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Kadiya del Carmen Calderón Alvarado

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MEMORIA PARA OBTENER EL GRADO DE DOCTOR.

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- A. González-Martínez; **K. Calderón**; A. Albuquerque; E. Hontoria; J. González-López; I.M. Guisado; F. Osorio. 2012. *Biological and technical study of a partial SHARON reactor at laboratory scale: Effect of hydraulic retention time*. Bioprocess Biosys. Eng. Accepted Manuscript; DOI: 10.1007/s00449-012-0772-7.

- Lopez-Lopez C., Martín-Pascual J., González-Martínez A., **Calderón K**., González-López J., Hontoria E., Poyatos JM. 2012. *Influence of filling ratio and carrier type in a moving bed biofilm reactor with pretreatment of electrocoagulation in wastewater treatment*. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng. 47:1759-67.

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Pies, ¿para que los quiero? si tengo alas para volar.....

Frida Kahlo

Que el corazón del universo esté en mi corazón,

Que mi corazón este en el corazón de la tierra,

Que el corazón de la tierra esté en mi corazón,

Y que mi corazón este en el corazón del universo.

Oración maya.

ÍNDICE

RESUMEN

RESUMEN

La presente tesis consiste en la agrupación de trabajos publicados basados en el estudio de la microbiota involucrada en los procesos depuradores para el tratamiento de aguas residuales urbanas mediante sistemas de biorreactores de membranas (MBR), así como de un reactor de biopelícula (MBBR) para su posible incorporación a los sistemas MBR.

Diferentes acercamientos a los dos tipos de MBR (aerobio y anaerobio) han sido realizados centrándose en los puntos clave de estos sistemas para el tratamiento de agua residual urbana. Se realizó en primer lugar una evaluación de la estructura de la biopelícula taponante formada en un sistema anaerobio MBR a escala piloto, mediante microscopía electrónica de barrido y dispersión de rayos X. Para su comparación, se realizaron controles con membranas alimentadas tanto con efluente del reactor anaerobio (UASB) como con agua residual procedente del decantador primario. La microbiota presente en las membranas taponadas fue además analizada por electroforesis en gel con gradiente de temperatura (TGGE) y secuenciación parcial de genes codificantes del 16S rRNA. Se observaron diferencias significativas en la estructura de la comunidad bacteriana entre las diferentes biopelículas taponantes analizadas en las membranas UASB, particularmente después de una limpieza química efectuada con NaClO, mientras que las comunidades de arqueas fueron más similares en todas las muestras y más resistentes a las limpiezas químicas. Las comunidades predominantes identificadas estuvieron relacionadas con Firmicutes y Alphaproteobacteria, así como *Methanosarcinales* y *Methanospirillaceae*.

Se analizaron también las características biológicas de un sistema aerobio MBR a escala piloto utilizando oxígeno puro como fuente de aireación, en comparación con la aireación convencional por aire, para el estudio de su influencia sobre la comunidad bacteriana presente en el fango del biorreactor y los niveles de sus actividades enzimáticas hidrolíticas. Así, se empleó el análisis de redundancia (RDA) para revelar la relación que existe entre los niveles enzimáticos y la variación de la fuente de aireación y otros parámetros operacionales, demostrándose un efecto significativo por el efecto de la concentración de sólidos suspendidos volátiles (VSS) y la temperatura, mas no por la influencia del oxígeno puro aplicado al fango del MBR. La estructura de la comunidad bacteriana fue examinada mediante TGGE, y no se observaron diferencias significativas en la diversidad analizada por el índice de Shannon-Wiener (*H'*) ni en la organización funcional de la comunidad determinada mediante el índice *Fo*; sin embargo, los estudios de fingerprinting realizados muestran cambios significativos de la estructura de la comunidad microbiana dependiendo de la fuente de aireación utilizada. En el global de los experimentos, se revela la presencia prevalente de poblaciones evolutivamente relacionadas con Alphaproteobacteria en el fango del biorreactor.

Por último, con la finalidad de realizar un estudio a escala piloto para una posible futura incorporación de los sistemas de soporte en suspensión (MBBR) al fango de los reactores MBR, se analizó la comunidad bacteriana desarrollada sobre tres diferentes materiales de soporte, que fueron analizados bajo tres diferentes tiempos de retención hidráulica (HRT) y porcentaje

de relleno (FR). Mediante análisis de redundancia (RDA) se determinó la relación entre las condiciones operacionales (tipo de soporte, HRT y FR) y la diversidad bacteriana de la biopelícula, demostrando que el único parámetro estadísticamente significativo es el FR, siendo el óptimo el de 50%. En estos sistemas, la comunidad predominante de las biopelículas consiste de Betaproteobacterias, seguida de Alpha- y Gammaproteobacteria.

1. INTRODUCCIÓN

La contaminación ambiental, el constante incremento de desechos tóxicos, el calentamiento global y la disminución en las reservas de los combustibles fósiles constituyen actualmente problemas prioritarios para las autoridades y gobiernos correspondientes (Kalia et al., 2008). Por ello, durante los últimos años se han adoptado políticas ambientales dirigidas al manejo de los mismos.

En materia de agua, el mal uso y manejo aplicado por el hombre ha contribuido a que la calidad de la misma sea cada vez menor. Así, el aumento en el desarrollo de industrias, el crecimiento de la población, el uso de fármacos y productos de limpieza así como las prácticas agrícolas han sido unos de los principales factores para que los recursos hídricos sufran un consumo desmesurado y estén actualmente tan deteriorados (Trapani et al., 2010).

El término "tratamiento de agua" es un concepto amplio que implica la combinación de procesos y operaciones unitarias para modificar la calidad de la misma hasta el grado requerido para el uso o disposición a la cual se destina (Jiménez-Cisneros, 2001). Además del carbono, otros de los nutrientes en materia de remoción con más interés en el tratamiento de aguas, son el nitrógeno y el fósforo (Metcalf y Eddy, 2003). Dentro de los sistemas biológicos, el de fangos activos resulta insuficiente para la remoción de estos nutrientes así como la de microorganismos patógenos (Tackett et al., 2003). En este sentido, cabe resaltar que tanto el fósforo como el nitrógeno en forma de amonio, resultan altamente tóxicos para los microorganismos acuáticos a causa de la eutrofización, y por la oxidación de amonio a nitrito (Plattes et al., 2007).

Dada la problemática presente en materia de agua, es necesario buscar soluciones a los procesos biológicos actuales para su tratamiento, que permitan cumplir no sólo con los límites establecidos de vertido y calidad, sino también que se amolden a las necesidades de la población por las variaciones de carga del agua residual, espacios reducidos para la ubicación de la planta y la mínima producción de fango.

Innumerables esfuerzos se han realizado en materia de legislación por las autoridades correspondientes para resolver el problema, sin embargo, las diferentes tecnologías generadas aún siguen presentando diversas limitaciones (Le-Minh et al., 2010; Le-Clech, 2010).

Una opción avanzada para el tratamiento de aguas residuales urbanas son los sistemas de membranas (Le-Clech, 2010; Dereli et al., 2012). Es por ello que para la realización de esta tesis se optó por el estudio de los sistemas de biorreactores de membranas, tanto anaerobio (AnMBR) como aerobio (MBR), y el sistema de biorreactor de membrana de lecho móvil (MBBR).

1. Biorreactores de Membrana (MBR).

1.1 Fundamento de los MBR

Con la creciente necesidad de mejorar la eficiencia en los procesos de tratamiento de agua residual tanto industrial como municipal, la tecnología de biorreactores de membranas (MBR) ha recibido considerable atención. Después de unas cuantas décadas de su existencia, los MBR pueden ahora considerarse como un sistema establecido para el tratamiento de agua residual que compite directamente con sistemas convencionales como el de lodos activos (Le-Clech, 2010).

Sin embargo, el proceso de MBR todavía sufre inconvenientes importantes, incluyendo los altos costos de operación debido al empleo de estrategias anti-taponamiento para mantener las condiciones de filtración requeridas en el sistema. Además, este uso específico de membranas no ha alcanzado su plena madurez todavía, pues tanto proveedores como usuarios de MBR aún carecen de experiencia en cuanto al funcionamiento a largo plazo del sistema. Más aún, mejoras en el diseño y operación de los MBR han sido desarrolladas en los últimos años, haciendo de los MBR una opción de elección para el tratamiento de aguas residuales y su reutilización (Le-Clech, 2010).

El proceso de biorreactor de membrana (MBR) es generalmente descrito como la combinación de un tratamiento de biodegradación por fango activo y la separación líquido/sólido por membranas de filtración. La presencia de membranas de micro o ultra-filtración permite significativas mejoras y ventajas cuando los MBR son comparados con el proceso de lodos activos, los cuales requieren de pasos limitantes como la sedimentación o la coagulación (Stephenson y Judd, 2000; Judd, 2006; Le-Clech, 2010). La utilización de membranas como proceso de separación hace que se incremente la calidad del efluente producido. Al haber una retención física completa de flóculos de bacterias y la mayoría de los sólidos suspendidos, los MBR pueden ofrecer una buena capacidad de desinfección, ofreciendo una reducción de coliformes alrededor de un promedio de 10^6 (Hirani et al., 2010).

La retención total del lodo activo en el biorreactor también permite la operación bajo alta concentración de sólidos suspendidos del licor mezcla (MLSS) y elevados tiempos de retención de sólidos (SRT), que posibilita a su vez el lento crecimiento de los microorganismos responsables de la degradación de contaminantes orgánicos específicos (especialmente, compuestos nitrogenados) y el bajo rendimiento de fango, resultando en una minimización de la producción del mismo. Por otra parte, las altas concentraciones de MLSS permiten así la reducción del tamaño del biorreactor (Rosenberger et al., 2002; Le-Clech, 2010), lo que unido a la ausencia de los decantadores permite la instalación y operación de las plantas en pequeños espacios.

La figura 1.1 muestra la operación general de los MBR comparada con la de lodos activos. El influente entra en el biorreactor, donde la biomasa microbiana, al igual que en el caso de los lodos activos, permite la degradación biológica de la materia orgánica. Posteriormente, la

mezcla es bombeada del biorreactor y, bajo presión, filtrada a través de la membrana. El agua filtrada es descargada del sistema, mientras que la biomasa microbiana es retenida en el biorreactor. De manera periódica el exceso de lodo se purga con el fin evitar que madure. Las membranas se limpian periódicamente, generalmente mediante lavado a contracorriente o retrolavado, y en algunas ocasiones mediante lavado químico o ambos.

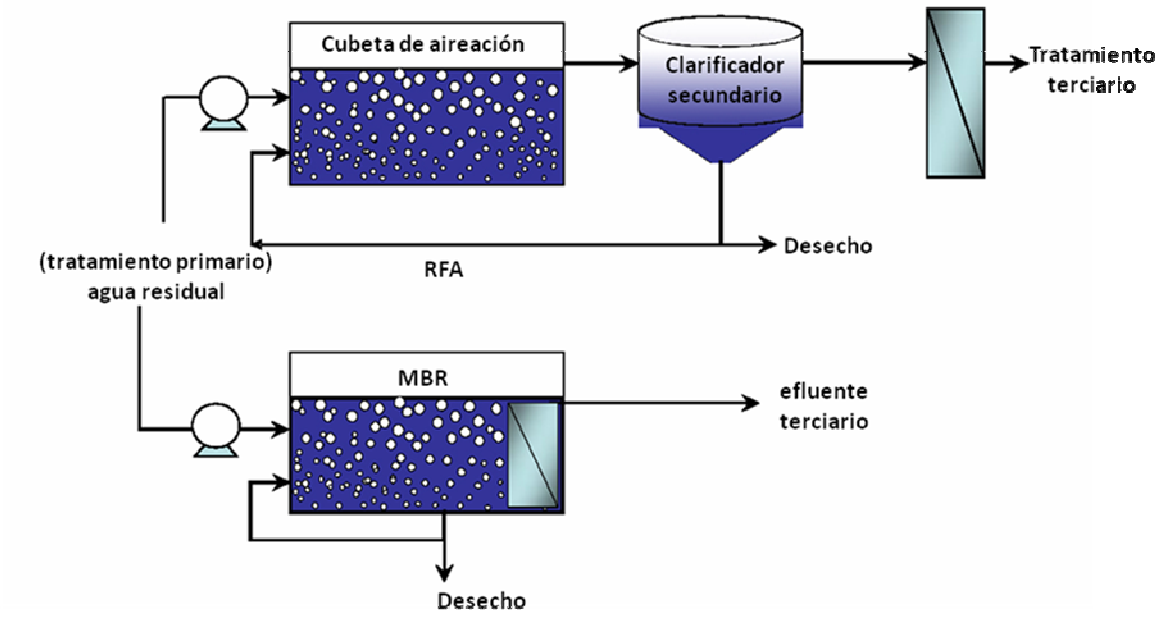

Fig. 1.1 Comparación entre el proceso convencional de fango activo y MBR. (Modificado de Le-Clech, 2010).

1.2 Diseño y tipo de MBR

Desde 1980 con la primera generación de los MBR, el diseño de los mismos ha sido crucial y está fundamentalmente basado en la configuración de la membrana. De esta manera los MBR se pueden clasificar en dos tipos;

a) *Biorreactor de membrana inmersa o sumergida (sMBR).* La unidad de membrana que realiza la separación física está inmersa en el tanque biológico. La fuerza impulsora a través de la membrana es alcanzada presurizando el biorreactor o creando presión negativa en el lado permeado de la membrana, que es lo más común (Rosenberger et al., 2002). Generalmente, se coloca un difusor de aire justo debajo del módulo de membrana para suministrar el aire necesario para homogeneizar el licor mezcla. Con esto también se consigue crear un régimen turbulento de aire en la capa límite de la membrana, que evita la formación de biopelícula que acaba por obstruir la membrana. En la figura 1.2 (a) se muestra un esquema de este tipo de biorreactor de membrana (Metcalf y Eddy, 2003).

b) *Biorreactor de membrana externa o de recirculación.* En este tipo de MBR la membrana se encuentra fuera del reactor biológico, y el licor de mezcla se recircula desde el biorreactor hasta el módulo de membrana por medio de una bomba capaz de generar cierta presión, aproximadamente 30 kPa (Manem y Sanderson, 1998) Fig. 1.2 (b). Este tipo de sistemas son raramente desarrollados a escala real principalmente por el alto consumo de energía que requiere la bomba de recirculación (Le-Clech, 2010).

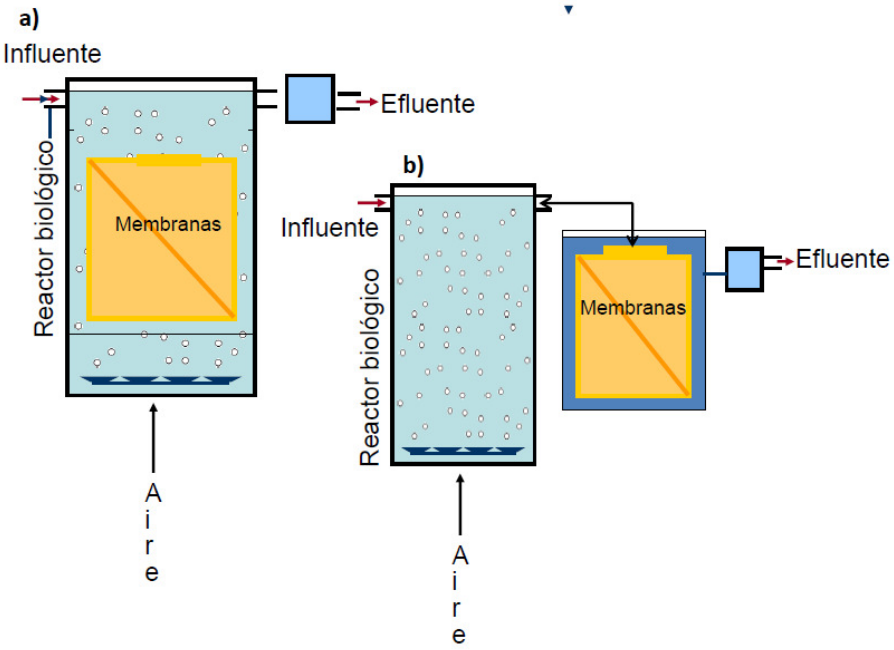

Fig. 1.2 Esquemas que representan las dos configuraciones de biorreactores de membrana. a) MBR integrado o sumergido y b) MBR externa o de recirculación.

Por otra parte, los MBR pueden subclasificarse como aerobios o anaerobios (AnMBR). Sobre estos últimos se tienen pocos estudios y la mayoría de ellos es sobre su empleo para tratamiento de agua residual industrial así como de la proveniente de destilerías de alcohol. Los AnMBR se definen como una combinación entre un reactor anaerobio acoplado a una unidad de membrana (Baek y Pagilla, 2003).

Estudios realizados por Harada et al., (1994) y Baek y Pagilla, (2003) sugieren que los AnMBR son adecuados para el tratamiento de agua residual que contiene alta carga orgánica. Las principales ventajas de los AnMBR son la posibilidad de recuperar energía gracias al biogás generado (metano) y la baja producción de lodos y bajos requerimientos nutricionales (Huang et al., 2010). Además, la remoción de nitrógeno, fósforo y amonio puede esperarse del proceso realizado con un AnMBR (Baek y Pagilla, 2003).

El principal problema de los sistemas es el taponamiento (fouling) generado por las sustancias orgánicas e inorgánicas y que no ha logrado ser completamente controlado. Este problema se presenta en los dos tipos de MBRs, pero por estudios realizados se ha observado que es

considerablemente mayor en los sistemas anaerobios por las altas concentraciones de materia coloidal final y precipitación inorgánica (Saddoud y Sayadi, 2007). Para tratar de controlar el taponamiento, la mayoría de los MBR aerobios trabajan con membranas sumergidas, sin embargo, son procesos más costosos en su operación debido a la alta energía necesaria para promover la aireación (Berube y Soucy, 2004).

2. Taponamiento y control de taponamiento en los sistemas MBR

Uno de los principales inconvenientes de los MBR reside en el indeseable depósito de materiales en la superficie de las membranas. Este fenómeno de taponamiento o fouling ha sido estudiado por diferentes grupos durante las últimas décadas.

Fouling o taponamiento es el término dado a la disminución de la capacidad de filtración en la membrana debido a la acumulación sobre su superficie de partículas coloidales, sólidos suspendidos (flóculos de materia orgánica, microorganismos y partículas inertes) y material soluble (materiales disueltos provenientes del agua residual y productos microbianos solubles (SMP) excretados por la biomasa activa) (Le-Clech et al. 2006; Miura et al., 2007; Drews, 2010).

Una primera disminución del flujo de permeado viene causada por la concentración-polarización. Este término se utiliza para describir la tendencia del soluto a acumularse en la membrana. A medida que el permeado atraviesa la membrana, los solutos que contenía se quedan en las proximidades de su superficie, este soluto forma una capa de líquido estancado cuya velocidad tiende a cero. Por otro lado, los sólidos en suspensión son transportados hacia la superficie de la membrana, formando una capa que reducirá la permeabilidad hidráulica y el flujo de permeado y al mismo tiempo incrementará la presión transmembrana (TMP) (Herrera-Robledo, 2007; Meng et al., 2009).

El fenómeno de taponamiento puede caracterizarse por el siguiente mecanismo: (1) la adsorción de solutos o coloides dentro de, o sobre las membranas y el acercamiento de los microorganismos a la superficie taponante; (2) la deposición de los flóculos del fango en la superficie de la membrana; (3) la formación de la capa taponante en la superficie de la membrana; (4) el desprendimiento de la materia taponante atribuida principalmente a las fuerzas de corte donde están involucradas fuerzas de Van der Waals y electrostáticas, así como el oxígeno disuelto; (5) cambios espaciales y temporales de la composición de la materia taponante durante largos periodos de operación (e.g., el cambio de la comunidad bacteriana y componentes biopoliméricos en la capa taponante) (Ridgway y Flemming, 1996; Marselina et al. 2008; Meng et al., 2009) Fig. 1.3.

