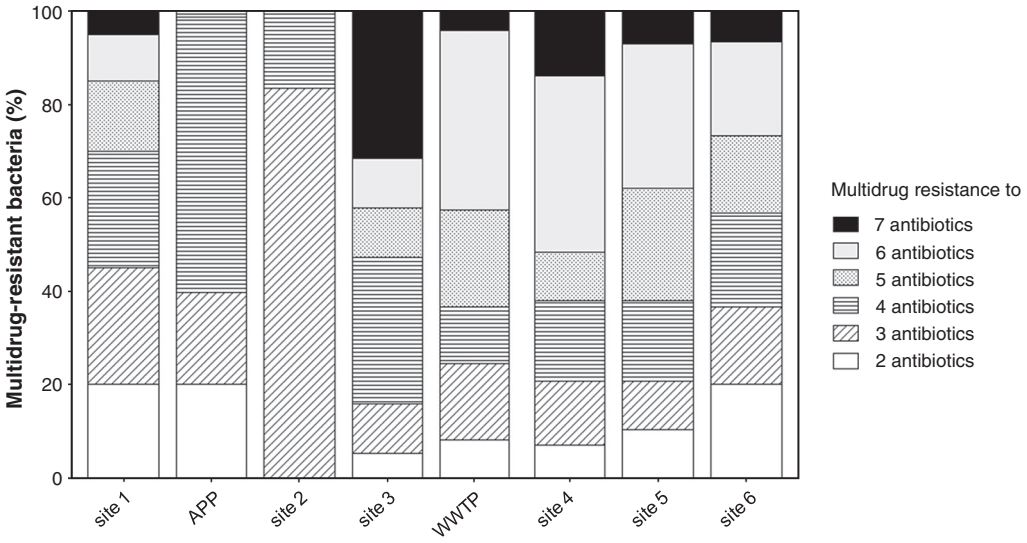

Fig. 4. Percentages of antibiotic-resistant *E. coli* isolates to different antibiotics in water samples collected at each sampling site.

protection they provide against light (Davies-Colley et al., 1999) and against predation by protozoa (Davies and Bavor, 2000). Several studies have demonstrated a strong correlation between bacterial concentration in sediment and water of different environments (freshwater shores and beaches) (Junco et al., 2001; Alm et al., 2003; de Oliveira and Pinhata, 2008), indicating the existence of a continuous flow of microorganisms between the two environments, including the exchange of genetic material such as ARGs. All these factors may be affecting the resistance rates to cephalexin and amoxicillin which were high in sediment samples collected at sites 2 and 3 but subsequently decreased along the river.

In general, water treatment plants reduce the number of bacteria between 1 and 3 logs in the incoming water (Hirata et al., 1993; Reinthaler et al., 2003); however, this reduction may not be accompanied by a reduction in the number of antibiotic-resistant bacteria: on the contrary, the percentage of antibiotic-resistant bacteria increases (da Costa et al., 2006). In this study, despite fecal bacteria concentrations being very similar in APP and WWTP effluents, their contribution to the increase in cephalexin and amoxicillin resistance were completely different. It is possible that *Enterobacteriaceae* released by the APP have had a high rate of genetic exchange with the autochthonous bacterial populations or that ARGs have reached the environment through other bacterial species or bacteriophages (Roszak and Colwel, 1987; Ash et al., 2002), which may explain why resistance increased strongly at the sampling sites after receiving the APP discharges. The expected high concentration of antibiotics in the APP and the working temperature of the reactor (about 25–27 °C) could be selecting activated sludge bacteria with a high flow of ARGs but low ability to survive in the river. In fact, the relative abundance of the bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} genes, which confer resistance on β-lactam antibiotics such as cephalexin and amoxicillin, was higher in water samples collected at the APP discharge point than that found at the first sampling site; however the abundance of these genes decreased at the sites after receiving those discharges. Likewise, the relative abundance of these ARGs, especially the $bla_{CTX\text{-}M}$ and bla_{SHV} genes, also increased in the sediment samples collected at the sites after receiving the WWTP discharges.