Los tres factores principales que afectan al taponamiento por las características de la membrana, la biomasa y las condiciones de operación son (Le-Clech, 2010):

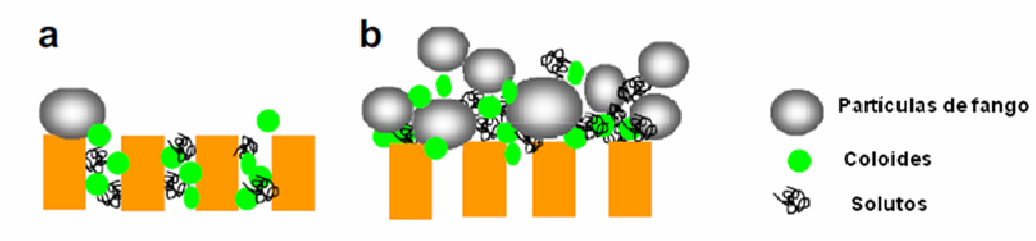

Fig. 1.3 Proceso de taponamiento de membranas en MBRs; (a) bloqueo de poro y (b) capa taponante (Meng et al., 2009)

* La configuración, el material, la hidrofobicidad, la porosidad y el tamaño del poro de la membrana.

* Los sólidos suspendidos del licor mezcla (MLSS), las sustancias poliméricas extracelulares (EPS), el tamaño de los flóculos, y la materia disuelta.

* Las condiciones de operación; el flujo, la aireación, el tiempo de retención celular (SRT), el tiempo de retención hidráulico (HRT), y la presión transmembrana (TMP).

El taponamiento puede ser reversible e irreversible. El irreversible es el producido por las partículas que penetran en la membrana y quedan retenidas en ella, produciendo una disminución del flujo que no es posible aumentar con lavados físicos de la membrana, y generalmente es parcialmente eliminado bajo regímenes de limpieza con agentes químicos. Mientras que el reversible es el formado por la deposición de partículas sobre la superficie de la membrana, pudiendo ser eliminado mediante protocolos de limpieza físicos (Metcalf & Eddy, 2003).

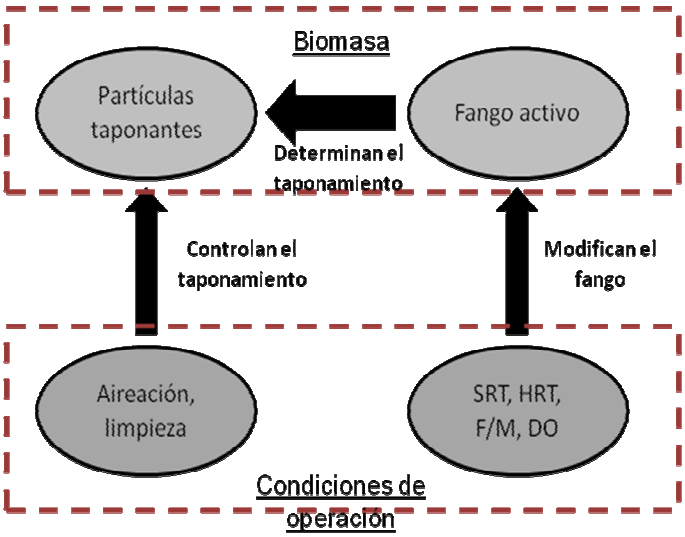

Fig. 1.4 Esquematización de los factores que afectan el taponamiento y su control (modificada de Meng et al., 2009)

Para prevenir la continua acumulación de sólidos sobre la superficie de las membranas, se lleva a cabo el retrolavado (o backwashing) de las mismas. La frecuencia de limpieza dependerá de las condiciones de operación (tiempo de operación, características del agua de alimentación, del flujo de permeado, entre otros). Por lo tanto, el retrolavado es un proceso completamente autocrítico, es decir, que depende de lo que el sistema tarda para bajar en rendimiento o taponarse. Para la mayoría de los casos, el retrolavado tiene lugar cada 30-60 minutos de trabajo y dura un tiempo de 1 a 3 minutos (Buckley y Jacangelo, 1998) cuando el sistema se encuentra en funcionamiento. Si el retrolavado es insuficiente para restaurar el caudal, entonces se lleva a cabo la limpieza química de las membranas. Después de la limpieza química, se alcanza una parcial o plena restauración del caudal a través de la membrana. La limpieza química de las membranas es requerida cada vez que el flujo de permeado disminuye un 20% respecto al original (Mulder, 2003), esto viene a ser en la mayoría de los casos una vez cada 3-6 meses (Till y Mallia, 2001). Se utiliza normalmente hipoclorito de sodio (NaClO en una concentración hasta 0.3%), aunque es común que se utilicen limpiadores alcalinos para la eliminación del taponamiento orgánico, mientras que la limpieza ácida (como el ácido cítrico) se requiere para asegurar la eliminación de precipitados inorgánicos. La limpieza química de las membranas se realiza parando el flujo de alimentación.

Por otra parte, el aire administrado a los sistemas MBR aeróbicos, es generalmente usado para controlar el taponamiento. A pesar de que pocos estudios han indicado muy buenos resultados con un burbujeo de pequeño tamaño, la mayoría de la literatura hace referencia a burbujas de gran tamaño que crean relativamente mayor turbulencia y por lo tanto, presentan una mejor opción anti-taponamiento (Prieske et al., 2008).

2.1 Aireación y transferencia de oxígeno

Como cualquier sistema de tratamiento aeróbico, el oxígeno es requerido en el proceso de MBR para mantener la existencia de la biomasa viva y degradar contaminantes y otros compuestos nitrogenados. El tamaño del flóculo y la concentración de biomasa son dos de los factores más importantes que influyen en la cantidad de oxígeno que puede ser transferida para la degradación biológica.

La eficiente remoción de contaminantes orgánicos es fuertemente afectada por la transferencia de oxígeno entre la burbuja y la solución; y el factor α (i.e. velocidad de oxígeno transferido en agua limpia, dividida entre la velocidad transferida en licor mezcla) es generalmente utilizada para referir el nivel de oxígeno transferido en los MBR. Es ampliamente reportado que la concentración de MLSS tiene un efecto negativo en el factor α, limitando la operación del MBR bajo elevados SRT (i.e. una alta concentración que resulta en una alta viscosidad del licor mezcla) (Judd, 2006).

3. MBR con incorporación de lecho móvil (MBBR).

Con la finalidad de mejorar la calidad del agua residual tratada y cumplir con lo establecido por las regulaciones ambientales, es necesario implementar tecnologías avanzadas para el

tratamiento que se requiere (Trapani et al., 2010). Los procesos biológicos basados en biopelículas han demostrado ofrecer soluciones satisfactorias para la remoción de compuestos orgánicos y nitrógeno del agua residual, evitando algunos de los problemas asociados al proceso de fangos activos, como reactores de gran tamaño, la necesidad de tanques de sedimentación y recirculación de la biomasa (Luostarinen et al., 2006; McQuarrie y Boltz, 2011). Los reactores de biopelícula en lecho móvil (MBBRs) han sido ampliamente utilizados para tratar agua residual tanto municipal como industrial. Esta tecnología permite alcanzar tasas de remoción de demanda bioquímica de oxígeno (DBO_5) y nitrógeno similares a las alcanzadas por los procesos basados en fangos activos, con la ventaja de utilizar un tanque de menor volumen (Andreottola et al., 2000). Los MBBRs pueden ser operados en fase anóxica o aerobia con el libre movimiento de flotación de soportes plásticos con biopelícula. Estos sistemas incluyen un reactor de biopelícula sumergida y una unidad de separación líquido-sólido.

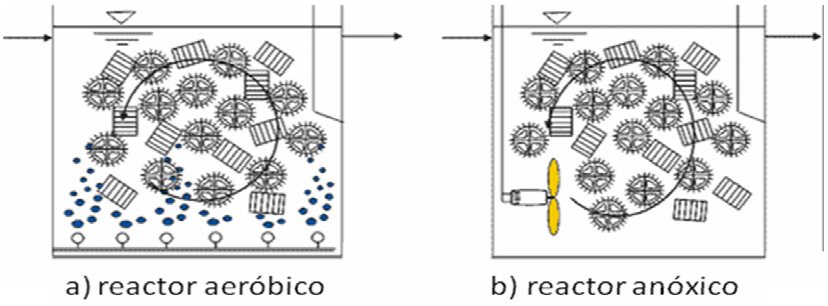

Fig. 1.5 Esquema de un MBBR aerobio (a) y un MBBR anóxico (b).

El objetivo de los sistemas MBBR es alcanzar el crecimiento de la biomasa como una biopelícula sobre pequeños soportes que tienen menor densidad que el agua. Estos se mantienen constantemente en el tanque y tienen la capacidad de moverse libremente en el reactor sin recirculación de fango (Ødegaard, 2008; Jaroszynski et al., 2012). Los soportes de la biopelícula son fabricados en diferentes materiales y diseños y son accesibles comercialmente. El principio de los sistemas MBBR tiene la posibilidad de incorporar los soportes al tanque del biorreactor en los sistemas MBR para ofrecer una tecnología conjunta.

Existen diversos estudios en este tipo de sistemas basados en su mecanismo y remoción desde el punto de vista técnico. Sin embargo, son escasos los estudios basados en investigar la composición de la microbiota involucrada en la biopelícula, y el mecanismo por el cual las variaciones operacionales pueden influir en su estructura está poco descrito aunque resulta crucial para la optimización de estos sistemas (Ciesielski et al., 2010; McQuarrie y Boltz, 2011; Calderón et al., 2012b).

4. Importancia del estudio de la dinámica de comunidades microbianas.

El tratamiento biológico de aguas residuales es una industria multibillonaria en dólares que tiene gran aplicación biotecnológica a nivel mundial. La eficiencia de los procesos biológicos de

tratamiento de aguas residuales está básicamente condicionada por la aclimatación y estabilidad del consorcio microbiano presente. Por ello, la falta de control en el rendimiento del proceso o la calidad del producto final suele ser consecuencia del escaso conocimiento sobre la influencia que tienen los factores ambientales o biológicos en la estructura y dinámica de las comunidades microbianas responsables de llevar a cabo las diferentes reacciones de depuración (Wang et al., 2012).

La estabilidad de un consorcio microbiano abarca un amplio espectro de definiciones. Estas se dividen en dos componentes: las propiedades funcionales del consorcio y el cambio a través del tiempo de la composición de la comunidad (dinámica). Frecuentemente sólo se evalúan los parámetros funcionales, pero recientemente se han hecho intentos para relacionar la funcionalidad del proceso de ingeniería con la estructura de la comunidad microbiana (Wittebolle et al., 2008; Marzorati et al., 2008; Fernández et al., 1999).

Los métodos dependientes de cultivo utilizados tradicionalmente para caracterizar la estructura de las comunidades microbianas en el tratamiento de desechos residuales han sido con el paso del tiempo cada vez más rezagados. Esto en parte se debe a que la diversidad microbiana en estos sistemas es tan compleja y viable pero no cultivable, que sólo se permite conocer alrededor del 2% de la totalidad de la comunidad (Forney et al., 2004; Sánz J.L y Köchling T, 2007). En este sentido, la diversidad Procariota conocida representa sólo la punta de un iceberg, y en las últimas dos décadas se ha hecho manifiesta la necesidad de introducir nuevas técnicas, llamadas herramientas moleculares independientes de cultivo, que proporcionen el complemento necesario a los métodos microbiológicos tradicionales para poder obtener información más precisa sobre la importancia de la diversidad microbiana en el mantenimiento de los ecosistemas (Molina-Muñoz et al., 2007).

Las técnicas moleculares de desarrollo relativamente reciente han sido aplicadas en las últimas décadas a la identificación de los grupos microbianos específicos en las comunidades mixtas, a diferentes niveles filogenéticos, lo cual reduce el tiempo y simplifica el experimento (Liu et al., 2002). Aún más recientemente, la nueva era de la metagenómica ha permitido comprender la composición genética y funciones de las comunidades microbianas complejas. Dichas técnicas aplicadas al análisis de comunidades microbianas se pueden englobar en tres grandes grupos;

a) El primero incluye aquellos métodos que se basan en la amplificación de genes concretos (normalmente el gen codificante del ARNr 16S en el caso de *Bacteria* y *Archaea*), seguido de su clonación y secuenciación, de forma que se consiga la identificación de la mayoría de los microorganismos o filotipos presentes en una determinada comunidad. En este grupo quedaría incluido también el análisis del metagenoma o biblioteca genómica cuasi completa de toda la comunidad.

b) El segundo grupo abarca las técnicas basadas en la amplificación del ácido desoxirribonucleico (ADN), tanto de forma dirigida (SSCP, Single strand conformation polymorphism; TGGE y DGGE, temperature/denaturing gradient gel electrophoresis)

como “aleatoria” (RAPD, Random amplified polymorphic DNA), y su posterior evaluación. Normalmente, con esta estrategia se pretende conseguir un perfil o “huella genética” comparable entre distintos estadios del proceso o entre diferentes condiciones.

c) El tercer grupo incluye todas las técnicas basadas en la hibridación de ácidos nucléicos debido a su similitud.

Por lo tanto, los tres grupos de técnicas moleculares descritas no son mutuamente excluyentes, sino que son y deben ser consideradas como complementarias (Dabert et al., 2002; Sánz J.L y Köchling T., 2007).

En 1993 nuevas metodologías de huella genética fueron integradas a la microbiología ambiental por Muyzer, la electroforesis en gel con gradiente de desnaturalización (DGGE) y su homólogo, la electroforesis en gel con gradiente de temperatura (TGGE). Estas técnicas permiten la separación de los fragmentos de ADN de tamaño similar pero con distinta secuencia de pares de bases (Gómez-Villalba et al., 2006). El TGGE se utiliza para obtener un perfil que representa la estructura genética y la diversidad de una comunidad microbiana en un ambiente especifico con una alta versatilidad, fiabilidad y reproducibilidad (Muyzer y Smalla, 1998). En general, la intensidad relativa de cada banda y su posición en el perfil de TGGE representan la abundancia relativa de una población dominante en particular. Por lo que se puede monitorear temporal y espacialmente los cambios en las comunidades microbianas y así pueden ser calculadas la diversidad y dinámica de las mismas (Mertens et al., 2005; Wittebole et al., 2005).

Sin embargo, hay algunas desventajas asociadas a las técnicas moleculares que deben ser tomadas en cuenta para proveer una interpretación válida de los resultados (Head et al., 1998). La mayor limitación está relacionada con el muestreo, la recuperación de ácidos nucleicos y la elección del método de extracción de ADN, la cual tiene una influencia significativa en la generación de perfiles de comunidades microbianas, en términos de la detección de ribotipos.

La amplificación de los genes codificantes del ARNr 16S por PCR es otra fuente de sesgo, que puede afectar a los resultados de la cuantificación de diversidad; diferencias pequeñas en las secuencias de regiones conservadas pueden resultar en la amplificación selectiva de algunas secuencias, también considerando que la llamada universalidad de los cebadores utilizados comúnmente no es absoluta (Ben-Dov et al., 2006). Para el TGGE, el número e intensidad de las bandas en un gel, no necesariamente da una imagen exacta de la comunidad microbiana, debido a que un organismo puede producir más de una banda por los múltiples y heterogéneos operones codificantes del ARNr (el número de copias de genes del ARNr en el genoma de diferentes organismos puede variar entre 1 y 14) (Head et al., 1998). Además, las secuencias parciales del gen codificante del ARNr 16S no siempre permiten la discriminación

entre especies y una banda puede representar dos o más especies, con secuencias parciales idénticas.

También, si los genes codificantes del ARNr 16S de una mezcla se presentan en concentraciones muy diferentes, las secuencias menos abundantes no son amplificadas lo suficiente para ser visualizadas como bandas en un gel de TGGE. De igual forma, se ha demostrado que secuencias heteroduplex, originadas por la recombinación de hebras individuales complementarias, derivadas de diferentes fuentes y normalmente generadas durante los últimos ciclos del PCR, no migran tanto como los fragmentos normales porque éstos se desnaturalizan a diferentes temperaturas, alterando la fiabilidad del patrón (Boon et al., 2002; Muyzer y Smalla, 1998).

Es por estas deficiencias, inherentes a todas las técnicas moleculares, que los parámetros calculados de las huellas genéticas deben ser interpretados como un indicador y no como una medida absoluta del grado de diversidad de una comunidad microbiana (Marzorati et al., 2008). Además debe considerarse que las huellas genéticas basadas en ADN solo nos dan una idea de las células más abundantes, ya sean activas, dominantes, atenuadas o muertas, por lo que con este tipo de estudio es imposible conocer la funcionalidad de cada grupo microbiano (Boon et al., 2002).

4.1 Diversidad procariota en aguas residuales

Hace más de una década, Hugenholtz et al. (1998) publicaron un estudio de la diversidad procariota en aguas residuales encontrando que ciertos grupos filogenéticos bacterianos tienden a predominar en determinados ambientes, e incluso se puede hablar de microorganismos o grupos filogenéticos característicos de un ambiente concreto.

En este sentido, dentro del tratamiento de aguas residuales basadas en fangos, estudios realizados por Witzig et al. (2002) revelaron que el grupo predominante en la comunidad de fangos de los sMBR pertenece a la clase β-Proteobacteria. Este grupo es frecuente en hábitats naturales y artificiales tales como fangos activos (Manz et al., 1994; Wagner y Loy, 2002). Otros estudios indican que, en cambio, aparecen en muy baja representación las bacterias Gram-positivas con alto contenido en G+C, las clases δ-Proteobacteria y ε-Proteobacteria, y miembros del cluster Cytophaga-Flavobacterium (Ahmed et al., 2007). El grupo más representativo tras las β-Proteobacterias son las α-Proteobacterias y las γ- Proteobacteria (Luxmy et al., 2000).

Por otra parte, las bacterias oxidadoras de amonio (*Nitrosomonas y Nitrosospira*), bacterias nitrificantes de fase I, no aparecen altamente representadas y son bastante comunes en los sistemas de lodos activos con actividad nitrificante (Wagner y Loy, 2002). Las especies de *Nitrospira* (nitrificante de fase II) están representadas en mayor número que *Nitrobacter*, por lo que se considera que *Nitrospira* spp. es más importante en el proceso de la oxidación del nitrito de los MBR (Schramm et al., 1998; Witzig et al., 2002).

Más recientemente, en un trabajo publicado por Wang et al. (2012), se analizó la diversidad bacteriana en 14 sistemas de tratamiento de agua residual para identificar si es que hay un "core" microbiano común en estos sistemas empleando herramientas más sensibles, como la secuenciación masiva en paralelo. Los grupos filogenéticos bacterianos encontrados en mayor abundancia fueron: Proteobacteria en la clase β predominantemente (21-52%), seguido de las clases α-Proteobacteria (7-48%), γ-Proteobacteria (8-34%) y δ-Proteobacteria (2-18%).

Así mismo, Hu et al. (2012), analizaron la comunidad microbiana de 12 plantas de tratamiento de agua municipal con diferentes procesos y la tendencia encontrada fue que el Phylum dominante resultó ser Proteobacteria para algunas muestras y para otras los Bacteroidetes.

Por todo lo anterior cabe resaltar que desde los estudios iniciales en el ámbito del tratamiento de las aguas residuales, ya se mostraban ciertas tendencias en el predominio de grupos bacterianos. Sin embargo, a pesar de que haya dominio de una población característica, la estructura de la comunidad microbiana de los sistemas de tratamiento de agua puede variar bastante dependiendo de las condiciones operacionales, sobre todo cuando éstas se modifican por la limitación de nutrientes presentes (Stamper et al., 2003; Zhang et al., 2011; Wang et al., 2012).

4.2 Diversidad microbiana en biopelículas para el tratamiento de agua residual

Las biopelículas son comunidades microbianas formadas por grupos de células retenidas en una matriz de sustancias poliméricas extracelulares (EPS), que ha sido producida por los propios microorganismos, hidratada y de densidad variable, que es continuamente permeada por canales de diversos tamaños (Costerton et al., 1999). Como se ha descrito anteriormente, la formación de las biopelículas está dada en una secuencia de pasos.

Las biopelículas desempeñan un papel muy importante en el tratamiento de agua residual, ya que forman la base de diversos tratamientos tanto aerobios como anaerobios y se caracterizan por su gran eficiencia y viabilidad (Fernández et al., 2007). Consecuentemente, la eficiencia del proceso es el resultado de la diversidad microbiana presente en la biopelícula. Es por esto que existe gran variedad de estudios respecto al crecimiento de biopelículas y su consolidación en sistemas de tratamiento de aguas, enfocado siempre a los parámetros operacionales, factores fisicoquímicos y propiedades del soporte (Choo y Lee, 1996). Los reportes de actividad, adhesión y biomasa de los microorganismos en biopelículas basados en técnicas de ecología microbiana siguen siendo aún escasos (Fernández et al., 2007; Ciesielski et al., 2010).

5. Biodiversidad de las actividades enzimáticas en los sistemas de tratamiento de agua residual

El agua residual de cualquier naturaleza, contiene aproximadamente entre el 40% al 60% de materia orgánica particulada. Considerando estos altos niveles de partículas orgánicas, el proceso de hidrólisis enzimática en los tratamientos biológicos de aguas residuales es muy importante para completar la mineralización de la misma (Goel et al., 1998).

Para hidrolizar las partículas orgánicas que en su mayoría son proteínas, carbohidratos y lípidos (Nielsen et al., 1992, Raunkjær et al., 1994), los microorganismos sintetizan y secretan diferentes enzimas hidrolíticas. De este modo, dependiendo del sistema (i.e., anaerobio, aerobio, anóxico), la tasa de hidrólisis bajo diferentes condiciones de aceptor de electrones puede afectar a la distribución de productos hidrolizables disponibles bajo estas fases, afectando el rendimiento de remoción de nutrientes (Henze et al., 1995; Goel et al., 1998).

La literatura reportada en este aspecto indica que hay tres diferentes puntos basados en los diferentes aceptores de electrones para que se lleve a cabo la hidrólisis.

1. Hay una falta de consenso en cuanto a la velocidad de hidrólisis bajo diferentes condiciones de aceptores de electrones.

2. La mayoría de los estudios se han centrado en sistemas únicos enzimáticos.

3. Hay pocos estudios realizados para entender la razón de fondo para que haya o no cambios en la tasa de hidrólisis bajo condiciones diferentes de aceptores de electrones.

La comprensión y conocimiento de las actividades bioquímicas y de la comunidad microbiana que componen el fango activo es de vital importancia para entender el funcionamiento de los sistemas de MBR y mejorarlos.

El fango activo del biorreactor se caracteriza por la formación de flóculos de microorganismos procariotas, eucariotas, protozoos, nematodos, rotíferos y bacteriófagos (Rittmann y McCarty, 2001). Estos flóculos se mantienen en suspensión por aireación haciendo que el licor mezcla sea homogéneo. Para el estudio biológico de un sistema concreto y establecer cómo determinados factores modifican la eficiencia del mismo, el conocimiento de las comunidades microbianas que integran el sistema, así como los procesos biológicos que se dan en este ambiente, son necesarios y fundamentales. En este sentido, el estudio de las modificaciones en las actividades enzimáticas, resulta muy útil porque son un indicador para evaluar la fisiología de las especies de la comunidad en el fango (Molina-Muñoz et al., 2007, 2010; Calderón et al., 2012a).

La localización de las enzimas extracelulares aún no está clara, sin embargo, algunos estudios han demostrado que estas enzimas extracelulares están asociadas a las sustancias poliméricas extracelulares (EPS) (Conrad et al., 2000; Guellil et al., 2001; Gessesse et al., 2003,).

Algunas de las actividades enzimáticas más importantes y aplicadas en los procesos biológicos de depuración son: fosfatasas, glucosidasas, proteasas y esterasas, debido a que la composición química del agua residual presenta una fracción orgánica mayoritariamente formada por carbohidratos y proteínas, en cuya hidrólisis juegan un papel muy importante las actividades enzimáticas descritas. Los valores de ciertas actividades enzimáticas pueden ser aplicados como indicadores de poblaciones específicas, como medida de biomasa activa e

indicadores de procesos específicos en el fango activo, como la eliminación de materia orgánica, nitrógeno y fósforo (Nybroe et al., 1992; Goel et al., 1998).

Así, las fosfatasas son enzimas que hidrolizan ésteres de fosfato, liberando grupos fosfato al medio. Existen dos tipos de fosfatasas que presentan diferencias en lo referente al valor de pH óptimo de acción y en cuanto a la preferencia frente a determinados sustratos; las fosfatasas ácidas y alcalinas (Goel et al., 1998).

Las glucosidasas (α-glucosidasas y β-glucosidasas) hidrolizan disacáridos procedentes de la degradación de polisacáridos. La primera de ellas hidroliza el enlace α-1,4 glicosídico en maltosa y glucosa, mientras que la segunda hidroliza la celobiosa (Goel et al., 1998).

Las proteasas rompen enlaces peptídicos de proteínas dando lugar a péptidos de distinto tamaño.

Las esterasas son enzimas bastante inespecíficas involucradas en la degradación de polímeros. Esta actividad se utiliza para determinar la degradación heterotrófica existente en el fango. Se pueden obtener distintas fracciones de esta enzima (esterasa extracelular, periplásmica y particulada) (Boczar et al., 2001).

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2. OBJETIVOS

El incremento en la demanda de recursos hídricos, principalmente causado por un crecimiento desmesurado en la población, ha llevado a la necesidad de considerar y ejecutar alternativas para su reutilización. La necesidad principal manifestada a nivel mundial, sobre todo por países que sufren sequía, está incrementando la eficiente remoción de contaminantes del agua residual que permitan su reuso directo cumpliendo con la calidad exigida por las normativas establecidas, evitando así causar daños a la salud pública y al medio ambiente. Por ello, en los últimos 40 años se ha producido un aumento considerable en el desarrollo de las tecnologías aplicadas para el tratamiento de aguas residuales.

En este contexto, y como objetivo principal de este trabajo, se ha realizado una serie de estudios dirigidos a analizar, evaluar y comparar la diversidad de las comunidades microbianas en distintos sistemas para el tratamiento de agua residual urbana, con especial atención a la relación entre los cambios observados en las comunidades microbianas y las condiciones de trabajo (parámetros operacionales y otras variables que influyen sobre el proceso). Se han estudiado plantas de tratamiento basadas en la tecnología MBR, operadas bajo condiciones aerobias y anaerobias respectivamente, así como variantes de lecho en suspensión y lecho fluidificado.

Esta tesis pretende responder a tres hipótesis fundamentalmente:

Si se asume que las biopelículas son el principal causante de taponamiento de las membranas en la tecnología MBR, ¿cómo está compuesta la comunidad procariota en un sistema AnMBR?

Si el oxígeno puro al ser utilizado como fuente de aireación en un sistema sMBR para el tratamiento de agua residual urbana, ¿tiene implicación sobre la actividad hidrolítica, estructura y dinámica de la comunidad bacteriana presente en el fango del reactor biológico?

Si los parámetros operacionales son cruciales para el funcionamiento de un sistema MBBR, ¿cómo es la relación entre dichos parámetros y la estructura de la comunidad bacteriana en la biopelícula responsable de la remoción?

Para su desarrollo hemos marcado los siguientes objetivos:

1. Analizar la estructura de la comunidad procariota en las biopelículas taponantes en un sistema AnMBR de membranas acopladas a escala piloto.

2. Analizar la influencia del oxígeno puro aplicado como fuente de aireación en un sistema sMBR convencional (biorreactor de lecho en suspensión) a escala piloto para el tratamiento de agua residual municipal, basado en el estudio de la estructura y dinámica de la comunidad bacteriana presente en el fango del biorreactor.

3. Estudiar la evolución de las actividades enzimáticas hidrolíticas extracelulares en el sistema sMBR convencional y sus variaciones según las condiciones de trabajo (carga orgánica, TRH y temperatura) y la influencia del oxigeno puro aplicado al tratamiento.

4. Estudiar la diversidad bacteriana y su eficiencia en el proceso depurador de un sistema de lecho móvil (MBBR) bajo la influencia de diferentes parámetros operacionales como tipo de relleno, TRH y porcentaje de relleno, para su posible integración a sistemas MBR.

4. RESULTADOS

CAPÍTULO I

Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment.

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Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment

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ABSTRACT

The structure of the biofouling layers formed on a pilot-scale membrane-coupled upflow anaerobic sludge blanket bioreactor (UASB) used to treat urban wastewater was analyzed by scanning electron microscopy and electron-dispersive X-ray microanalysis. For comparison, control samples of the membranes were fed either UASB effluent or raw wastewater in a laboratory-scale experiment. Microbial diversity in the fouling materials was analyzed by temperature gradient gel electrophoresis (TGGE) combined with sequence analysis of partial 16S rRNA. Significant differences in structure of the Bacteria communities were observed amongst the different fouling layers analyzed in the UASB membranes, particularly following a chemical cleaning step (NaClO), while the Archaea communities retained more similarity in all samples. The main Bacteria populations identified were evolutively close to Firmicutes (42.3%) and Alphaproteobacteria (30.8%), while Archaea were mostly affiliated to the Methanosarcinales and Methanospirillaceae. Sphingomonadaceae-related bacteria and methanogenic Archaea were persistently found as components of biofouling, regardless of chemical cleaning.

1. Introduction

Anaerobic wastewater treatments offer a good alternative to the conventional activated sludge processes due to their greater tolerance of an organic pollutant load, their higher efficiency due to their lower requirements of energy, the possibility of operating at shorter hydraulic retention times (HRT), and the opportunity for energy recovery by reutilizing the methane generated in the anaerobic processes (Li et al., 2010). Micro- and ultrafiltration membranes have been widely applied for the tertiary treatment of wastewater and as part of submerged membrane bioreactors (MBRs) (Fane and Chang, 2002). However, one of the drawbacks limiting the use of membrane technologies is the undesirable accumulation of materials on the surface of the membranes, due to the growth of microbial biofilms and the subsequent gathering of different types of organic and inorganic materials. This phenomenon, known as biofouling, is a major problem regarding the wide application of membrane technologies for the treatment and reutilization of wastewater as it generates technical failures which increase the overall cost of the process. Its principal effects are the reduction of the permeate efflux and an increase in transmembrane pressure, consequently causing a higher energy use and increasing the frequency of the required chemical cleaning operations of the membranes, both of which shorten membrane lifespans (Ridgway and Flemming, 1996; Herrera-Robledo et al., 2010).

Research in the last decade has been focused on studying the biofouling phenomenon from multidisciplinary approaches, although these efforts have been largely dedicated to membrane technologies applied in aerobic wastewater treatment (Huang et al., 2008; Ivnitsky et al., 2007; Miura et al., 2007). Knowledge based on the ecology of the microbial communities involved in biofouling, and the mechanisms by which plant operation may influence their structure and dynamics are regarded as of crucial importance for the optimization of MBR technology via the development of fouling control strategies (Herzberg et al., 2010; Huang et al., 2008; Miura et al., 2007). The purpose of this work was to analyze the structure and diversity of the prokaryotic communities in the biofouling layers of a pilot-scale membrane-coupled upflow anaerobic sludge blanket bioreactor (UASB) used to treat urban wastewater, by a combination of scanning electron microscopy (SEM), electron-dispersive X-ray microanalysis (EDX), and molecular fingerprinting tools. Polymerase chain reaction coupled to temperature gradient gel electrophoresis (PCR-TGGE) was used to study the structure of the microbial community in the fouling material, and identification of the dominant bacterial and archaeal

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populations involved was achieved by DNA sequencing of the prevalent TGGE bands.

2. Methods

2.1. Description of the pilot-scale membrane-coupled upflow anaerobic sludge blanket bioreactor (UASB)

The experiments were developed using a pilot-scale membrane-coupled UASB, installed at the wastewater treatment facilities on the campus of the National Autonomous University of Mexico, Mexico City. A schematic diagram of the experimental plant is displayed in Fig. 1. The cylindrical anaerobic bioreactor (4 m height, 0.53 m diameter, 849 L operating volume) received the influent wastewater (0.141 m^3/h) by means of a peristaltic pump (Masterflex IP 77410-10). The UASB was operated at an HRT of 6 h, with 100% biomass retention. The UASB effluent exit was connected to an external membrane unit equipped with six membrane modules, manufactured by Koch Membrane Systems (USA). Each module (3 m length and 0.038 m diameter) consisted of seven tubular ultrafiltration membranes (0.0127 m diameter) made of fluoride polyvinylidene (FPVD), with a pore size of 100,000 Da. The total filtrating surface was 5.02 m^2. The membrane modules underwent chemical cleaning every 30 days (720 h of operation) by backflushing with a 300 mg/L NaClO solution, as described by Cid León (2007).

A control membrane biofouling experiment was also set up at the laboratory scale for comparison. The system consisted of a peristaltic pump, a recirculation tank and a membrane module (22 cm long). The membrane was fed for 8 h with either the UASB effluent (Treatment I) or raw wastewater (Treatment II).

The full-sized membranes used in the pilot-scale UASB for 2400 h of operation were analyzed both before and after the chemical cleaning step (named Treatment III and Treatment IV, respectively). Furthermore, the chemically-cleaned membranes were additionally fed raw wastewater for 8 h, and the resulting biofouling layers were also analyzed (Treatment V). The tubular membrane modules were dismantled from the experimental plant after the corresponding treatments, opened, and the membranes removed and divided into four pieces along their total length. Each separated piece was cut in 15 cm fragments and immediately stored at −80 °C.

2.2. Physico-chemical analysis of the wastewater

The influent raw wastewater, the treated effluent from the UASB, and the final effluent generated after filtration through the coupled membrane modules were analyzed. Samples were taken in triplicate over seven consecutive days and the following parameters were measured: total and soluble chemical oxygen demand (COD, mgO_2/L), total and suspended solids (TS and TSS, mg/L), total fixed solids (TFS, mg/L), total volatile solids (TVS, mg/L), suspended fixed solids (FSS, mg/L), and suspended volatile solids (VSS, mg/L). Total dissolved solids (TDS, mg/L), fixed dissolved solids (FDS, mg/L) and volatile dissolved solids (VDS, mg/L) were calculated by subtraction. All analyses were performed in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 2001).

2.3. Scanning electron microscopy (SEM) and electron-dispersive X-ray microanalysis (EDX)

The fouling layers developed on the membranes were analyzed by SEM. Individual pieces of the membranes were fixed with glutaraldehyde (5% v/v) in 0.2 M sodium cacodylate buffer (pH 7.1), washed, and post-fixed in OsO_4, before being dehydrated with graded ethanol solutions (10, 30, 50, 70, 90, and 100% ethanol). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The samples were transferred to fresh 100% ethanol, and critical point-dried from liquid carbon dioxide at 36.1 °C and 7.37 Pa, using a Samdri 780B apparatus (Tousimis, Rockville, USA). Samples were coated with gold before being examined by electron microscopy. Micrographs were taken with a Jeol JSM 5310LV microscope (Jeol Ltd., Tokyo, Japan) and analyzed by the software provided with the equipment. Regular surfaces of fouling layer samples (150 × 150 μm) were analyzed by EDX using a Pentafet EDX Si/Li detector (Oxford Instruments, Abingdon, UK) coupled to the SEM, and with the help of the ISIS Link v. 1.04[a] software.

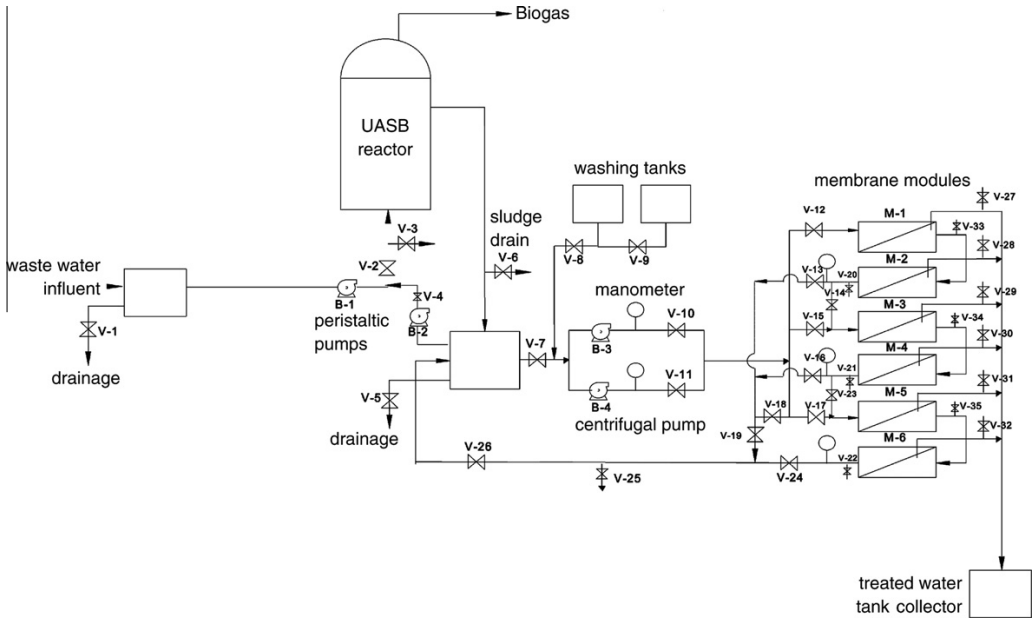

Fig. 1. Diagram of the pilot-scale membrane-coupled upflow anaerobic sludge blanket bioreactor (UASB) used throughout the study.

2.4. DNA extraction

The DNA was extracted from sample pieces of the biofouled membranes (*ca.* 300 mg) using the Fast ID DNA Isolation kit (Genetic ID, IA, USA), helped by mechanical disruption of the fouling material using glass beads.

2.5. PCR amplification of partial bacterial and archaeal 16S rRNA genes

Two-step approaches were used for PCR amplification, as previously described by other authors for TGGE or DGGE fingerprinting (Gómez-Silván et al., 2010; Molina-Muñoz et al., 2009). One microliter (2–5 ng) of the DNA extracted from the fouling material was used as a template for all PCRs. The primers used are listed in Table 1. The HPLC-purified oligonucleotides were purchased from Sigma, and AmpliTaq Gold polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used for all PCRs, performed in a Perkin–Elmer 2400 Thermo Cycler (Perkin–Elmer, Waltham, MA, USA).

The universal primers fD1 and rD1 were used to amplify the bacterial 16S rRNA gene in almost its full length (Weisburg et al., 1991). Subsequently, 1 μL of the first PCR product was used as a template for a nested PCR using the universal primers GC-P1 and P2, targeting the V3 hypervariable region of the 16S rRNA gene (Muyzer et al., 1993). Conditions for each of the PCR reactions were kept as previously described (Molina-Muñoz et al., 2009). The primers selected for the amplification of Archaeal 16S rRNA genes were ARCH915 and UNI-b-rev (hypervariable regions V6–V8, 492 bp) (Yu et al., 2008). For the second two-step PCR, a GC clamp was added to primer ARCH915, and the PCR reaction was set as described previously (Gómez-Silván et al., 2010). All primers were manufactured by Sigma–Aldrich (St. Louis, MO, USA). All final PCR products were cleaned and/or concentrated (when required) using Microcon YM100 cartridges (Eppendorf, Hamburg, Germany). Two to five microliters (60–100 ng DNA) were loaded into each well for TGGE.

2.6. TGGE analysis

TGGE was performed using a TGGE Maxi system (Whatman-Biometra). For Bacteria fingerprinting, the denaturing gels (6% polyacrylamide (37.5:1 acrylamide:bisacrylamide), 20% deionized formamide, 2% glycerol and 8 M urea) were made and run with 2× Tris–acetate-EDTA buffer. The temperature gradient was optimized at 43–63 °C (Molina-Muñoz et al., 2009). For Archaea fingerprinting, the denaturing gels (6% polyacrylamide (37.5:1 acrylamide:bisacrylamide), 20% deionized formamide, 2% glycerol and 7.47 M urea) were made and run with 2× Tris–acetate-EDTA buffer. All chemicals (molecular biology grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The temperature gradient was optimized at 45–54 °C (Gómez-Silván et al., 2010). All gels were run at 125 V for 18 h. The bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Different PCR reactions were tested and different TGGE gels were run to check the reproducibility of the results.