WWTPs are considered hot spots for the emergence and spread of antibiotic resistance, because they provide an ideal environment for gene transfer, as bacteria are in continuous direct contact with antibiotics and antibiotic-resistant bacteria (Rizzo et al., 2013; Marti et al., 2014). In this study the percentage of antibiotic resistance in *E. coli* in the WWTP effluent (Table 1) was negatively related ($r^2 = 0.53$, $p = 0.07$) to how long antibiotics have been used over decades, considering that penicillin and streptomycin have been used since the 40s, streptomycin

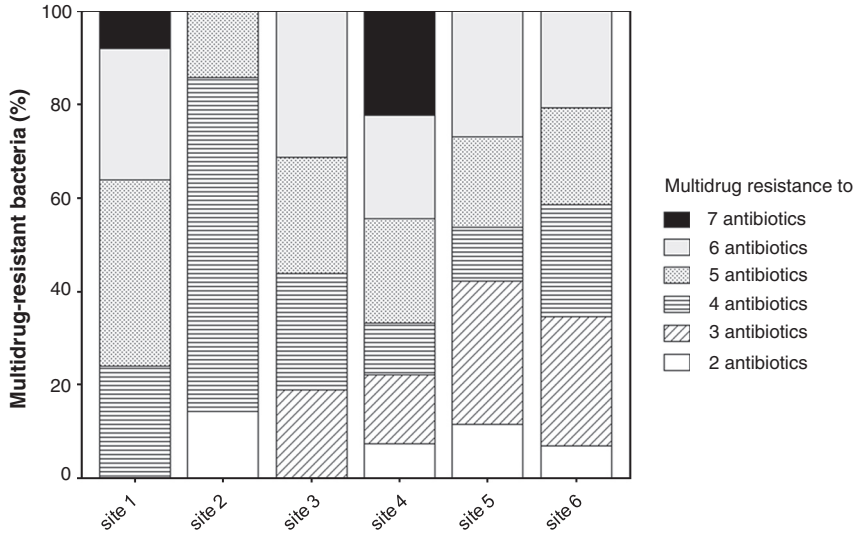

Fig. 5. Percentages of antibiotic-resistant *E. coli* isolates to different antibiotics in sediment samples collected at each sampling site.

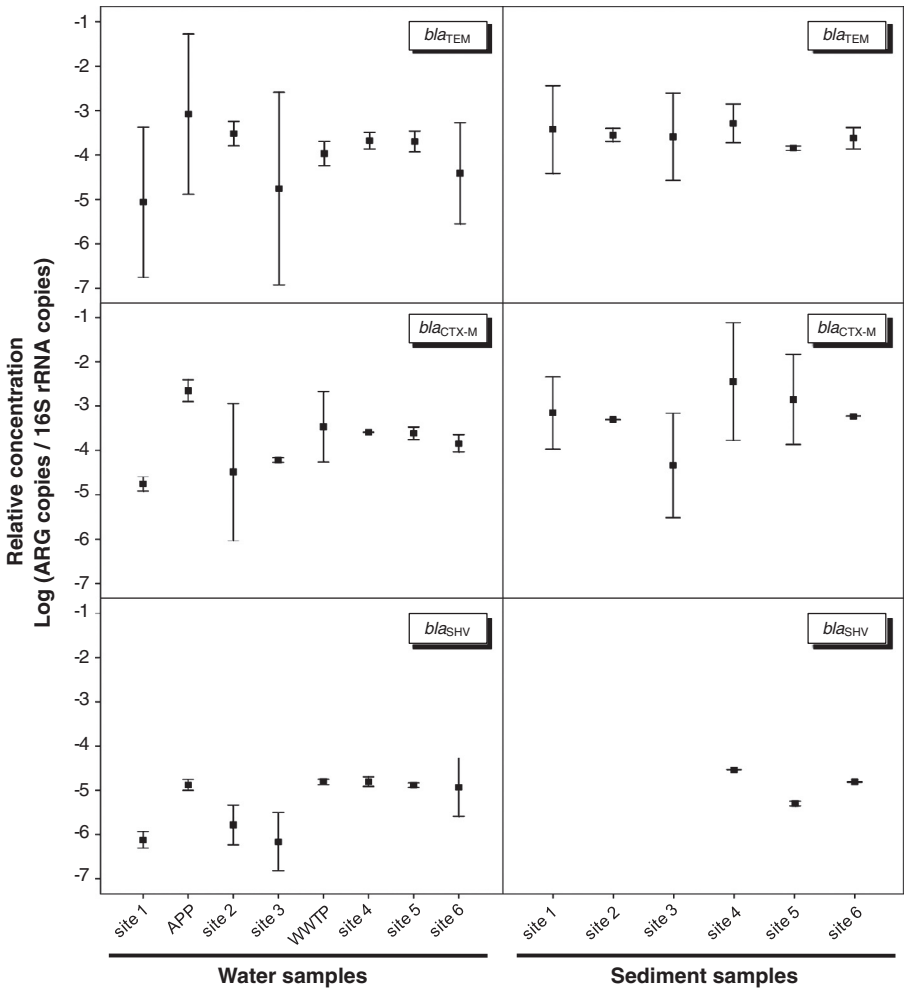

Fig. 6. Relative concentration of ARGs in water and sediment samples collected at each sampling site. Mean values of ARGs are represented by black squares with their standard errors.

and erythromycin since the 50s, ampicillin and doxycycline since the 60s and azithromycin since the 90s. This supports the relationship between the years of use of antibiotics and bacterial resistance (Alonso et al., 2001).

Additionally, highly multidrug-resistant bacteria were detected in both water and sediment samples at the sampling sites, even before receiving the treatment plant discharges. However, the frequency of multidrug-resistant bacteria (more than 4 antibiotics) increased at the sites after receiving those discharges. The distribution of multidrug-resistant bacteria in water samples was very similar between the first and last sampling sites. This result provides evidence that, despite the concentration of bacteria tending to increase in both water and sediment samples after receiving the discharges as previously observed in rivers (Goñi-Urriza et al., 2000), the percentage of multidrug-resistant bacteria tends to remain constant in the river once selective pressure decreases.

5. Conclusions

This study demonstrates that both APP and WWTP discharges increased antibiotic resistance in fecal bacteria located at the nearest sampling site downstream of the discharges. However, antibiotic resistance decreased as the distance to the effluents increased, probably because of the 'dilution effect' on antibiotics and therefore their selective pressure. Multidrug-resistant bacteria were also detected, their percentages being related to the type of water treatment process. Moreover, the high survival of bacteria released by the discharges and the high proportion of antibiotic-resistant bacteria and ARGs in these discharges suggest that treatment plants (APP and WWTP) may be an important pathway for the spread of antibiotic resistance in the environment. Efficient and effective treatment of APP and WWTP discharges should, therefore, be considered as the first priority for counteracting the emergence and spread of antibiotic resistance.

Acknowledgments

This study was supported by "Fundación MAPFRE". Ricardo Sidrach-Cardona was awarded a grant by the Spanish Ministry of Science and Innovation through project CTM2008-06676-C05-03. José Luis Balcázar acknowledges the Ramon y Cajal research fellowship (RYC-2011-08154) from the Spanish Ministry of Economy and Competitiveness. The authors thank Amando Escudero and Diego Junquera for their help in field sampling.

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8 Otros artículos publicados

1. **Pedescoll, R. Sidrach-Cardona, M. Hijosa-Valsero, E. Bécares, Design parameters affecting metals removal in horizontal constructed wetlands for domestic wastewater treatment, Ecological Engineering, Volume 80, July 2015, Pages 92-99, ISSN 0925-8574, http://dx.doi.org/10.1016/j.ecoleng.2014.10.035.**

As regards constructed wetlands (CWs), there is a great deal of research on metals removal, although comparison of different parameters under the same conditions is scarce. The aim of this study was to determine the most important factors affecting the removal efficiency and dynamics of metals and metalloids according to different configurations of horizontal CWs. An experimental plant, including the most commonly used CWs, was analysed for several metals (Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn and Zn). Arsenic, which was under the detection limits at the influent, presented a release in those wetlands with subsurface flow (SSF) and followed the same pattern as iron and manganese. The presence of vegetation and flow type were key design factors affecting metals removal from urban wastewater. Free water surface (FWS) flow provided favourable conditions for the removal of As, Fe and Mn, which are sensitive to redox changes, whereas SSF slightly enhanced the removal of other metals, such as Cu or Pb. On the other hand, vegetation was not able to maintain steady oxidised conditions to guarantee redox dependent metals removal by combination with oxides in SSF systems. In contrast, constant reduced conditions promoted the long-term removal of metals by sulphide combination and precipitation.