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 μL of filtered and autoclaved water, and directly used for re-amplification with the appropriate primers. The PCR products were electrophoresed in agarose gels and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The recovered DNA was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, CA, USA).

2.7. Analysis of TGGE fingerprints

The band patterns generated by TGGE were normalized, compared and clustered using the Gel Compar II v. 5.101 software (Applied Maths, Belgium). Range weighted richness indices (*Rr*), which provide an estimation of the level of microbial diversity in environmental samples, were calculated based on the total number of bands in each TGGE pattern (*N*) and the temperature gradient (°C) between the first and the last band of each pattern (*Tg*), as described by Marzorati et al. (2008). The resulting values were divided by 100 (Gómez-Silván et al., 2010) to keep an order of magnitude analogous to that of the *Rr* index as originally described for DGGE by Marzorati et al. (2008).

For cluster analysis, the TGGE profiles were compared using a band assignment independent method (Pearson product-moment correlation coefficient), as well as a method based on band presence/absence (Dice coefficient). In band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied. Dendrograms relating band pattern similarities were automatically calculated with UPGMA algorithms (Unweighted pair group method with arithmetic mean). The significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients.

The relative intensities of particular bands were also calculated using Gel Compar II, expressed as percentages of the total band intensity in each particular TGGE lane. To render a graphical representation of the evenness of the bacterial and archaeal communities in the different samples, Pareto-Lorenz distribution curves were drawn based on the TGGE fingerprints, as previously described (Marzorati et al., 2008). The bands in each TGGE lane were ranked from high to low based on intensity levels. The cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands. The curves were numerically interpreted by the functional organization index (*Fo*), given by the horizontal *y*-axis projection on the intercept with the vertical 20% *x*-axis line (Marzorati et al., 2008). The calculation of the *Fo* indexes allows for the evaluation of the functional redundancy of the microbial communities analyzed by fingerprinting methods (Marzorati et al., 2008).

2.8. Phylogenetic and molecular evolutionary analyses

The DNA sequences were analyzed using the biocomputing tools provided online by the National Center for Biotechnology

Table 1
Primers used for the PCR amplification of partial 16S rRNA genes from Bacteria and Archaea. The GC-clamps added for TGGE separation are marked in bold.

Primers	Sequence (5′ → 3′)	Hypervariable region	References
fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	Almost full length 16S, bacteria	Weisburg et al. (1991)
rD1	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC	Almost full length 16S, bacteria	Weisburg et al. (1991)
P1	ACGGGGGGCCTACGGGAGGCAGCAG	V3, bacteria	Muyzer et al. (1993)
GC-P1	**CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC**ACGGGGGGCCTACGGGAGGCAGCAG	V3, bacteria	Muyzer et al. (1993)
P2	ATTACCGCGGCTGCTGG	V3, bacteria	Muyzer et al. (1993)
ARCH915F	AGGAATTGGCGGGGGAGCAC	V6, Archaea	Yu et al. (2008)
GC-ARCH915F	**CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC**AGGAATTGGCGGGGGAGCAC	V6, Archaea	Yu et al. (2008)
UNI-b-REV	GACGGGCGGTGTGTRCAA	V6, Archaea	Yu et al. (2008)

Table 2
Physico-chemical characterization of the wastewater treated in the experimental plant and of the effluent water generated after the UASB and the membrane treatments. COD: chemical oxygen demand; TS: total solids; TFS: total fixed solids; TVS: total volatile solids; TSS: total suspended solids; FSS: fixed suspended solids; VSS: volatile suspended solids; TDS: total dissolved solids; FDS: fixed dissolved solids; VDS: volatile dissolved solids. Values are averages ± SD of triplicate measures over seven consecutive days.

Parameters	Wastewater	UASB effluent	% Removal	Membrane effluent	% Removal
COD (mgO_2/L)	425 ± 138	123 ± 18	71	33 ± 8	92
Soluble COD (mgO_2/L)	212 ± 20	94 ± 17	56	27 ± 7	88
TS (mg/L)	624 ± 32	385 ± 25	38	350 ± 29	50
TFS (mg/L)	317 ± 25	254 ± 22	20	236 ± 26	32
TVS (mg/L)	307 ± 33	131 ± 17	55	114 ± 13	68
TSS (mg/L)	70 ± 9	19 ± 7	73	1 ± 0	99
FSS (mg/L)	16 ± 6	6 ± 2	62	1 ± 0	94
VSS (mg/L)	54 ± 7	13 ± 4	75	0 ± 0	100
TDS (mg/L)	554	366	34	349	37
FDS (mg/L)	301	248	18	235	22
VDS (mg/L)	253	118	53	114	55

Information (http://www.ncbi.nlm.nih.gov). The BLASTn program (Altschul et al., 1997) was used for sequence similarity analysis. The ClustalX v. 2.0.3 software (Jeanmougin et al., 1998) was used for alignment of the DNA sequences. Putative chimeric sequences were discarded after being analyzed by the Black Box Chimera Check software, B2C2 (Gontcharova et al., 2010). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences are HQ662274–HQ662309. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Kumar et al., 2001). A *p*-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm. The bootstrap test was conducted to infer the reliability of branch order, with a round of 1000 reassemblings. Bootstrap values below 50% are not shown in the tree.

2.9. Statistics

STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) was used for calculating the analyses of variance (ANOVA) and multiple-range tests (Student's *t*-test). A significance level of 95% ($p < 0.05$) was selected.

3. Results and discussion

3.1. Efficiency of the UASB bioreactor in wastewater treatment

The results of the physico-chemical characterization of the raw influent wastewater and the effluent water after the treatment are summarized in Table 2. The results show that the UASB treatment was efficient in removing 71% of the total COD and over 70% of total and volatile suspended solids in the raw influent wastewater. The subsequent membrane treatment improved COD removal to 92% and completely eliminated VSS in the treated water. The system eliminated 99% of the TSS in the influent wastewater, while the removal of TDS was less efficient (37%). The membrane treatment further increased the removal of FDS (4%) and VDS (3%) achieved after the UASB treatment. The final effluent generated by the system fulfilled the quality standards required by the municipal wastewater regulation in Mexico (NOM-003-SEMARNAT-1997, 1998).

3.2. Analysis of the fouling layers by SEM and EDX

Image analysis of the fouling layers by SEM is shown in supplementary material (Fig. S-1). SEM revealed that new membranes, examined before being mounted in the laboratory-scale or the pilot-scale UASB systems, were not free of microorganisms and their surfaces already bore a small number of bacteria, particularly rod-shaped cells (Fig. S-1A). Analysis of the membrane surfaces after the different treatments studied showed changes in the structure of the fouling layer. Control membranes (Treatment I) displayed extensive colonization of their surfaces after being fed the UASB effluent for 8 h (Fig. S-1B and C). Microorganisms of diverse morphologies were observed including cocci, straight and incurvated bacilli, and spirilla (Fig. S-1C). Cells were already embedded in large amounts of extracellular polymeric substances, indicating the formation of a biofilm. Similar results were observed in membranes from Treatment II (Fig. S-1D), demonstrating that in both laboratory experiments a dense fouling layer had developed on the membrane surfaces after 8 h of operation.

Images of samples analyzed on membranes from the UASB after 2400 h of plant operation (Treatment III) showed a mature biofilm layer. Again, bacteria of different morphologies were embedded in the exopolymeric matrix (Fig. S-1E), and large crystal structures were also visible trapped in the cake layers (Fig. S-1F). EDX analysis demonstrated that the crystals were composed of Ca and Mg carbonates, and they also contained Cu oxides and sulfates. According to the chemical analysis of the water treated by the membrane-coupled UASB, the content of FDS was reduced at the membrane filtration step by 4% (Table 1), effectively indicating the retention of inorganic materials on the membrane surface. Removal of FDS by membrane filtration in connection to interactions with the fouling layer has been previously reported in AnMBR (Herrera-Robledo et al., 2010). Negatively-charged organic groups in exopolymeric substances chemically interacted with dissolved cations, causing their immobilization by biosorption (Cho and Fane, 2002; Flemming and Wingender, 2001). Biofilms also contributed to the precipitation of cations by their reaction with microbial metabolic end-products such as sulfides, carbonates and phosphates, which can bind or chemically modify metal species, resulting in the precipitation of bio-inorganic metal complexes (Harrison et al., 2007).

The SEM images of membrane surfaces taken after the chemical cleaning (Treatment IV) were characterized by the presence of spherical structures not observed in any other treatment (Fig. S-1G). The EDX analysis demonstrated the presence of an important fraction of fluoride (F = 14.51%) in these structures, suggesting that they originated from the material comprising the membrane (fluoride polyvinylidene). The SEM of membranes sampled from Treatment V showed the formation of a new biofilm over the chemically-cleaned membranes when they were additionally operated with raw wastewater for 8 h (Fig. S-1H).

Although many studies have focused on the application of anaerobic membrane bioreactors (AnMBRs) for wastewater reclamation (Herrera-Robledo et al., 2010; Huang et al., 2008), few efforts have been directed toward the specific study of the microorganisms involved in the generation of biofouling in these systems (Gao et al., 2010). In contrast, biofouling-related microbial communities have been well characterized in aerobically operated MBRs and reverse osmosis water purification systems by means of electronic microscopy studies and the use of molecular tools (Bereschenko et al., 2008; Chen et al., 2004; Herzberg et al., 2010; Huang et al., 2008; Miura et al., 2007; Pang and Liu, 2007). Even though these previous works were undertaken in water filtration systems of a diverse nature, working under a variety of operation conditions and using membranes of different pore sizes, an overall conclusion of most of the studies is the complex nature and heterogeneity of the microbial communities which developed on the biofouling layers. The SEM analyses performed in the present study are in agreement with these previous findings.

3.3. Analysis of the structure of bacterial communities by TGGE fingerprinting. Phylogenetic study of the DNA sequences of the prevalent TGGE bands

The PCR-TGGE approach demonstrated clear and significant differences of the community structure of Bacteria in the biofouling layers between the five treatments studied (Fig. 2). The Pearson coefficient-based analysis allowed the identification of four clusters corresponding to the different treatments analyzed, except for the majority of the samples retrieved from the membranes in Treatment III and Treatment V, which formed a single cluster displaying over 85% similarity of the composition of their communities. In contrast, the Bacteria fingerprints of samples of fouling material retrieved from membranes right after the chemical cleaning step (Treatment IV) significantly clustered away from the rest of samples, at only 20% similarity. Fingerprints corresponding to Treatments I and II (laboratory-scale experiments) formed their own separate clusters. The bacterial community of the biofilms which developed on the control membranes was thus clearly influenced by the effluent origin (raw wastewater or UASB effluent). Cluster analysis based on the Dice coefficient yielded equivalent results to the Pearson-based clustering (Fig. 2B).

Image analysis with Gel Compar II detected a total of 37 unique band classes in the TGGE fingerprints of Bacteria among the 26

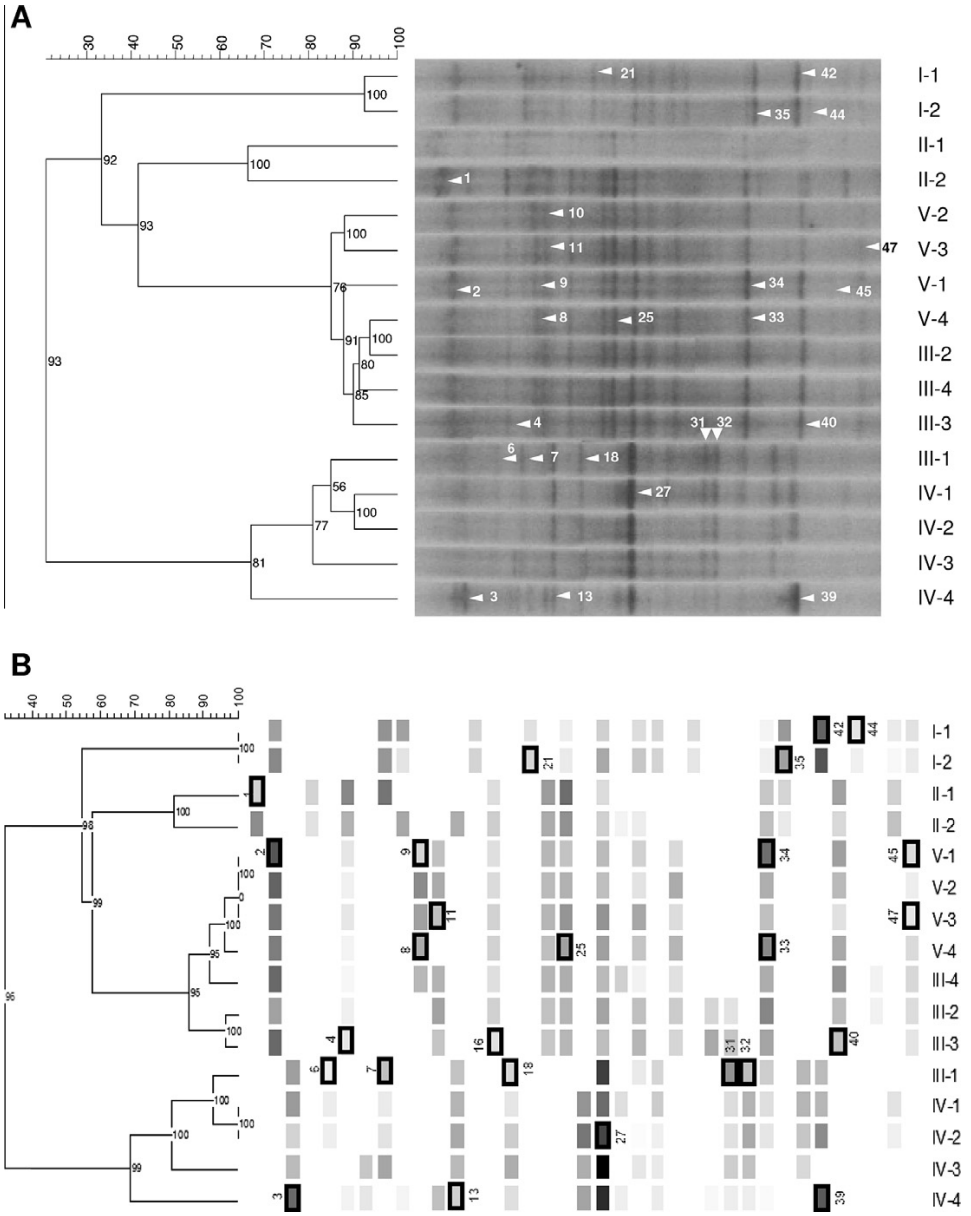

Fig. 2. Community structure of the Bacteria in the biofouling layers analyzed by TGGE profiling. (A) Pearson coefficient-based analysis of the band patterns generated from all membrane samples analyzed. (B) Dice coefficient-based analysis of band patterns generated from all samples analyzed with presence/absence matrix. /□: bands separated by TGGE which were re-amplified and sequenced in order to perform the phylogenetic study, shown in Fig. 3.

Table 3
Average range-weighed richness (*Rr*) and functional organization (*Fo*) indices of the bacterial communities in membrane samples from the five treatments analyzed in this study. Values marked with the same lower-case letter (a, b, c) indicate a statistically non-significant difference (Student's *t*-test, $p < 0.05$).

Treatment	*Rr*	*Fo*
I	20^{c}	44^{ab}
II	15^{abc}	35^{a}
III	17^{bc}	36^{a}
IV	16^{ab}	50^{b}
V	12^{a}	36^{a}

samples analyzed. Calculation of the *Rr* indices showed significant differences (ANOVA analysis, $p < 0.05$) between the five different treatments studied (Table 3). Treatment I samples displayed the highest average values of the index, while the lowest *Rr* was found for samples from Treatment V. Construction of the Pareto-Lorenz curves of the bacterial community profiles in the five treatments analyzed allowed for the calculation of the *Fo* indices (Table 3). It was observed for Treatment IV that 20% of the bands (number based) corresponded to 50% on average of the cumulative band intensities, while the bacterial community in Treatments II, III and V displayed a significantly lower evenness, according to the Student's *t*-test ($p < 0.05$). These results demonstrated the higher specialization of the bacterial community in the biofilm after the chemical cleaning step.

Important shifts in the species composition of the Bacteria communities were observed in the pilot-scale UASB membranes following the chemical cleaning step. Gel Compar II analysis detected several band classes which were exclusive to the post-chemical cleaning stage (i.e. those represented by sequences 3, 6, 13, 18, 31, 32, and 39), while band classes occurring in the majority of samples from Treatments III and V were absent in the chemically-cleaned membranes (i.e. those represented by sequences 4, 8, 9, 11, 25, 40, 45, and 47). These data showed that the bacterial communities in the biofilms consisted of populations displaying different degrees of susceptibility to the antimicrobial compound NaClO. When the membranes were fed raw wastewater for 8 additional hours after chemical cleaning (Treatment V), the community structure was restored similar to that of the pre-chemical cleaning stage (Treatment III), even though the nature of the effluent origin was shown to influence the diversity of the biofouling community, according to the laboratory-scale control experiment (Fig. 2A). These results suggest that the bacterial populations more resistant to the NaClO treatment provided the basic biofilm structure for membrane fouling, and that specific groups of bacteria were preferentially selected to join the preexisting biofilm when operation was resumed after chemical cleaning, regardless of the nature of the influent (UASB effluent or raw wastewater).

Twenty-six bands selected from the TGGE fingerprints targeting Bacteria were successfully amplified and sequenced, representing 57% of the recognized band classes. The phylogenetic affiliations of the sequences and their closest relatives (cultured and uncultured) are provided in detail in Supplementary material (Table S-1). The phylogenetic tree (Fig. 3) shows that most of the identified populations were evolutively related to the Firmicutes (46.1% of sequences). Nine of these operational taxonomy units (OTUs) (sequences 4, 9, 11, 16, 33, 34, 35, 40, and 45) were phylogenetically close to the genera *Clostridium*, *Frigovirgula* and *Eubacterium* (Clostridiales), while sequence 1 was close to *Enterococcus devrieseii*. The band class represented by sequences 33 and 34 was present in nearly all analyzed samples, including the laboratory-scale experiments. Alphaproteobacteria accounted for 30.8% of the identified sequences and most were related to the Sphingomonadales (sequences 6, 7, 13, 18, and 27). It is worth noting that sequence 27 was representative of a band class dominant in the biofilm for all of the treatments analyzed in the study. Of the identified sequences, 11.5% were related to Betaproteobacteria of the Comamonadaceae. Two bands exclusive to Treatment I (sequences 21, and 44) represented populations phylogenetically close to sulfate-reducing Deltaproteobacteria. Only one identified OTU (sequence 2) was found to be related to *Arcobacter cryaerophilus* (Epsilonproteobacteria). This band class was common to all membranes fed UASB effluent in both the laboratory-scale experiment and the pilot-scale bioreactor, excluding Treatment IV.

According to our results, Firmicutes were identified as the dominant group of Bacteria involved in biofouling in the pilot-scale UASB, particularly in the samples of membranes from Treatments III and V. This finding can be explained as an effect of the colonization of the membrane surface by microorganisms washed down from the UASB sludge. Firmicutes are widespread components of the bacterial community in anaerobic sludge systems (Gao et al., 2010; Patil et al., 2010). Li et al. (2010) reported that they were the dominant bacterial group (30%) in sludge from an anaerobic bioreactor treating swine wastewater. In a study conducted on a laboratory-scale AnMBR fed artificial sewage sludge, Gao et al. (2010) found that Firmicutes were also present in biofouling layers, although at a lesser relative abundance compared to sludge. In particular, members of the Clostridiales are responsible for the processes of hydrolysis and fermentation of organic matter during anaerobic digestion in UASBs treating wastewater, and they display an ability to become part of heterogeneous biofilms formed under such conditions (Fernández et al., 2007). In the present study, it is worth noting that several bands corresponding to Clostridiales-related bacteria, which were universally present in the membrane samples of Treatments III and V (represented by sequences 4, 8, 9, 11, and 40), disappeared from the membrane fouling fingerprints after being subjected to the chemical cleaning step (Fig.2B), indicating that these populations became numerically reduced following this maintenance operation, and that they were replaced in importance by other populations with a better capacity to retain membrane attachment.