2. **Pedescoll, A. Sidrach-Cardona, R. Sanchez, J. C. Becares, E. 2013. Evapotranspiration affecting redox conditions in horizontal constructed wetlands under Mediterranean climate: Influence of plant species. ECOLOGICAL ENGINEERING 58, 335-343. DI 10.1016/j.ecoleng.2013.07.007**

The aim of this study was to conduct a comparative evaluation of evapotranspiration (ET) rates for eight different mesocosm constructed wetlands (CWs), and the relationship with redox potential (E-H). Inflow, outflow and E-H were measured over 4 years in winter and summer campaigns as well as over 24 h on selected days in summer. Vegetation was the main design parameter which affected water loss in the wetlands (on average, ET in planted wetlands was 4 times higher than in unplanted ones), and *Typha angustifolia* was more active than *Phragmites australis* (mean daily ET - expressed as the average of ET rate measured every 2 h in selected days in summer - was 36.8 +/- 2.3 mm d(-1) and 23.0 +/- 1.9 mm d(-1) for hydroponic wetlands planted with cattail and common reed, respectively), although P. australis water use efficiency was lower. Positive relationships were found between ET and E-H for planted wetlands. Cattail presented a stronger linear regression than common reed, demonstrating that ET and consequently redox conditions are plant species-dependent.

3. **Pedescoll, A. Sidrach-Cardona, R. Sanchez, J. C. Carretero, J. Garfi, M. Becares, E. 2013. Design configurations affecting flow pattern and solids accumulation in horizontal free water and subsurface flow constructed wetlands. WATER RESEARCH 47-3, 1448-1458 DI 10.1016/j.watres.2012.12.010**

The aim of this study was to evaluate the effect of different horizontal constructed wetland (CW) design parameters on solids distribution, loss of hydraulic conductivity over time and hydraulic behaviour, in order to assess clogging processes in wetlands. For this purpose, an experimental plant with eight CWs was built at mesocosm scale. Each CW presented a different design characteristic, and the most common CW configurations were all represented: free water surface flow (FWS) with different effluent pipe locations, FWS with floating macrophytes and subsurface flow (SSF), and the presence of plants and specific species (*Typha angustifolia* and *Phragmites australis*) was also considered. The loss of the hydraulic conductivity of gravel was greatly influenced by the presence of plants and organic load (representing a loss of 20% and c.a. 10% in planted wetlands and an overloaded system, respectively). Cattail seems to have a greater effect on the development of clogging since its below-ground biomass weighed twice as much as that of common reed. Hydraulic behaviour was greatly influenced by the presence of a gravel matrix and the outlet pipe position. In strict SSF CW, the water was forced to cross the gravel and tended to flow diagonally from the top inlet to the bottom outlet (where the inlet and outlet pipes were located). However, when FWS was considered, water preferentially flowed above the gravel, thus losing half the effective volume of the system. Only the presence of plants seemed to help the water flow partially within the gravel matrix.

4. **Garfi, Marianna. Pedescoll, Anna. Becares, Eloy. Hijosa-Valsero, Maria. Sidrach-Cardona, Ricardo. Garcia, Joan. 2012. Effect of climatic conditions, season and wastewater quality on contaminant removal efficiency of two experimental constructed wetlands in different regions of Spain. SCIENCE OF THE TOTAL ENVIRONMENT 437, 61-67. DI 10.1016/j.scitotenv.2012.07.087**

The aim of this study was to examine the effects of climate, season and wastewater quality on contaminant removal efficiency of constructed wetlands implemented in Mediterranean and continental-Mediterranean climate region of Spain. To this end, two experimental horizontal subsurface flow constructed wetlands located in Barcelona and Leon (Spain) were compared. The two constructed wetland systems had the same experimental set-up. Each wetland had a surface area of 2.95 m(2), a water depth of 25 cm and a granular medium of D-60 = 73 mm, and was planted with *Phragmites australis*. Both systems were designed in order to operate with a maximum organic loading rate of 6 g(DBO) m(-2) d(-1). Experimental systems operated with a hydraulic loading rate of 28.5 and 98 mm d(-1) in Barcelona and Leon, respectively. Total suspended solids, biochemical oxygen demand and ammonium mass removal efficiencies followed seasonal trends, with higher values in the summer (97.4% vs. 97.8%; 97.1% vs. 962%; 99.9% vs. 88.9%, in Barcelona and Leon systems, respectively) than in the winter (83.5% vs. 74.4%; 73.2% vs. 60.6%; 19% vs. no net removal for ammonium in Barcelona and Leon systems, respectively). During the cold season, biochemical oxygen demand and ammonium removal were significantly higher in Barcelona system than in Leon, as a result of higher temperature and redox potential in Barcelona. During the warm season, statistical differences were observed only for ammonium removal. Results showed that horizontal subsurface flow constructed wetland is a successful

technology for both regions considered, even if winter seemed to be a critical period for ammonium removal in continental climate regions.

5. **María Hijosa-Valsero, Ricardo Sidrach-Cardona, Eloy Bécares, Comparison of interannual removal variation of various constructed wetland types, Science of The Total Environment, Volume 430, 15 July 2012, Pages 174-183, ISSN 0048-9697, http://dx.doi.org/10.1016/j.scitotenv.2012.04.072.**

Seven mesocosm-scale (1 m2) constructed wetlands (CWs) of different configurations were operated outdoors for thirty-nine months under the same conditions to assess their ability to remove organic matter and nutrients from urban wastewaters. CWs differed in some design parameters, namely the presence of plants, the species chosen (i.e., *Typha angustifolia* or *Phragmites australis*), the flow configuration (i.e., surface flow or subsurface flow) and the presence/absence of a gravel bed. It was observed that, in general, removal efficiencies decreased with the aging of the system and that seasonality had a great influence on CWs. A comparison was made in order to figure out which kind of CW was more efficient for the removal of every pollutant in the long term. Planted systems were clearly better than unplanted systems even in winter. Efficiency differences among CWs were not extremely great, especially after a few years. However, some types of CWs were more adequate for the removal of certain pollutants. The effect of the aging on the main parameters involved in pollutant removal in CWs (temperature, pH, conductivity, dissolved oxygen concentration and redox potential) was assessed. The efficiency of CWs should not be evaluated based on short monitoring periods (1–2 years) after the start-up of the systems, but on longer periods.

6. **Reyes-Contreras, Carolina. Hijosa-Valsero, Maria. Sidrach-Cardona, Ricardo. Bayona, Josep M. Becares, Eloy. 2012. Temporal evolution in PPCP removal from urban wastewater by constructed wetlands of different configuration: A medium-term study. CHEMOSPHERE 88-2, 161-167. DI 10.1016/j.chemosphere.2012.02.064**

Pharmaceuticals and personal care products (PPCPs) are widely distributed in urban wastewaters and can be removed to some extent by constructed wetlands (CWs). The medium-term (3-5 years) behaviour of these systems regarding PPCP removal is still unknown. Seven mesocosm-scale (1 m(2)) CWs of different configurations were operated outdoors for 39 months under the same conditions to assess their PPCP removal ability and temporal evolution. CWs differed in some design parameters, namely plant presence, species chosen (Typha angustifolia vs Phragmites australis), flow configuration and presence/absence of gravel bed (floating macrophytes surface flow, FM-SF; free-water surface flow, FW-SF; free-water subsurface flow, FW-SSF; or conventional horizontal subsurface flow, SSF). PPCP efficiencies decreased throughout time and performance differences among CWs disappeared with the systems aging. This could be due to a homogenization process in the systems caused by detrimental factors like saturation, clogging and shading. Winter efficiencies were lower than summer ones for salicylic acid, caffeine, methyl dihydrojasmonate, galaxolide and tonalide, and seasonal biological activities seem key factors to explain this fact. Maximal removal efficiencies were achieved in an unplanted-FW-SSF for ketoprofen (47-81%), naproxen (58-81%) and salicylic acid (76-98%); in an unplanted-SSF for caffeine (65-99%); in a Phragmites-FM-SF for ibuprofen (49-96%) and diclofenac (16-68%); in a Typha-FM-SF for carbamazepine (35-71%); and in a Typha-FW-SSF for methyl dihydrojasmonate (71-96%), galaxolide (67-82%) and tonalide (55-74%). Photodegradation could be involved in ketoprofen, naproxen, ibuprofen and

diclofenac removal. Carbamazepine and diclofenac were moderately removed by the most efficient CWs studied. Carbamazepine might be eliminated by vegetal uptake.