Our data also identified Alphaproteobacteria members as prevalent populations harbored in the biofouling layers. Five identified alphaproteobacterial sequences were phylogenetically close to the Sphingomonadales (Fig. 3). Sphingomonads have often been described being involved in biofouling in ultra- and microfiltration aerobic MBRs aimed at wastewater treatment (Huang et al., 2008; Miura et al., 2007), as well as in reverse osmosis water purification plants (Bereschenko et al., 2008; Chen et al., 2004), where they were characterized as key microorganisms pioneering the initiation of biofilms causing biofouling (Pang et al., 2005). They are also commonly present in biofilms formed in drinking water pipes (Balkwill et al., 2003; Huang et al., 2008). These findings are consistent with the well-known ability of Sphingomonads to colonize solid surfaces, where they usually adhere strongly regardless of the surface nature, favored by their swarming and twitching mobility and the production of abundant exopolymers (Balkwill et al., 2003; Huang et al., 2008). In the present study, the analysis of TGGE fingerprints showed that four of the band classes shifting and/or increasing in abundance in the biofouling layer following the chemical cleaning step corresponded to Sphingomonadaceae-related organisms (represented by sequences 6, 13, 18, and 27). These results demonstrate the relevance of Sphingomonads on the formation of persistent biofilms leading to biofouling.

With regard to other bacterial populations involved in fouling identified in our work, two Rhizobiales-related populations were also found to be prevalent in the chemically-cleaned membranes (sequences 31 and 32). Bacteria related to this group have been often found in fouled reverse osmosis membranes by both

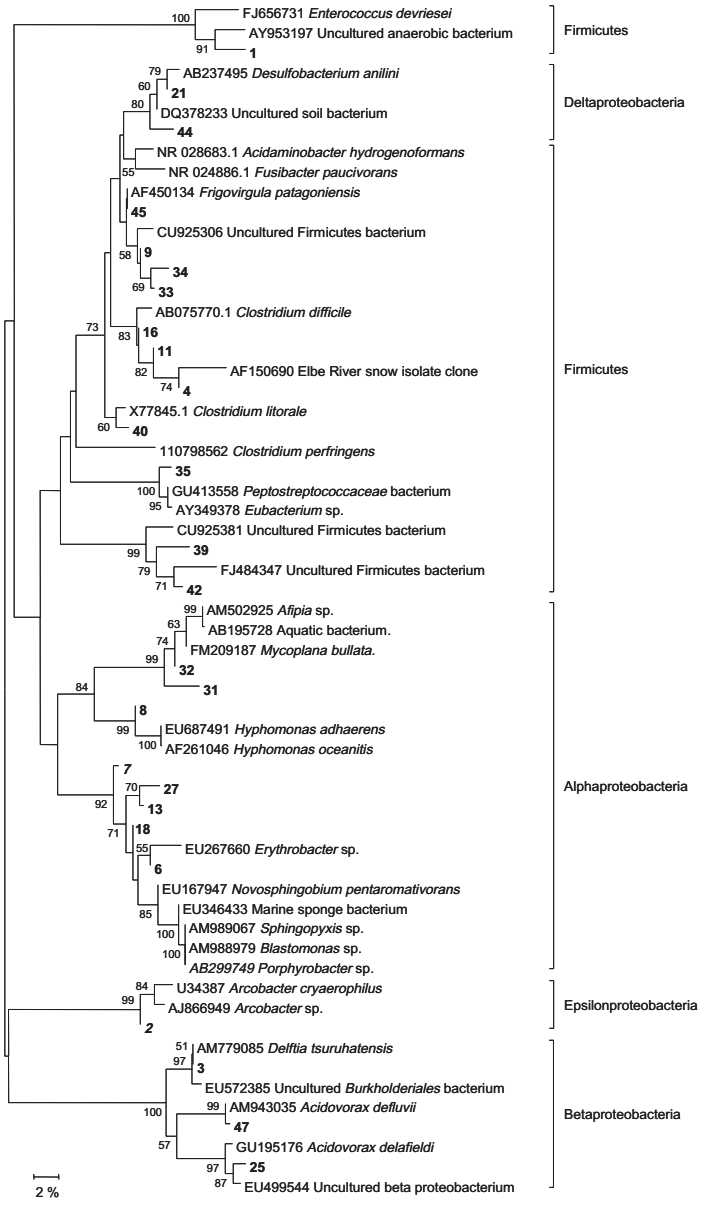

Fig. 3. Neighbor-joining phylogenetic tree showing the positions of 26 bacterial sequences from re-amplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on *ca.* 200 nt length of sequences. The scale bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes.

cultivation-dependent and independent methods, and they were reported as being ecologically relevant for the treatment of secondary effluents (Chen et al., 2004; Pang and Liu, 2007). Betaproteobacteria were described as one major component in most studies which analyzed the diversity of bacterial communities involved in mature biofouling development, both in aerobic MBRs and reverse osmosis systems (Bereschenko et al., 2008; Herzberg et al., 2010; Huang et al., 2008; Miura et al., 2007; Pang and Liu, 2007). In contrast, in the present study performed in an AnMBR, only 11.5% of the identified fingerprinting band sequences were related to the Comamonadaceae, being this group displaced in abundance by Alphaproteobacteria and Firmicutes. Betaproteobacteria are well known as major inhabitants of freshwater environments and the Comamonadaceae in particular are recognized as part of the cosmopolitan βI cluster, universally distributed throughout continental freshwaters (Glöckner et al., 2000). Only one of the identified band classes, represented by sequence 2, was related to Epsilonproteobacteria, displaying an evolutive proximity to the fecal contamination indicator *Arcobacter cryaerophilus*. *Arcobacter* spp. are also reported as being able to establish on surfaces and form biofilms under anaerobic conditions (Fernández et al., 2007).

3.4. Analysis of the structure of Archaea communities by TGGE fingerprinting. Phylogenetic study of the DNA sequences of the prevalent TGGE bands

The Pearson-based cluster analysis of archaeal TGGE fingerprints revealed that all samples retrieved from the membranes in

the UASB had a very similar community structure, since all of them clustered together with a 90% similarity, even including those which corresponded to the chemically-cleaned membranes. Samples of membranes retrieved from the laboratory-scale experiment which received UASB effluent (Treatment I) also developed a similar archaeal community, while those receiving raw wastewater (Treatment II) developed significantly different community profiles (Fig. 4). The *Rr* values ranged from 6 to 9 and the *Fo* indices from 38 to 52, showing no significant differences between treatments according to ANOVA ($p < 0.05$).

Eleven bands were amplified and sequenced from TGGE fingerprints targeting Archaea, revealing that populations closely related to the Methanospirillaceae were prevalent in the five treatments studied (63% of identified sequences), followed by populations related to *Methanosaeta* spp. (Fig. 5, Table S-1). The sequence representative of a prominent band class found in the TGGE profiles (sequence A2) had a high sequence identity with *Methanosaeta thermophilus*. Other Archaea found dominant in the fouling biofilm were related to *Methanocalculus pumilus* (sequence A4) and *Methanobrevibacter* (sequence A14). Regardless of the characteristics of the membrane samples analyzed in the study, all of the archaeal populations identified in the TGGE profiles from the pilot-scale UASB were found to be phylogenetically close to the methanogenic Archaea, indicating that these communities remained largely undisturbed by the chemical cleaning step. Methanogenic Archaea are frequent inhabitants of sludge in UASBs (Liu and Whitman, 2008; Gao et al., 2010) and also of biofilms formed in aerobic wastewater treatment systems (Gómez-Silván et al., 2010). The unique characteristics of the archaeal cell envelope (Matai et al., 2001) may contribute to the persistence of these organisms on membrane surfaces, in spite of the chemical cleaning operation.

Although previous studies on the community composition of fouling layers in AnMBRs have focused on Bacteria rather than on Archaea (Gao et al., 2010; Lin et al., 2010), their results are in agreement with the findings presented here concerning the distinct nature of the composition of the microbial communities, and suggest the pioneering role of particular populations on biofilm initiation and persistence. The results presented here evidence the need to improve the process of membrane cleaning in order to achieve an efficient control of the biofouling phenomena in AnMBRs. Advanced methods suggested by several authors include modifications of membrane materials, dispersal of biofilms by nitric oxide, enzymatic disruption of exopolymers, bacteriophages, quorum-quenching and energy uncoupling strategies (Le-Clech et al., 2006; Gao et al., 2010; Xiong and Liu, 2010). Although these alternative methods have a higher cost than the conventional combination of backflushing and NaClO treatment, they offer significant advantages such as their higher efficiency and the development of less microbial resistances, contributing to a better control of biofouling and the extension of the useful life of the membranes.

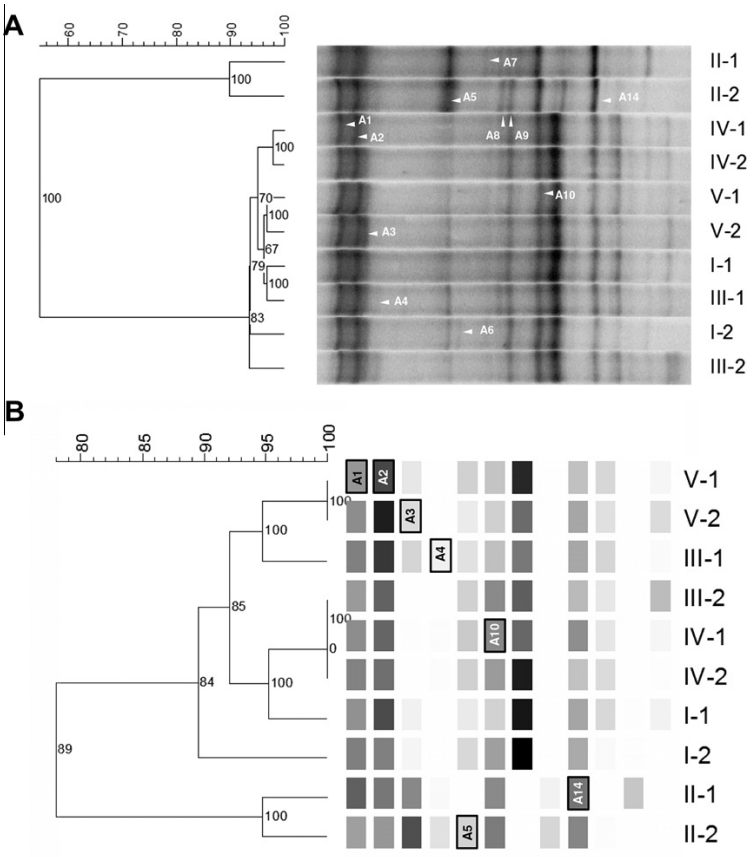

Fig. 4. Community structure of Archaea in the biofouling layers analyzed by TGGE profiling. (A) Pearson coefficient-based analysis of the band patterns generated from all membrane samples analyzed. (B) Dice coefficient-based analysis of band patterns generated from all samples analyzed with presence/absence matrix. /□: bands separated by TGGE which were re-amplified and sequenced in order to perform the phylogenetic study, shown in Fig. 5.

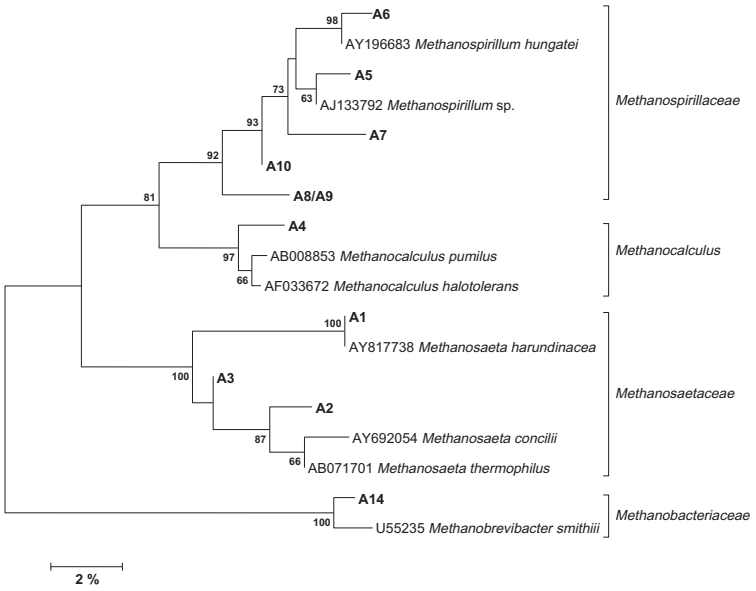

Fig. 5. Neighbor-joining phylogenetic tree showing the positions of 11 archaeal sequences from re-amplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on *ca.* 450 nt length of sequences. The scale bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes.

4. Conclusions

The results of our study are in agreement with previous works which focused on biofouling in aerobic and anaerobic membrane systems, pointing to the universal importance of particular microbial groups in the generation of this unwanted phenomenon. Chemical cleaning did not completely remove membrane biofouling in the analyzed UASB system, and the populations which remained attached supported the re-growth of the biofilm, leading to the regeneration of a similar community structure. The efficiency of backflushing and NaClO treatment as antifouling strategies was limited by the complex structure of the biofilm communities. The use of alternative antifouling strategies, particularly those specifically directed towards microbial groups shown to be resistant to standard chemical cleaning methods (i.e. Sphingomonadaceae bacteria and methanogenic Archaea), seems to be a promising approach for improving biofouling control and prevention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.01.007.

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Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment.

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Appendix A. Supplementary material

Table S-1. Phylogenetic affiliation of 26 bacterial and 10 archaeal populations found prevalent in the biofouling layers by TGGE fingerprinting, based on the partial sequence of their 16S rRNA genes (V3 hypervariable region) obtained from re-amplified TGGE bands.

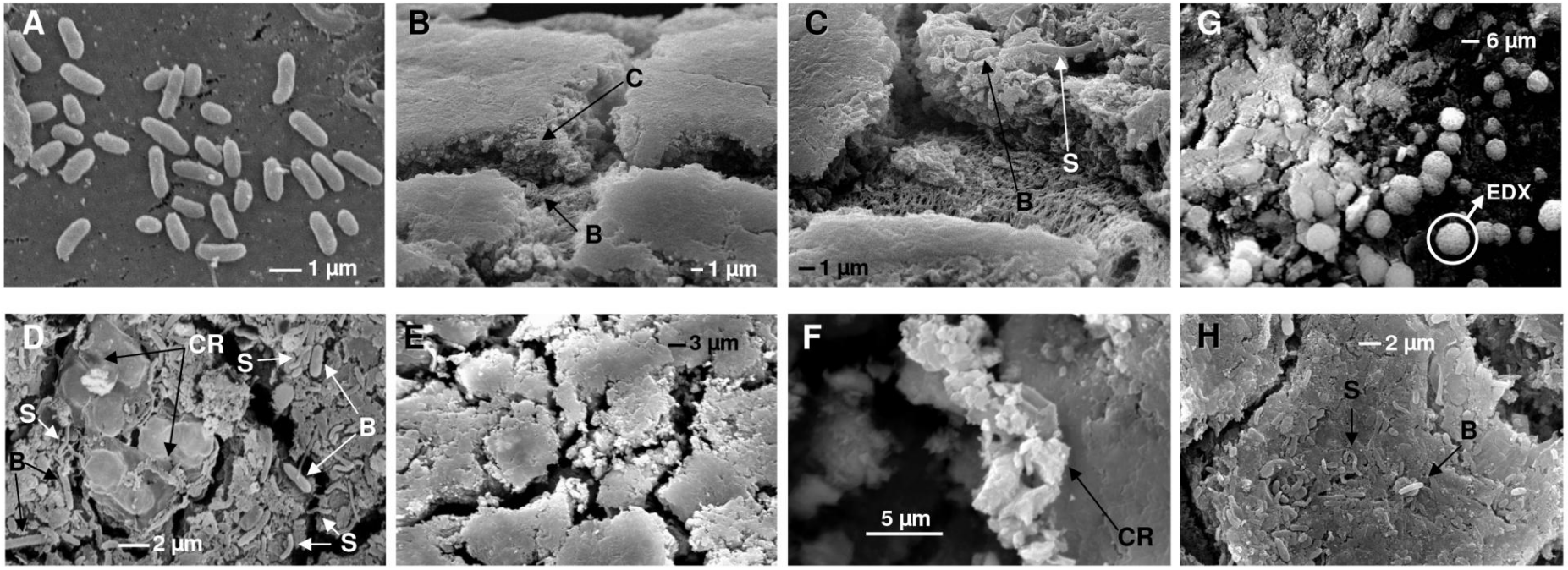
Sequence name	Closest phylogenetic affiliation (class/ family)	Overlap (nt)	Most similar described organism and data base accession No.	Identity (%)
1	Firmicutes/ Enterococaceae	123	AY953197 Uncultured anaerobic bacterium FJ656731 *Enterococcus devriesei*	98 98
2	Epsilonproteobacteria/Campylobacteriaceae	80	AJ866949 *Arcobacter* sp. U34387 *Arcobacter cryaerophilus*	100 100
3	Betaproteobacteria/ Burkholderiales	123	AM779085 *Delftia tsuruhatensis* EU572385 Uncultured *Burkholderiales* bacterium	100 100
4	Firmicutes/ Clostridiales	98	AF150690 Elbe River snow isolate clone EF662878.1 Uncultured *Clostridium* sp.	100 100
6	Alphaproteobacteria/Sphingomonadales	101	EU267660 *Erythrobacter* sp. EU167947 *Novosphingobium pentaromativorans*	100 100
7	Alphaproteobacteria/Sphingomonadales	93	*AB299749 Porphyrobacter* sp. EU346433 Marine sponge bacterium	100 100
8	Alphaproteobacteria/Hyphomonadaceae	100	EU687491 *Hyphomonas adhaerens* AF261046 *Hyphomonas oceanitis*	99 100
9	Firmicutes/ Clostridiales	103	CU925306 Uncultured Firmicutes bacterium AF450134 *Frigovirgula patagoniensis*	100 100
11	Firmicutes/ Clostridiales	98	AB075770.1 *Clostridium difficile*	98

Table S-1 (continued)

Sequence name	Closest phylogenetic affiliation (class/ family)	Overlap (nt)	Most similar described organism and data base accession No.	Identity (%)
13	Alphaproteobacteria/Sphingomonadales	104	EU267660 *Erythrobacter* sp. EU346433 Marine sponge bacterium	100 100
16	Firmicutes/ Clostridiales	107	AF150690 Elbe River snow isolate clone AB075770.1 *Clostridium difficile*	98 99
18	Alphaproteobacteria/Sphingomonadales	100	EU267660 *Erythrobacter* sp.	100
21	Deltaproteobacteria/ Desulfobacteriales	122	AB237495 *Desulfobacterium anilina* DQ378233 Uncultured soil bacterium	100 99
25	Betaproteobacteria/ Comamonadaceae	141	GU195176 *Acidovorax delafieldi* EU499544 Uncultured beta proteobacterium	98 98
27	Alphaproteobacteria/ Sphingomonadales	121	EU267660 *Erythrobacter* sp. *AB299749 Porphyrobacter* sp.	100 99
31	Alphaproteobacteria/ Rhizobiales	114	FM209187 *Mycoplana bullata.*	98
32	Alphaproteobacteria/ Sphingomonadales	100	AM502925 *Afipia* sp.	100
33	Firmicutes/ Clostridiales	116	AF450134 *Frigovirgula patagoniensis*	98
34	Firmicutes/ Clostridiales	118	AF450134 *Frigovirgula patagoniensis* AB075770.1 *Clostridium difficile*	100 98
35	Firmicutes/ Clostridiales	122	AY349378 *Eubacterium* sp. GU413558 *Peptostreptococcaceae* bacterium	100 100
39	Firmicutes/ Clostridiales	123	CU925381 Uncultured Firmicutes bacterium FJ484347 Uncultured Firmicutes bacterium	98 98
40	Firmicutes/ Clostridiales	116	X77845.1 *Clostridium litorale* FJ764316.1 Uncultured Clostridiaceae bacterium	98 97
42	Firmicutes/ Clostridiales	120	FJ484347 Uncultured Firmicutes bacterium FJ269074.1 Iron-reducing bacterium enrichment culture clone	100 100
44	Firmicutes/ Clostridiales	124	AB237495 *Desulfobacterium anilini* DQ378233 Uncultured soil bacterium	100 99

Table S-1 (continued)				
Sequence name	**Closest phylogenetic affiliation (class/ family)**	**Overlap (nt)**	**Most similar described organism and data base accession No.**	**Identity (%)**
45	Firmicutes/ Clostridiales	121	AF450134 *Frigovirgula patagoniensis* CU925306 Uncultured Firmicutes bacterium	100 100
47	Betaproteobacteria/ Burkholderiales	141	AM943035 *Acidovorax defluvii*	99
A1	Methanosarcinales/Methanosaetaceae	374	AY817738.1 *Methanosaeta harundinacea* AB329664.1 Uncultured bacterium	100 100
A2	Methanosarcinales/Methanosaetaceae	374	AY692054 *Methanosaeta concilii* CU915880.1 Uncultured Methanosarcinales archaeon	98 98
A3	Methanosarcinales/Methanosaetaceae	328	AY817738 *Methanosaeta harundinacea* GU135461.1 Uncultured Methanosaeta sp	98 97
A4	Methanomicrobiales/Methanocalculus	305	AB008853 *Methanocalculus pumilus* EU721753.1 Uncultured *Methanocalculus* sp.	98 98
A5	Methanomicrobiales/Methanospirillaceae	360	AJ133792 *Methanospirillum* sp. AY196683 *Methanospirillum hungatei*	98 98
A6	Methanomicrobiales/Methanospirillaceae	410	AY196683 *Methanospirillum hungatei* EU888814.1 Uncultured *Methanospirillum* sp.	99 99
A7	Methanomicrobiales/Methanospirillaceae	378	AJ133792 *Methanospirillum* sp.	99
A8/A9	Methanomicrobiales/Methanospirillaceae	351	AJ133792 *Methanospirillum* sp.	97
A10	Methanomicrobiales/Methanospirillaceae	304	AJ133792 *Methanospirillum* sp. AY196683 *Methanospirillum hungatei*	98 98
A14	Methanobacteriales/Methanobacteriaceae	361	U55235 *Methanobrevibacter smithiii* AY454731.1 Uncultured *Methanobrevibacter* sp.	99 98

Figure S-1. SEM analysis of the fouling material accumulated on the membranes after the different treatments. A: New hollow-fiber UF membrane showing bacillar microorganisms on its surface at time zero. B and C: Partially fouled membrane, after 8 h of being fed UASB effluent (Treatment I). Coccoid (C), bacillar (B) and spiral (S) bacterial morphologies are shown. D: Partially fouled membrane after 8 h of being fed with raw wastewater (Treatment II), crystals (CR) are also observed. E, F: Mature biofilm on the surface of a heavily fouled UF membrane after 2400 h of operation (Treatment III). Bacillar (B) and spiral (S) bacterial morphologies are abundant and large crystals observed (CR): G: Surface of the UF membrane operated for 2400 hours, right after chemical cleaning with NaClO (Treatment IV). H. The biofilm which reformed over the chemically-cleaned membranes (Treatment V) additionally operated with raw wastewater for 8 h.



CAPÍTULO II

SUBCAPÍTULO II.1

Bacterial community structure and enzyme activities in a membrane bioreactor (MBR) using pure oxygen as an aeration source

Kadiya Calderón, Alejandro González-Martínez, Camino Montero-Puente, Patricia Reboleiro-Rivas, José M. Poyatos, Belén Juárez-Jiménez, María Victoria Martínez-Toledo, Belén Rodelas.

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Bacterial community structure and enzyme activities in a membrane bioreactor (MBR) using pure oxygen as an aeration source

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ABSTRACT

A pilot-scale membrane bioreactor was used to treat urban wastewater using pure oxygen instead of air as a source of aeration, to study its influence on bacterial diversity and levels of enzyme activities (acid and alkaline phosphatases, glucosidase, protease, and esterase) in the sludge. The experimental work was developed in two stages influenced by seasonal temperature. Operational parameters (temperature, pH, BOD_5, COD, total and volatile suspended solids) were daily monitored, and enzyme activities measured twice a week. Redundancy analysis (RDA) was used to reveal relationships between the level of enzyme activities and the variation of operational parameters, demonstrating a significant effect of temperature and volatile suspended solids. Bacterial diversity was analyzed by temperature-gradient gel electrophoresis of PCR-amplified partial 16S rRNA genes. Significant differences in community structure were observed between both stages. Sequence analysis revealed that the prevalent Bacteria populations were evolutively close to Alphaproteobacteria (44%), Betaproteobacteria (25%) and Firmicutes (17%).

1. Introduction

Since the development of the activated sludge process in the early 20th century, aeration is required in wastewater treatment plants (WWTPs) to transfer oxygen to the biologically active mass of microorganisms, in order to achieve an adequate removal of organic matter. Different types of aeration are employed, depending on specific treatment requirements (Mueller et al., 2002). Aeration systems involve an important contribution to wastewater plant construction and operation cost (Mueller et al., 2002; Germain and Stephenson, 2007).

The use of pure oxygen as a substitute for air in the activated sludge process was proposed in the 1940s and put into commercial use in 1970 (Shammas and Wang, 2009). The main advantages found when pure oxygen is used instead of air in conventional activated sludge processes are: increased oxygen mass transfer, smaller installations due to a lower required tank volume, reduced power requirements, decreased sludge production, reduced bulking and foaming problems, ability to treat high-strength wastewaters, improved biokinetics, and faster treatment rates at high suspended solid concentrations (Brindle et al., 1998; Mueller et al., 2002; Shammas and Wang, 2009; Zupančič and Roš, 2008). These advantages balance out the significant cost of oxygen generation equipment or the purchase of oxygen tanks.

Membrane bioreactor (MBR) systems for wastewater treatment are based on the combination of the activated sludge process and membrane technology to separate the particulate material from water, avoiding the requirement of the secondary clarifier (Chang et al., 2011). For the last 40 years, MBR technology is one of the most used to treat industrial, domestic and municipal wastewater for the numerous advantages offered such as excellent effluent quality, low sludge production, small configuration, and flexibility for future expansion and upgrade (Miura et al., 2007; Gómez-Silván et al., 2010). In aerobic MBRs, aeration is used not only to administrate dissolved oxygen to the microbial biomass, but also to help keeping solids in suspension and minimize membrane fouling (Germain and Stephenson, 2007; Calderón et al., 2011).

Use of pure oxygen as a source of aeration in MBRs allows the achievement of high removal rates of organic matter (over 90% reduction of COD and BOD_5) (Rodríguez et al., 2010, 2011). However, the effect of pure oxygen aeration on the biology of bacterial communities in MBR sludge remains poorly characterized. During the formation of the activated sludge, microorganisms use their enzymes to hydrolyze and degrade the organic matter, mostly composed by carbohydrates and proteins (Burgess and Pletschke, 2008). Evaluating microbial diversity and enzyme activities through the operating phase of MBRs is essential to achieve the

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biological characterization of the complex microcosm which is carrying out the waste treatment. Variations of the enzyme activities are well used as an indicator to evaluate the physiology of the mixed species community in the sludge (Molina-Muñoz et al., 2007, 2010).

The aim of this work was to analyze the influence of pure oxygen applied as a source of aeration to a pilot-scale MBR on the bacterial community structure and its enzyme activities. Molecular fingerprinting tools (polymerase chain reaction coupled to temperature gradient gel electrophoresis, PCR-TGGE) were used in parallel to enzyme activity measures.

2. Methods

2.1. Description of the pilot scale MBR plant

The pilot-scale experimental plant used in this study was described in detail elsewhere (Rodríguez et al., 2010). The system worked with real domestic wastewater, supplied by Estación Depuradora Oeste Puente de Los Vados (Granada), run by EMASAGRA, S.A. (Spain). The wastewater was pumped from the primary settling tank of the municipal facility into the pilot-scale bioreactor, which was installed nearby. The plant consisted of two separated cylindrical bioreactors. The first tank (358 l operating volume and 1.85 m height) was aerated with pure oxygen (supplied by AL Air Liquide S.A., Madrid, Spain). A porous diffuser was used to generate fine bubbles, providing an adequate oxygen transfer. Dissolved oxygen (DO) was kept at 2.8 mg/l throughout the experimental set. The second tank (89 l operating volume and 1.10 m height) sheltered two submerged ultrafiltration hollow-fiber membrane units (fluoride polyvinylidene, PVTF, 1.86 m^2 and of 0.04 μm pore size) manufactured by Zenon (GE Water and Process Technologies, Fairfield, USA). The membranes were continuously aerated with tangential air flows (6.8 m^3/h) to avoid clogging on their surfaces and maintain solids in suspension. The membrane tank and sludge bioreactor were separated to avoid the interference that would be produced by the superficial aeration cleaning system in the evaluation of pure oxygen aeration effects on microbial communities. A recirculation of 141 l/h was established to keep a constant concentration of total suspended solids (TSS) in both bioreactors. The effluent water was suctioned mechanically and collected in a 25-l tank, to be used for the periodical back flushing of the membrane modules. Control devices were available to monitor the membrane pressure, temperature, pH, and the concentration of dissolved oxygen in the activated sludge.

The experimental work in the plant was divided in two stages, from March 23rd 2009 to April 30th 2009 (stage I) and from June 15th 2009 to July 11th 2009 (stage II). The average temperature was 15.8 ± 2.33 °C during stage I and 26.5 ± 1.7 °C during stage II. A hydraulic retention time (HRT) of 18 h was set in both the experiments. Both stages of plant operation were initiated with a chemical cleaning of the membrane with sodium hypochlorite, and subsequently operation in the bioreactor was started at the inflow water TSS concentration (100 mg/l) with no previous inoculation; thus, the microorganisms inhabiting the sludge were those present in the influent wastewater. A TSS concentration within the 4000–5000 g/l range was maintained in both stages. In stage I, continuous purge of the MBR (38.5 l/day) was required after stabilization of the system, to keep TSS into the desired range (average TSS value of 4880 ± 1307 mg/l). In stage II, the system became stable reaching an average TSS of 4400 ± 757 mg/l and no purges were needed.

2.2. Physico-chemical analysis

The water samples for the analytical determinations were obtained every 24 h from the primary settling tank, biological reactor and permeate. Chemical oxygen demand (COD), biological oxygen demand at 5 days (BOD_5), total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to the Standard methods for the examination of water and waste water (APHA, 2005). The pH was measured using a Crison pH 25 pH-meter (Crison Instruments S.A., Barcelona, Spain).

2.3. Enzyme activities

Enzyme activities phosphatase, glucosidade, protease and esterase were analyzed two times per week in the sludge of the pure oxygen-aerated biological tank. Samples (ca. 200 ml) were taken in sterile flasks, placed in ice, and immediately transferred to the laboratory. Experiments to measure enzyme activities were always conducted in triplicate. Phosphatase determination was performed by the method of Goel et al. (1998), using *p*-nitrophenyl phosphate (0.1%), which is converted by the enzyme to *p*-nitrophenol. Different buffers were used for measuring acid and alkaline phosphatases (acetate–acetic, pH 4.8, or carbonate–bicarbonate, pH 9.6, respectively). Glucosidases were measured by the colorimetric method (Goel et al., 1998), with Tris–HCl buffer using 1% *p*-nitrophenyl α-D-glucopyranoside as substrate for the reaction. For both phosphatases and glucosidases, standard curves were constructed using known concentrations of *p*-nitrophenol. Protease activity was calculated by the colorimetric method described by Cadoret et al. (2002). Total esterase activity was measured using the method by Boczar et al. (2001). Substrates used in the assays were *p*-nitrophenyl esters of acetate, butyrate, caproate, caprilate, laurate, and palmitate. All chemicals required for enzyme activity analysis were provided by Sigma–Aldrich (St. Louis, MO, USA).

2.4. DNA extraction and PCR amplification of partial bacterial 16S rRNA genes

The DNA was extracted from sludge samples (ca. 200 mg) taken from the pure oxygen-aerated biological tank, using the FastDNA Spin Kit for Soil and the FastPrep24 apparatus (MP-BIO, Germany).

Two-step approaches were used for PCR amplification, as previously described by other authors for TGGE or DGGE fingerprinting (Calderón et al., 2011; Molina-Muñoz et al., 2009). One microliter (2–5 ng) of the DNA extracted was used as a template for all PCRs. The HPLC-purified oligonucleotides were purchased from Sigma, and AmpliTaq Gold polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used for all PCRs, performed in a Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). Primers and conditions for each of the PCR reactions were previously described (Molina-Muñoz et al., 2009). The final PCR products were cleaned and/or concentrated (when required) using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). Eight microliters (60–100 ng DNA) were loaded into each well for TGGE.

2.5. TGGE

TGGE was performed using a TGGE Maxi system (Whatman-Biometra, Goettingen, Germany). The denaturing gels (6% polyacrylamide (37.5:1 acrylamide:bisacrylamide), 20% deionized formamide, 2% glycerol and 8 M urea) were made and run with 2× Tris–acetate–EDTA buffer. All chemicals required were purchased from Sigma–Aldrich (St. Louis, MO, USA). The temperature gradient was optimized at 43–63 °C (Molina-Muñoz et al., 2009). Gels were run at 125 V for 18 h. The bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Different PCR reactions were tested and different TGGE gels were run to check the reproducibility of the results.

Table 1
(A) Operational parameters of experiments conducted in the pilot scale MBR during stages I and II. (B) Enzyme activities measured in the sludge during stages I and II. All enzyme activities are given as mM min^{-1} g^{-1} VSS. Values shown are averages ± SD. LSD: least significant difference (Student's *t* test, $p < 0.05$).

	TSS (mg/L) sludge	VSS (mg/L) sludge	BOD_5 (mg O_2/L) influent water	COD (mg/L) influent water	*T* (°C)	pH influent water
(A)						
Stage I	4880 ± 1307	4220 ± 1050	382 ± 54	543 ± 56	14.6 ± 2.1*	7.42 ± 0.17
Stage II	4400 ± 757	3529 ± 1062	427 ± 88	682 ± 202	26.5 ± 1.0*	7.53 ± 0.20
LSD ($p < 0.05$)	1177	1108	73	141	1.85	0.19

Samples	Acid phosphatase	Alkaline phosphatase	Glucosidase	Protease	Esterase
(B)					
Stage I					
1	2.69 ± 0.11	2.68 ± 0.09	0.41 ± 0.04	1.29 ± 010	0.98 ± 0.36
2	5.05 ± 0.24	6.72 ± 0.30	0.77 ± 0.01	2.58 ± 0.05	1.25 ± 0.10
3	11.50 ± 1.08	11.54 ± 0.42	0.93 ± 0.01	1.11 ± 0.08	0.45 ± 0.13
4	4.05 ± 0.06	7.52 ± 0.04	0.41 ± 0.01	1.94 ± 0.22	0.37 ± 0.00
5	1.99 ± 0.00	3.19 ± 0.15	0.19 ± 0.01	1.35 ± 0.06	0.30 ± 0.06
6	5.56 ± 0.40	4.88 ± 0.06	0.45 ± 0.01	2.41 ± 0.11	0 ± 0.10
7	9.75 ± 0.78	12.68 ± 2.90	0.56 ± 0.01	1.33 ± 0.19	0.59 ± 0.14
8	0.24 ± 0.01	11.89 ± 1.14	0.60 ± 0.01	1.43 ± 0.40	0.63 ± 0.03
9	1.21 ± 0.12	5.75 ± 1.00	1.50 ± 0.06	2.06 ± 0.03	0 ± 0.04
10	3.22 ± 0.06	9.26 ± 0.17	1.82 ± 0.00	1.78 ± 0.11	0.50 ± 0.17
Stage II					
11	21.82 ± 0.31	10.64 ± 0.46	2.96 ± 0.11	16.47 ± 0.30	0.06 ± 0.08
12	16.19 ± 0.01	7.55 ± 0.60	1.45 ± 0.05	4.72 ± 0.34	0.19 ± 0.03
13	0.41 ± 0.04	9.71 ± 0.81	1.02 ± 0.06	7.20 ± 0.03	0.35 ± 0.31
14	2.33 ± 1.59	22.86 ± 0.72	0.78 ± 0.01	6.57 ± 0.18	0 ± 0.14
15	2.45 ± 0.26	13.36 ± 0.17	0.80 ± 0.01	6.33 ± 0.13	0 ± 0.25
16	2.08 ± 2.66	13.46 ± 0.05	1.07 ± 0.04	6.73 ± 0.14	0 ± 0.03
17	1.93 ± 0.37	3.31 ± 0.34	0.88 ± 0.04	3.35 ± 0.10	0 ± 0.17
Averages stage I	4.53	7.61	0.76	1.72*	0.51*
Averages stage II	6.74	11.55	1.28	7.33*	0.09*
LSD ($p < 0.05$)	6.41	5.00	0.67	2.85	0.33

* Significant differences between stage I and stage II ($p < 0.05$).

2.6. Analysis of TGGE fingerprints

The band patterns generated by TGGE were normalized, compared and clustered using the Gel Compar II v. 5.101 software (Applied Maths, Belgium). For cluster analysis, the TGGE profile was compared using a band assignment independent method (Pearson product-moment correlation coefficient), as well as a method based on band presence/absence (Dice coefficient). In band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied (Calderón et al., 2011). Dendrograms relating band pattern similarities were automatically calculated with UPGMA algorithms (*Unweighted pair group method with arithmetic mean*). Significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients.

Based on the TGGE fingerprints, several theoretical indices were calculated to analyze population richness and evenness in the MBR sludge samples. The Shannon–Wiener index of diversity, H' (Shannon and Weaver, 1963), was calculated for each TGGE lane using the function:

$$H' = -\sum_{i=1}^{S} pi \ln pi$$

where *S* represents the total number of bands in a given TGGE lane, and *pi* represents the relative intensity of a given band in the whole densitometric curve of the corresponding lane. Relative intensities of bands were calculated using Gel Compar II.

Range weighted richness indices (*Rr*), which provide an estimation of the level of microbial diversity in environmental samples, were calculated based on the total number of bands in each TGGE pattern (*N*) and the temperature gradient (°C) between the first and the last band of each pattern (Tg), as described by Marzorati et al. (2008). The resulting values were divided by 100 (Gómez-Silván et al., 2010) to keep an order of magnitude analogous to that of the *Rr* index as originally described for DGGE by Marzorati et al. (2008).

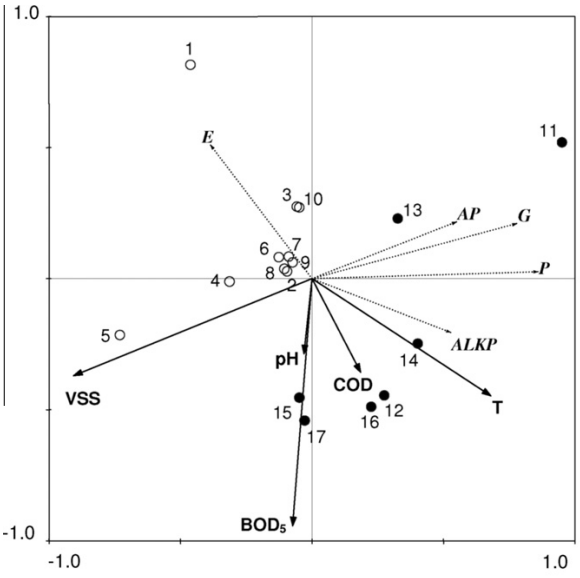
To render a graphical representation of the evenness of the bacterial communities in the different samples, Pareto–Lorenz distribution curves were drawn based on the TGGE fingerprints, as previously described (Marzorati et al., 2008). The bands in each TGGE lane were ranked from high to low based on intensity levels. The cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands. The curves were numerically interpreted by the functional organization index (*Fo*), given by the horizontal *y*-axis projection on the intercept with the vertical 20% *x*-axis line (Marzorati et al., 2008). The calculation of the *Fo* indexes allows for the evaluation of the functional redundancy of the microbial communities analyzed by fingerprinting methods (Marzorati et al., 2008).