7. **Hijosa-Valsero, Maria. Sidrach-Cardona, Ricardo. Martin-Villacorta, Javier. Cruz Valsero-Blanco, M. Bayona, Josep M. Becares, Eloy. 2011. Statistical modelling of organic matter and emerging pollutants removal in constructed wetlands. BIORESOURCE TECHNOLOGY 102-8, 4981-4988. DI 10.1016/j.biortech.2011.01.063**

Multiple regression models, clustering tree diagrams, regression trees (CHAID) and redundancy analysis (RDA) were applied to the study of the removal of organic matter and pharmaceuticals and personal care products (PPCPs) from urban wastewater by means of constructed wetlands (CWs). These four statistical analyses pointed out the importance of physico-chemical parameters, plant presence and chemical structure in the elimination of most pollutants. Temperature, pH values, dissolved oxygen concentration, redox potential and conductivity were related to the removal of the studied substances. Plant presence (*Typha angustifolia* and *Phragmites australis*) enhanced the removal of organic matter and some PPCPs. Multiple regression equations and CHAID trees provided numerical estimations of pollutant removal efficiencies in CWs. These models were validated and they could be a useful and interesting tool for the quick estimation of removal efficiencies in already working CWs and for the design of new systems which must fulfil certain quality requirements.

8. **Hijosa-Valsero, Maria. Fink, Guido. Schluesener, Michael P. Sidrach-Cardona, Ricardo. Martin-Villacorta, Javier. Ternes, Thomas. Becares, Eloy. 2011. Removal of antibiotics from urban wastewater by constructed wetland optimization. CHEMOSPHERE 83-5, 713-719. DI 10.1016/j.chemosphere.2011.02.004**

Seven mesocosm-scale constructed wetlands (CWs), differing in their design characteristics, were set up in the open air to assess their efficiency to remove antibiotics from urban raw wastewater. A conventional wastewater treatment plant (WWTP) was simultaneously monitored. The experiment took place in autumn. An analytical methodology including HPLC-MS/MS was developed to measure antibiotic concentrations in the soluble water fraction, in the suspended solids fraction and in the WWTP sludge. Considering the soluble water fraction, the only easily eliminated antibiotics in the WWTP were doxycycline (61 +/- 38%) and sulfamethoxazole (60 +/- 26%). All the studied types of CWs were efficient for the removal of sulfamethoxazole (59 +/- 30-87 +/- 41%), as found in the WWTP, and, in addition, they removed trimethoprim (65 +/- 21-96 +/- 29%). The elimination of other antibiotics in CWs was limited by the specific system-configuration: amoxicillin (45 +/- 15%) was only eliminated by a free-water (FW) subsurface flow (SSF) CW planted with *Typha angustifolia*; doxycycline was removed in FW systems planted with *T. angustifolia* (65 +/- 34-75 +/- 40%), in a *Phragmites australis*-floating macrophytes system (62 +/- 31%) and in conventional horizontal SSF-systems (71 +/- 39%); clarithromycin was partially eliminated by an unplanted FW-SSF system (50 +/- 18%); erythromycin could only be removed by a *P. australis*-horizontal SSF system (64 +/- 30%); and ampicillin was eliminated by a *T. angustifolia*-floating macrophytes system (29 +/- 4%). Lincomycin was not removed by any of the systems (WWTP or CWs). The presence or absence of plants, the vegetal species (*T. angustifolia* or *P. australis*), the flow type and the

CW design characteristics regulated the specific removal mechanisms. Therefore, CWs are not an overall solution to remove antibiotics from urban wastewater during cold seasons. However, more studies are needed to assess their ability in warmer periods and to determine the behaviour of full-scale systems.

9. **Hijosa-Valsero, M. Matamoros, V. Sidrach-Cardona, R. Pedescoll, A. Martin-Villacorta, J. Garcia, J. Bayona, J. M. Becares, E. 2011. Influence of design, physico-chemical and environmental parameters on pharmaceuticals and fragrances removal by constructed wetlands. WATER SCIENCE AND TECHNOLOGY 63-11, 2527-2534. DI 10.2166/wst.2011.500**