2.7. DNA reamplification and sequencing

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 μl of filtered and autoclaved water, and 3 μl of the resulting DNA suspensions were used for re-amplification with the appropriate primers. The PCR products were electrophoresed in agarose gels and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The recovered DNA was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, CA, USA).

2.8. Phylogenetic and molecular evolutionary analysis

The DNA sequences were analyzed and compared using the biocomputing tools provided online by the National Center for



Relationships between RDA ordination axes and environmental variables:

Variable	F1 (horizontal)	F2 (vertical)
Temperature	0.68	-0.41
VSS	-0.92	-0.37
COD	0.18	-0.36
BOD_5	-0.07	-0.95
pH	-0.03	-0.30
Cumulative % of total variation	41.4%	49.3%

Fig. 1. Redundancy analysis (RDA) ordination diagram (triplot) showing samples (numbered circles), operational parameters (straight arrows), and enzyme activity data (dotted arrows) during operation of the MBR in stages I and II. First axis is horizontal, second axis is vertical. *T*: temperature; COD: chemical oxygen demand in influent water; BOD_5: biological oxygen demand at 5 days in influent water; pH: pH of influent water; *P*: protease; *E*: esterase; *G*: glucosidase; *ALKP*: alkaline phosphatase; *AP*: acid phosphatase. Open circles: samples from stage I; solid circles: samples from stage II.

Biotechnology Information (http://www.ncbi.nlm.nih.gov). The BLASTn program (Altschul et al., 1997) was used for sequence similarity analysis. The ClustalX v. 2.0.3 software (Jeanmougin et al., 1998) was used for the alignment of the DNA sequences. Phylogenetic and molecular evolutionary analyzes were conducted using MEGA version 4 (Tamura et al., 2007). A *p*-distance based evolutionary tree was inferred using the Neighbor-Joining algorithm. The bootstrap test was conducted to infer the reliability of branch order, with a round of 1000 reassemblings. Bootstrap values below 50% are not shown in the tree.

2.9. Statistical analysis

STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) was used for calculation of analyses of variance (ANOVA). A significance level of 95% ($p < 0.05$) was selected.

Redundancy analysis (RDA), an ordination method of direct gradient analysis (ter Braak and Prentice, 1988), was performed to search for patterns in the set of biological parameters (enzyme activities) and to assess their relationship with the environmental data (operational parameters). The following nominal environmental variables were used: TSS and VSS concentrations in sludge, COD and BOD_5 of influent water, pH of influent water. Of these, TSS concentration had to be removed from the final analysis because of its strong linear correlation with VSS. RDA was chosen as the ordination method after initial analysis by detrended correspondence analysis (DCA) revealed that the enzyme activities data exhibited a linear, rather than unimodal, response to the environmental variables (Lepš and Šmilauer, 1999). All environmental variables were transformed to log ($x + 1$) except pH. The Monte Carlo permutation test was used to assess the statistical significance of the canonical axes. All the multivariate statistics were computed using the Canoco for Windows v. 4.5 software (ScientiaPro, Budapest, Hungary).

3. Results and discussion

3.1. Analysis of the influence of physico-chemical parameters on enzyme activities

Table 1 shows the average values of the physico-chemical parameters measured in the bioreactor and the enzyme activities of the sludge during the two stages of the study. ANOVA analysis showed that the only parameter displaying a significant difference between the two experimental stages was temperature (Table 1A). Overall, average extracellular activities reached higher values during stage II, with the exception of esterase. The observed differences were not statistically significant in the case of phosphatases and glucosidase (Table 1B).

Extracellular microbial enzymes are essential for the biological treatment of wastewater in order to fulfill the removal by mineralization of organic compounds, mostly proteins, carbohydrates and lipids (Burgess and Pletschke, 2008). Temporal variations of enzyme activity often obey to bacterial regulatory mechanisms, in response to the changes of the environmental conditions such as substrate concentration, availability of electron acceptors, pH or temperature (Boczar et al., 2001). The influence of the simultaneous variation of physico-chemical parameters (VSS concentration, temperature, pH, COD and BOD_5) on the enzyme activities measured in both stages was analyzed by RDA, and the triplot diagram generated is displayed in Fig. 1.

The first ordination RDA axis (horizontal) was mainly correlated to VSS and temperature and described a 41.4% of the total variability of the enzyme activities (72.1% of observed variability). The second ordination axis (vertical), which was strongly associated with BOD_5 concentration, described a 7.9% of the total variability (13.7% of observed variability). The triplot shows the clear segregation of the samples in two groups, belonging to each stage of operation (stage I, open circles; stage II, solid circles). According to the results of the Monte Carlo permutation test, VSS concentration and temperature ($p < 0.05$) were the major factors explaining variations of enzyme activity levels under the experimental conditions assayed in the study. All the enzyme activities correlated negatively to VSS concentration and positively to temperature, with the exception of esterase.

Temperature is an environmental factor with important influence on enzyme activities and the level of solubility of sludge in the bioreactor (Banister and Pretorius, 1998). The relevant role of temperature on the level of enzyme activities in MBR-based WWTPs has been studied by other authors in systems not aerated with pure oxygen. In this sense, Whiteley et al. (2002) demonstrated a positive correlation between temperature and proteolytic activity, which reached maximal levels at temperatures ranging 20–22 °C. Enzyme activities in an MBR WWTP analogous to the one used in this study but lacking aeration by pure oxygen were analyzed in previous work (Molina-Muñoz et al., 2007, 2010). These authors reported that seasonal temperature was the most influential factor on the level of enzyme activities protease, phosphatase and esterase of the sludge biomass, which were all favored by the increase in temperature, except glucosidase that reached the highest values during the winter season (Molina-Muñoz

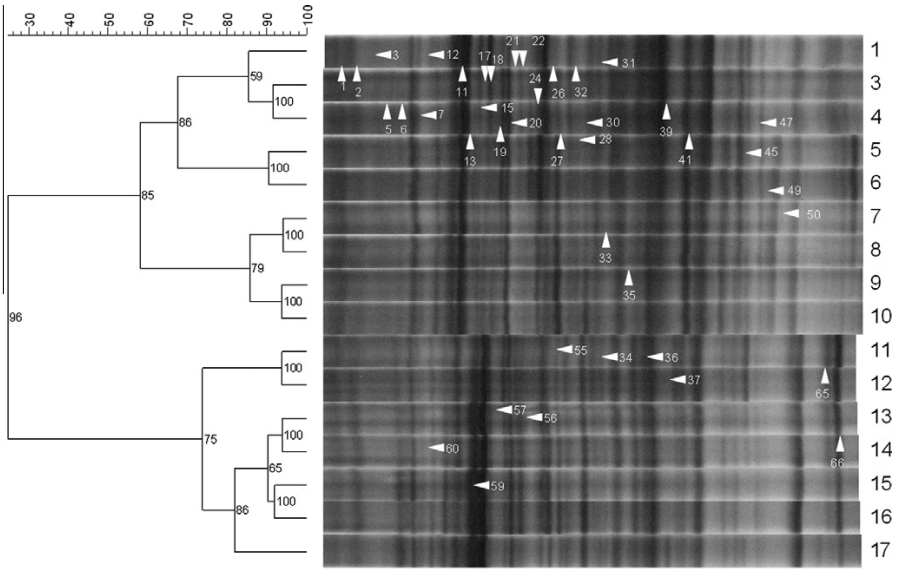


Fig. 2. Dendrogram generated by UPGMA clustering (Pearson correlation coefficient) of 16S rRNA based TGGE patterns from activated sludge samples taken during operation of the MBR in stages I and II, using Gel Compar II v. 4.601 (Applied Maths, Belgium). Profiles are based on the amplification and separation of the V3 hypervariable region of the 16S rRNA gene. Bands which were successfully amplified and sequenced for the phylogenetic analysis shown in Fig. 4 are numbered and marked with an arrowhead. The scale bar indicates the percentage of similarity. Numbers in nodes represent the cophenetic correlation coefficient values. Samples 1–10: stage I; samples 11–17: stage II.

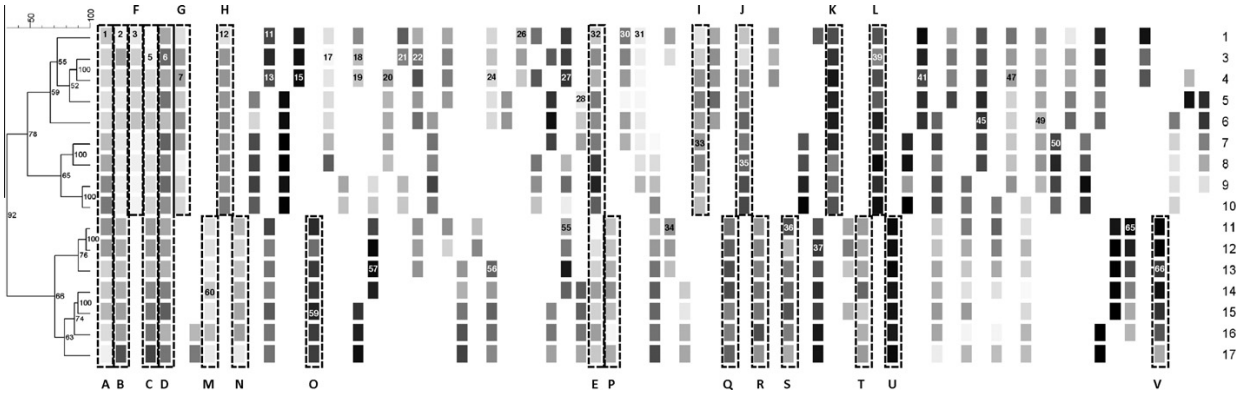


Fig. 3. Dendrogram generated by UPGMA clustering (Dice correlation coefficient) of 16S rRNA based TGGE patterns from activated sludge samples taken during operation of the MBR in stages I and II, using Gel Compar II v. 4.601 (Applied Maths, Belgium). The scale bar indicates the percentage of similarity. Numbers in nodes represent the cophenetic correlation coefficient values. Band classes universal to all samples or common only to a same stage are highlighted and named with letters A–V. TGGE bands which were successfully amplified and sequenced are numbered. Samples 1–10: stage I; samples 11–17: stage II.

et al., 2007, 2010). We did not observe a negative correlation between temperature and glucosidase activity, although it must be taken into consideration that the experiments described here were only developed during the spring and summer seasons.

Li and Chróst (2006) determined that VSS concentration in activated sludge is a representative parameter to estimate active biomass; however, an increase of VSS concentration does not necessarily correlate positively to the levels of enzyme activities (Macomber et al., 2005; Molina-Muñoz et al., 2007, 2010). In an MBR operated under full biomass retention conditions, positive correlation between the increase in VSS concentration and enzyme activities occurred, but decay was observed when threshold values of VSS concentration were reached (Molina-Muñoz et al., 2007). Explanations for the inhibition of enzyme activities at given VSS concentrations include feed-back inhibition by end-products and the accumulation of slowly biodegradable substances with inhibitory or toxic activities (Burgess and Pletschke, 2008; Molina-Muñoz et al., 2010).

3.2. Analysis of the structure of bacterial communities by TGGE fingerprinting

To evaluate changes in the composition of the bacterial community in the sludge, the PCR-TGGE technique was employed. This approach demonstrated clear and significant differences of the community structure of Bacteria among the two experimental stages studied (Fig. 2). The Pearson coefficient-based analysis allowed for the identification of two separated clusters corresponding to samples taken from the two different experimental stages, which clustered at a 25% similarity, indicating a low relationship between the composition of the two bacterial communities. Stage I samples are grouped at a 58% similarity, while the fingerprints of

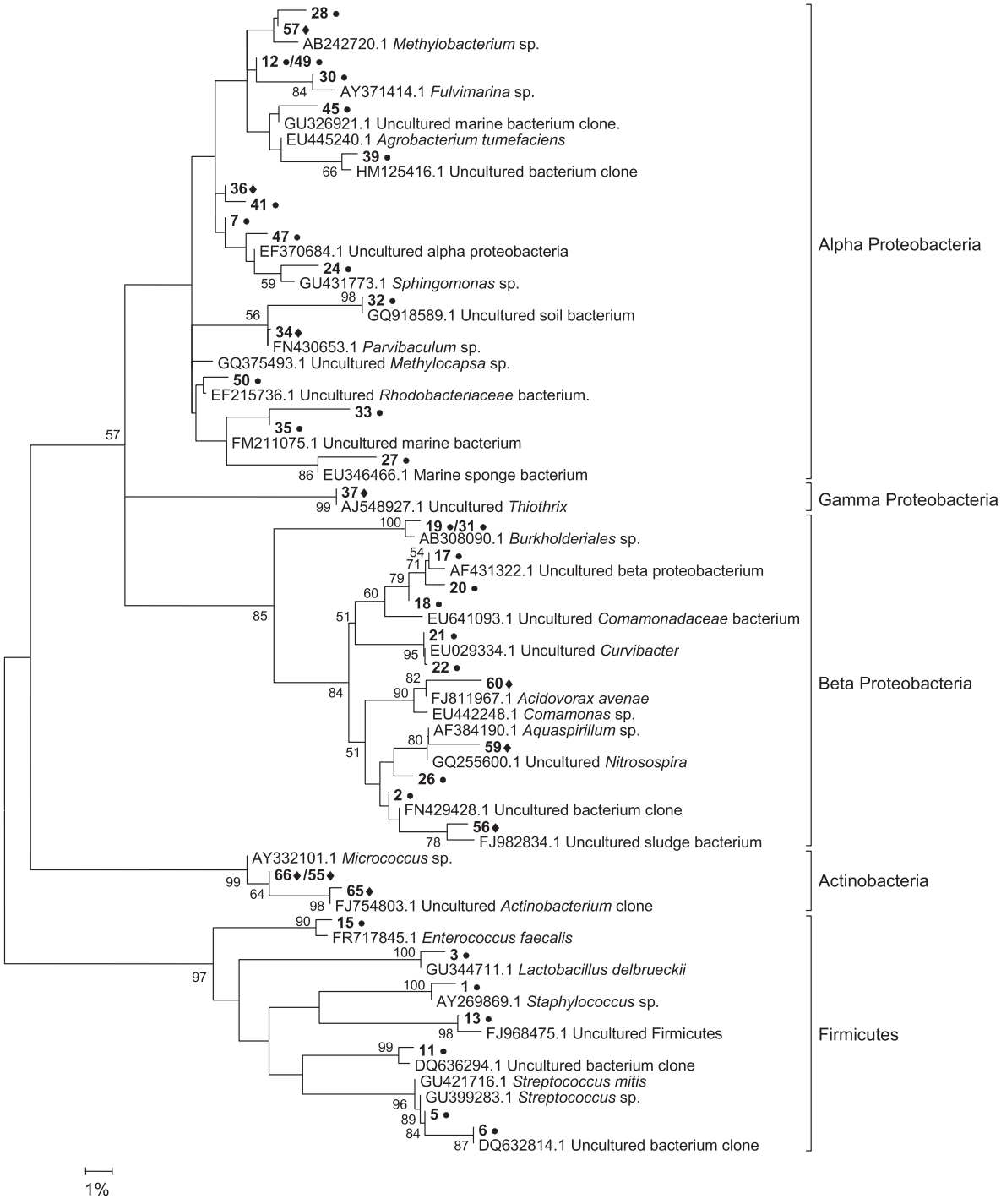


Fig. 4. Neighbor-Joining phylogenetic tree showing the positions of 41 Bacteria sequences from reamplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on ca. 200 nt length of sequences. The scale bar indicates a 1% divergence. Bootstrap values over 50% are shown in nodes. (●) Sequences from bands reamplified from stage I fingerprints; (♦) sequences from bands reamplified from stage II fingerprints.

samples taken during stage II displayed a higher similarity (74%). Cluster analysis based on the Dice coefficient showed equivalent results to the Pearson-based clustering (Fig. 3). A total of 75 band classes were identified in the bacterial fingerprints. Only

band classes A, B, C, D and E were common to both stages of operation, and were present in 100% of the analyzed samples. Seven band classes were universal in samples from stage I, while 10 band classes were common to all stage II samples (Fig. 3). The fingerprint analysis reveals that the bacterial community developed in the studied system was highly dynamic, as few populations were universally present in samples from both stages, or common to all samples from a same stage. These results are in agreement with previous work in a similar, conventionally aerated MBR (Molina-Muñoz et al., 2009).

Calculation of the *Rr* indices showed no significant differences (ANOVA analysis, $p < 0.05$) between the two different stages studied. Based on Marzorati et al. (2008), the bacterial community in the sludge had an average *Rr* = 70 in both stages studied, a value typical of very habitable environments characterized by a high microbial diversity, as it can be considered the mixed liquor of WWTPs. The average values of Shannon–Wiener index (H') were 3.22 ± 0.09 for stage I samples and 3.15 ± 0.03 for stage II samples, indicating a high level of population richness and evenness in both studies. The evenness of the bacterial community was also revealed by the calculation of the *Fo* indices, which also displayed similar values in both stages (*Fo* = 46%). According to Marzorati et al. (2008), *Fo* index values around 45% represent a balanced community, potentially able to preserve its functionality under changing environmental conditions.

3.3. Phylogenetic study of the DNA sequences of the prevalent TGGE bands

A total of 41 TGGE bands were successfully reamplified and sequenced from TGGE gels, corresponding to the dominant populations in the mixed liquor community and representing 49% of the total band classes recognized. A prevalence of Proteobacteria in the set of sequences analyzed was found for both experimental stages (Fig. 4). The main group of identifiable TGGE bands was related to Alphaproteobacteria (18 of 41 sequences, 44%). This group included sequence 32, representative of band class E (Fig. 3), found universal to all analyzed samples. The second group in order of abundance was Betaproteobacteria (25%), followed by Firmicutes (17%) although sequences evolutively close to this phylum were mainly identified in samples from stage I. Only one sequence was related to an uncultured Gammaproteobacteria, and 3 sequences were related to Actinobacteria (See Fig. 4).

The results presented in this paper differ from the current available knowledge on the average composition of the bacterial community in urban WWTPs, in particular, the relative abundances of Alphaproteobacteria in both stages of operation. Wagner and Loy (2002) reviewed data retrieved from several laboratory and full-scale WWTPs, as revealed by studies mainly derived from the screening of clone libraries and quantitative FISH, concluding that Proteobacteria dominated the composition of the bacterial communities (56%), being Betaproteobacteria the major component in most of the analyzed systems (29%), while Alphaproteobacteria, Actinobacteria and Firmicutes represented 11%, 7% and 2% of the OTUs, respectively. Similar conclusions were reached in analogous studies specifically focused on MBRs (Rosenberger et al., 2000; Molina-Muñoz et al., 2009). Recent work exploring bacterial diversity in full-scale urban WWTPs by means of phylogenetic microarrays (Xia et al., 2010) and high-throughput DNA sequencing (Sanapareddy et al. 2009; Ye et al., in press) supported the notion of the universal prevalence of Proteobacteria in WWTPs, although discrepancies regarding the predominance of the Beta subpopulation are reported, attributable to the particular biases introduced by the use of different cultivation-independent techniques (Eschenhagen et al., 2003; Xia et al., 2010). TGGE fingerprinting, as all PCR-based methods, may display a preference to major bacterial groups, possibly missing some variations of numerically minor but functionally relevant constituents of the community, hence further research is required to confirm the extent to which the use of pure oxygen influences bacterial community composition.

4. Conclusions

The studies carried out in the pilot-scale MBR WWTP aerated with pure oxygen showed that the levels of enzyme activities and the influence on these processes of operational parameters (VSS concentration and temperature) were analogous to those reported in previous studies regarding conventionally-aerated MBRs. H', *Rr* and *Fo* indices calculated from TGGE fingerprints were similar in both experimental stages and showed that the sludge bacterial community was equivalent in diversity and functionality to those developed in WWTPs using air. Differences in the relative abundance of prevalent populations were encountered, compared to the previous knowledge on bacterial diversity in WWTPs.

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CAPÍTULO II

SUBCAPÍTULO II.2

Comparative analysis of the enzymatic activities and the bacterial community structure based on the aeration source supplied to an MBR to treat urban wastewater.