The ability of several mesocosm-scale and full-scale constructed wetlands (CWs) to remove pharmaceuticals and personal care products (PPCPs) from urban wastewater was assessed. The results of three previous works were considered as a whole to find common patterns in PPCP removal. The experiment took place outdoors under winter and summer conditions. The mesocosm-scale CWs differed in some design parameters, namely the presence of plants, the vegetal species chosen (Typha angustifolia versus Phragmites australis), the flow configuration (surface flow versus subsurface flow), the primary treatment (sedimentation tank versus HUSB), the feeding regime (batch flow versus continuous saturation) and the presence of gravel bed. The full-scale CWs consisted of a combination of various subsystems (ponds, surface flow CWs and subsurface flow CWs). The studied PPCPs were ketoprofen, naproxen, ibuprofen, diclofenac, salicylic acid, carbamazepine, caffeine, methyl dihydrojasmonate, galaxolide and tonalide. The performance of the evaluated treatment systems was compound dependent and varied as a function of the CW-configuration. In addition, PPCP removal efficiencies were lower during winter. The presence of plants favoured naproxen, ibuprofen, diclofenac, salicylic acid, caffeine, methyl dihydrojasmonate, galaxolide and tonalide removal. Significant positive correlations were observed between the removal of most PPCPs and temperature or redox potential. Accordingly, microbiological pathways appear to be the most likely degradation route for the target PPCPs in the CWs studied.

10. Hijosa-Valsero, Maria. Sidrach-Cardona, Ricardo. Martin-Villacorta, Javier. Becares, Eloy. 2010. Optimization of performance assessment and design characteristics in constructed wetlands for the removal of organic matter. CHEMOSPHERE 81-5, 651-657. DI 10.1016/j.chemosphere.2010.08.010

Some of the most used constructed wetland (ON) configurations [conventional and modified free-water (FW) flow, surface flow, conventional horizontal subsurface flow (SSF) and soilless systems with floating macrophytes (FM)] were assessed in order to compare their efficiencies for the removal of organic pollutants [COD, filtered COD (FCOD), BOD and total suspended solids (TSS)] from urban sewage under the same climatic and wastewater conditions. The removal performance was calculated using three approaches: effluent concentrations, areal removed loads and mass removal. Results were very different depending on the approach, which indicates that the way to present CW efficiency should be considered carefully. All CW-configurations obtained BOD effluent concentrations below 25 mg L(-1) in summer, with a FW-CW with effluent leaving through the bottom of the tank being the only one maintaining low BOD effluent concentrations even in winter and under high organic loading conditions. In this kind of CW, the presence of plants favoured pollutant removal. SSF-CWs were the most efficient for the removal of COD. FM systems can be as efficient as some gravel bed CWs. *Typha angustifolia* worked better than *Phragmites australis*, at least when the systems were at the beginning of their operation period.

11. Hijosa-Valsero, Maria. Matamoros, Victor. Sidrach-Cardona, Ricardo. Martin-Villacorta, Javier. Becares, Eloy. Bayona, Josep M. 2010. Comprehensive assessment of the design configuration of constructed wetlands for the removal of pharmaceuticals and personal care products from urban wastewaters. WATER RESEARCH 44-12, 3669-3678. DI 10.1016/j.watres.2010.04.022

Seven mesocosm-scale constructed wetlands (CWs) of different configurations were operated outdoors for nine months to assess their ability to remove pharmaceuticals and personal care products (PPCPs) from urban wastewaters. CWs differed in some design parameters, namely the presence of plants, the species chosen (i.e., *Typha angustifolia* vs *Phragmites australis*), flow configuration (i.e., surface flow vs subsurface flow) and the presence of a gravel bed. A nearby conventional activated-sludge wastewater treatment plant (WWTP) fed with the same sewage was simultaneously monitored for comparison. The PPCPs ketoprofen, naproxen, ibuprofen, diclofenac, salicylic acid, carbamazepine, caffeine, galaxolide, tonalide and methyl dihydrojasmonate were monitored. The presence of plants favoured the removal of some PPCPs. The performance of the mesocosm studied was compound-dependant, soilless CWs showing the highest removal efficiency for ketoprofen, ibuprofen and carbamazepine, while free-water CWs with effluent leaving through the bottom of the tank performed well for the degradation of ketoprofen, salicylic acid, galaxolide and tonalide. Finally, subsurface horizontal flow CWs were efficient for the removal of caffeine. Significant linear correlations were observed between the removal of some PPCPs and temperature or redox potential. Hence, microbiological pathways appear to be the most probable degradation route for PPCPs in the CWs studied.