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Comparative analysis of the enzyme activities and the bacterial community structure based on the aeration source supplied to an MBR to treat urban wastewater

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HIGHLIGHTS

►A pilot-scale MBR was operated using pure O_2 or air as aeration sources ►Bacterial diversity and enzyme activities were compared under each aeration source ►Enzyme activity was influenced by temperature and VSS, not by the aeration source ►The aeration source significantly influenced the bacterial community structure ►VSS and temperature had a significant influence on the *H'* and *Fo* diversity indices

Keywords:
Pure oxygen
MBR
Enzyme activities
Bacterial diversity

ABSTRACT

A comparative analysis was performed in a pilot-scale membrane bioreactor (MBR) treating urban wastewater when it was supplied with either pure oxygen (O_2) or air, to assess the influence of each aeration source on the diversity and activity of the bacterial communities in the sludge. The MBR was operated in three experimental stages with different concentrations of volatile suspended solids (VSS) and temperature, and under both aeration conditions. Phosphatases, glucosidases, proteases, and esterases were tested as organic material removal markers in the sludge, and the bacterial community diversity was analyzed by amplification of partial 16S-rRNA genes followed by temperature-gradient gel electrophoresis fingerprinting. Redundancy analysis revealed that significant differences in the levels of enzyme activities, as well as the Shannon-Wiener's diversity (*H'*) and functional organization (*Fo*) indices, were only related to temperature and VSS, while the bacterial community structure was significantly influenced by the aeration source supplied in each experimental stage.

1. Introduction

Aerobic wastewater treatment (WWT) requires oxygen for the removal of organic matter, conducted by a microbial consortium, since it is essential for their maintenance and growth (Shammamas and Wang, 2009). Aeration systems involve an important contribution to wastewater plant construction and operation cost (Germain and Stephenson, 2007). In 1940 the use of pure oxygen (O_2) as a replacement for air was proposed for the conventional activated sludge (CAS) process and put into commercial use in 1970 (Shammas and Wang, 2009). By using pure O_2 instead of air, the driving force for oxygen mass transfer and the degree of oxygen saturation are significantly increased (Andreadakis, 1987). Other main advantages found when pure O_2 is used instead of air in CAS were; smaller installations due to a lower required tank volume, reduced bulking and foaming problems, reduced power requirements, decreased sludge production, ability to treat high-strength wastewaters, improved biokinetics, and faster treatment rates at high suspended solid concentrations (Esparza-Soto et al., 2006; Shammas and Wang, 2009). These advantages balance out the significant cost of oxygen generation equipment or the purchase of oxygen tanks. Moreover, CAS-based plants that use pure O_2 have been tested for the improved removal of emerging organic contaminants present in wastewater at low concentrations, such as antibiotics (Batt et al., 2007; Lietz and Meyer, 2006).

Membrane bioreactor (MBR) systems are based on the combination of the CAS process and membrane technology to separate the particulate material from water, avoiding the requirement of the secondary clarifier (Chang, 2010). For the numerous advantages offered such as excellent effluent quality, low sludge production, small configuration, and flexibility for future expansion and upgrade, MBR technology is nowadays one of the most used technologies to treat industrial and municipal wastewater (Calderón et al., 2012a; Rodriguez et al., 2012).

The possible advantages of the application of pure O_2 as aeration source in MBRs are poorly studied, and to date it has only been demonstrated that it allows the achievement of high removal rates of organic matter and nitrogen compounds (Rodriguez et al., 2011; 2012). Besides, although the effects of pure O_2 aeration on the biology of the bacterial communities in WWT plants plants have been pointed out before (Nelson and Puntenney, 1983), to date, they remain not well characterized (Calderón et al., 2012a).

The aim of this work was to compare the use of pure O_2 versus air applied as an aeration source to a pilot-scale MBR, with regard to their influence on the bacterial community composition and its enzyme activities. Molecular fingerprinting tools (polymerase chain reaction coupled to temperature gradient gel electrophoresis, PCR-TGGE) and

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sequence analysis of isolated TGGE bands were used to characterize the bacterial community structure and dynamics, in parallel to enzyme activity measures.

2. Methods

2.1. Description of the pilot-scale MBR plant

The pilot-scale experimental plant used in this study has beens described in detail elsewhere (Rodriguez et al., 2010). The system worked with real urban wastewater, supplied by Estación Depuradora Oeste Puente de Los Vados (Granada), run by EMASAGRA, S.A. (Spain). The wastewater was pumped from the primary settling tank of the municipal facility into the pilot-scale MBR plant, which was installed nearby. The plant consisted of two separated cylindrical bioreactors. The first tank (358 l operating volume and 1.85 m height) was aerated with either pure O_2 (supplied by AL Air Liquide S.A., Madrid, Spain) or air in the different phases of the study. A porous diffuser was used to generate fine bubbles, providing an adequate oxygen transfer. Dissolved oxygen (DO) was kept at 2.8 mg/l throughout the experimental set according to Rodriguez et al. (2010). The membrane was placed in a second tank separated from the bioreactor. A recirculation of 141 l/h was established to keep a constant concentration of total suspended solids (TSS) in both bioreactors. Control devices were available to monitor the transmembrane pressure (TMP), temperature, pH, and the concentration of dissolved oxygen in the activated sludge.

The experimental work in the plant was divided in three stages of operation (Table 1S), and each of the stages was subdivided in two phases, using first pure O_2 and subsequently air as aeration sources. A hydraulic retention time (HRT) of 12 h was set in all the experiments. There were no significant differences in pH nor chemical oxygen demand (COD) concentration in the influent water between the three stages, while significantly different temperatures were recorded in each of them (Table 1). A chemical cleaning of the membranes with sodium hypochlorite and citric acid (pH 2.0) was performed before the start of each of the three experimental stages, or in the event of TMP rising to the maximum level recommended by the membrane manufacturer.

Operation in the MBR system was initiated at the inflow water TSS concentration (150 mg/l) with no previous inoculation; thus, the microbial community in the sludge developed from that present in the influent wastewater. After the stabilization of the system, stages I and II were operated consecutively but keeping different conditions of biomass retention in the bioreactor, to compare the effect of pure O_2 versus air as aeration source when different concentrations of suspended solids were reached in the sludge of the MBR (Table 1). The average TSS values were 11056 ± 880 for stage I, and 7200 ± 1040 for stage II, requiring continuous purges of sludge (12.0 l/day and 6.8 l/day, respectively) to keep the TSS concentrations into the desired ranges. For stage III, the MBR was emptied and cleaned before being restarted at the inflow water TSS concentration (150 mg/l). When the system became stable, an average TSS of 3700 ± 600mg/l was reached, and maintained by a continuous purge of 32 l/day.

2.2. Physico-chemical analysis

The water samples for the analytical determinations were obtained every 24 h from the influent water, biological reactor and permeate. COD, total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). The pH was measured using a Crison pH 25 pH-meter (Crison Instruments S.A., Barcelona, Spain).

2.3. Enzyme activities

Enzyme activities phosphatase, glucosidade, protease and esterase were analyzed two times per week in the sludge of the biological tank as was described before (Calderón et al., 2012a). Experiments to measure enzyme activities were always conducted in triplicate. All chemicals required for enzyme activity analysis were provided by Sigma Aldrich (St. Louis, MO, USA).

2.4. DNA extraction and PCR amplification of 16S-rRNA genes

Total genomic DNA was extracted from sludge samples (ca. 200 mg) retrieved from the MBR twice a week, coinciding with enzyme activity measurements. The FastDNA Spin Kit for Soil and the FastPrep24 apparatus (MP-BIO, Germany) were used following the manufacturer‘s indications.

Two-step approaches were used for PCR amplification, using universal primers targeting the hypervariable V3 region of the 16S rRNA gene, as previously described by other authors for TGGE or DGGE fingerprinting (Calderón et al., 2012b). The final PCR products were cleaned and/or concentrated using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). Eight microliters (60–100 ng DNA) were loaded into each well for TGGE.

2.5. TGGE

TGGE was performed using a TGGE Maxi system (Whatman-Biometra, Goettingen, Germany). The denaturing gels [6% polyacrylamide (37.5:1 acrylamide:bisacrylamide), 20% deionized formamide, 2% glycerol and 8 M urea] were made and run with 2 × Tris–acetate-EDTA buffer. All chemicals required were purchased from Sigma Aldrich (St. Louis, MO, USA). The temperature gradient was optimized at 43–63 °C (Molina-Muñoz et al., 2009). Gels were run at 125 V for 18 h. The bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Different PCR reactions were tested and different TGGE gels were run to check the reproducibility of the results.

2.6. Analysis of TGGE fingerprints

The band patterns generated by TGGE were normalized, compared and clustered using the Gel Compar II v. 5.101 software (Applied Maths, Belgium). For cluster analysis, the TGGE profiles were compared using a method based on band presence/absence (Dice coefficient). In band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied (Calderón et al., 2012a). Dendrograms relating band pattern similarities were automatically calculated with the unweighted pair group method with arithmetic mean algorithms (UPGMA). The significance of the UPGMA clustering was estimated by calculating the cophenetic correlation coefficients.

Based on the TGGE fingerprints, several theoretical indices were calculated to analyze population richness and evenness in the MBR sludge samples. The Shannon-Wiener index of diversity, *H'* (Shannon and Weaver, 1963), was calculated for each TGGE lane using the function

$$H' = -\sum_{i=1}^{S} pi \ln pi$$

where *S* represents the total number of bands in a given TGGE lane, and *pi* represents the relative intensity of a given band in the whole densitometric curve of the corresponding lane. Relative intensities of bands were calculated using Gel Compar I.

Table 1. A. Operational parameters of experiments conducted in the pilot scale MBR during stages I, II and III. B. Enzyme activities measured in the sludge during stages I, II and III. All enzyme activities are given as mM min-1 g-1 VSS. Values shown are averages ± SD. LSD: least significant difference (Student's T test, $p<0.05$)

A)		Dates	Average T (°C)*	COD (mg/L) influent water	pH influent water	TSS (mg/L) sludge	VSS (mg/L) sludge
Stage I	Oxygen	April 20th to May 13th, 2010	19.0 ± 0.7[b]	379.2 ± 24.5[a]	8.1 ± 0.0[a]	10924 ± 273[c]	9381 ± 215[c]
	Air	May18th to May 27th, 2010	21.9 ± 1.0[b]	330.8 ± 36.8[a]	8.0 ± 0.1[a]	11350 ± 410[c]	9375 ± 324[c]
Stage II	Oxygen	June 10th to July 1st, 2010	23.7 ± 1.0[bc]	441.6 ± 30.0[a]	7.9 ± 0.1[a]	7600 ± 335[b]	6583 ± 265[b]
	Air	July 6th to July 15th, 2010	27.1 ± 1.0[c]	404.1 ± 36.8[a]	7.8 ± 0.1[a]	6720 ± 410[b]	5885 ± 324[b]
Stage III	Oxygen	September 28th to November 4th, 2010	17.4 ± 0.6[b]	406.3 ± 22.2[a]	8.2 ± 0.1[a]	3745 ± 247[a]	3288 ± 195[a]
	Air	November 9th to November 25th, 2010	13.0 ± 1.0[a]	361.9 ± 36.8[a]	8.2 ± 0.1[a]	3805 ± 410[a]	3000 ± 324[a]

B)		Acid Phosphatase*	Alkaline Phosphatase	Glucosidase	Protease	Esterase
Stage I	Oxygen	1.41 ± 0.30[a]	2.95± 0.44[a]	0.33 ± 0.05[a]	2.85 ± 0.30[a]	0.18 ± 0.07[a]
	Air	1.75 ± 0.45[a]	2.35 ± 0.67[a]	0.29 ± 0.07[a]	3.84 ± 0.45[a]	0.30 ± 0.11[a]
Stage II	Oxygen	2.01 ± 0.37[a]	3.51 ± 0.55[a]	0.31 ± 0.06[a]	3.00 ± 0.37[a]	0.72 ± 0.09[ac]
	Air	1.47 ± 0.45[a]	2.37 ± 0.67[a]	0.27 ± 0.07[a]	3.52 ± 0.45[a]	0.43 ± 0.11[ab]
Stage III	Oxygen	6.01 ± 0.27[c]	6.68 ± 0.40[b]	0.70 ± 0.04[b]	3.20 ± 0.27[a]	0.51 ± 0.07[bc]
	Air	4.36 ± 0.45[b]	8.26 ± 0.67[b]	0.76 ± 0.07[b]	3.39 ± 0.64[a]	0.66 ± 0.11[ac]

To render a graphical representation of the evenness of the bacterial communities in the different samples, Pareto-Lorenz distribution curves were drawn based on the TGGE fingerprints, as previously described (Marzorati et al., 2008). The bands in each TGGE lane were ranked from high to low based on intensity levels, and the cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands, as described elsewhere (Calderón et al., 2012a). The curves were numerically interpreted by the functional organization index (*Fo*), given by the horizontal y-axis projection on the intercept with the vertical 20% x-axis line. The calculation of the *Fo* indices allows for the evaluation of the functional redundancy of the microbial communities analyzed by fingerprinting methods (Marzorati et al., 2008)

2.7. DNA reamplification and sequencing.

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 µL of filtered and autoclaved water, and 3 µl of the resulting DNA suspensions were used for re-amplification with the appropriate primers. The PCR products were electrophoresed in agarose gels and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The recovered DNA was directly used for automated sequencing in an ABI PRISM 3130XL Genetic Analyzer (Life Technologies, CA, USA).

2.8. Phylogenetic and molecular evolutionary analysis.

The DNA sequences were analyzed and compared using the biocomputing tools provided online by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The BLASTn program (Altschul et al., 1997) was used for sequence similarity analysis. The ClustalX v. 2.0.3 software (Jeanmougin et al., 1998) was used for the alignment of the DNA sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). A p-distance based evolutionary tree was inferred using the Neighbor-Joining algorithm. The bootstrap test was conducted to infer the reliability of branch order, with a round of 1000 reassemblings. Bootstrap values below 50% are not shown in the tree.

2.9. Statistical analysis.

STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) was used for calculation of analyses of variance (ANOVA). A significance level of 95% ($p<0.05$) was selected.

Redundancy analysis (RDA), an ordination method of direct gradient analysis (Leps and Smilauer, 2003), was performed to search for patterns in the different sets of biological parameters and to assess their relationship with the environmental variables (operational parameters). The biological parameters analyzed throughout the whole set of samples were: 1) enzyme activities, 2) presence/absence of band classes in the TGGE profiles, and 3) values calculated of the Shannon-Wiener (*H'*) and functional organization (*Fo*) indices. The following environmental variables were used: TSS and VSS concentrations in sludge, COD of influent water, pH of influent water and temperature (T), and source of aeration (pure O_2 versus air). From these, TSS concentration had to be removed from the final analysis because of its strong linear correlation with VSS. RDA was chosen as the ordination method after initial analysis by detrended correspondence analysis (DCA) revealed that all the biological parameters exhibited a linear response to the environmental variables, rather than an unimodal response (Lepš and Šmilauer, 2003). All environmental variables were transformed to log (x+1). The Monte Carlo permutation test was used to assess the statistical significance of the canonical axes. All the multivariate statistics were computed using the Canoco for Windows v. 4.5 software (ScientiaPro, Budapest, Hungary).

3. Results and Discussion

3.1 Analysis of the influence of physico-chemical parameters on enzyme activities.

Table 1A shows the average values of the physico-chemical parameters measured in the bioreactor and the enzyme activities of the sludge acid phosphatase (AP), alkaline phosphatase (AlkP), glucosidase (G), esterase (E) and protease (P) during the three stages of the study. Overall, average extracellular activities reached higher values during stage III, with the exception of protease and esterase. The observed

differences were statistically significant ($p<0.05$) between stages in the case of phosphatases and glucosidase (Table 1B).

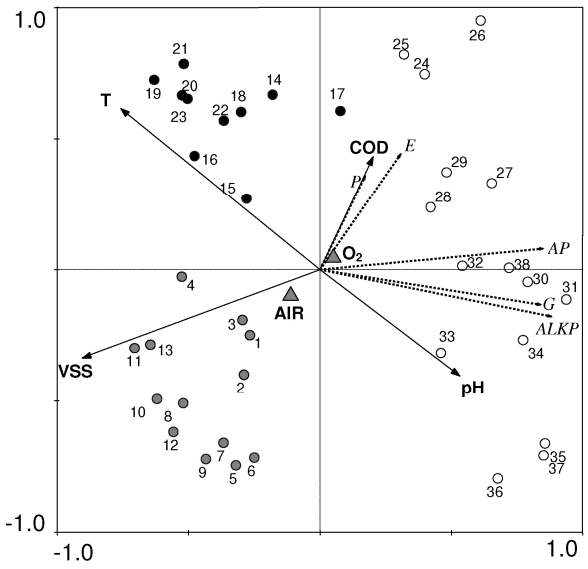
RDA was performed to relate the enzyme activities in the sludge of the MBR, aerated using either pure O_2 or air, to the variations of the different physico-chemical parameters (VSS concentration in the sludge, temperature, pH and COD of influent water). According to the results of the Monte Carlo permutation test, the significant factors explaining the variations of the enzyme activities were temperature ($p<0.05$) and VSS concentration ($p<0.05$), while the aeration source did not contribute significantly to explain the variation of the enzyme activities. An ordination plot was generated, consisting of a triplot of the significant environmental variables, species (enzyme activities) and samples (Fig. 1.). Strong species-environment correlation was found for the first ordination axe (horizontal) ($r=0.906$), which was mainly correlated to the temperature and VSS concentration, and described a 46.9% of the total variance of specie data and an 83.1% of the variance of the species-environment relation. The second ordination axis (vertical) ($r=0.527$), which was mostly associated with temperature, described a 7.7% of the total variance of species data and 13.7% of the variance of the species-environment relation. The triplot shows the clear segregation of the samples in three different groups, belonging to each stage of operation (stage I, gray circles; stage II, black circles; and stage III, white circles). The samples from the stage III are dispersed along the 2nd ordination axe, clearly denoting the temperature influence. All the enzyme activities correlated negatively to VSS concentration, and both phosphatases and glucosidase also correlated negatively to temperature. Hence, RDA analysis confirmed that the significantly higher activities observed for these hydrolases on stage III samples (Table 1B) were attributed to the lower temperatures and VSS concentrations registered during that experimental stage.

Temperature is an environmental factor with important influence on enzyme activities and the level of solubility of sludge in WWTs (Banister and Pretorius, 1998). The effect of seasonal temperature on the level of enzyme activities was explored by Molina-Muñoz et al. (2007, 2010) in studies carried out in a conventionally-aerated MBR, concluding that while protease and phosphatases reached the highest levels at temperatures over 17°C, glucosidase activity was favored at lower temperatures. The negative correlation between hydrolytic activities and VSS concentration has been previously reported in MBRs supplied with either air or pure O_2 (Calderón et al., 2012a; Molina-Muñoz et al., 2007, 2010). The latter could be explained for the inhibition of enzyme activities at given VSS concentrations, including feed-back inhibition by end-products and the accumulation of slowly biodegradable substances with inhibitory or toxic activities (Burgess and Pletschke, 2008; Molina-Muñoz et al., 2007, 2010).

To the best of our knowledge, no previous studies have compared the enzyme activities in a same WWT plant under pure O_2 versus air. In an earlier study, Goel et al. (1998) measured α-glucosidase and protease activities in the aerobic, anoxic and anaerobic phases of an activated sludge bench-scale system, finding no significant differences in the activities and only detecting a marginal difference in enzyme synthesis rates depending on the availability of electron acceptors. Several authors (Esparza-Soto et al., 2006; Shammas and Wang, 2009) had reported higher removal rates of organic matter when pure O_2 is used instead of air. According to the results described here, this improvement is not related to an enhancement of the depolymerization of the macromolecules in the bioreactor.

3.2 Analysis of the structure of bacterial communities by TGGE fingerprinting.

Cluster analysis based on the Dice coefficient allowed for the identification of significant differences in the community structure of Bacteria among the three experimental stages evaluated (Fig. 2). A clear segregation was observed in two main clusters (similarity below 45%), being the first one composed of the samples belonging to stages I and II, and the second of samples taken during stage III. The analysis also confirmed the separated clustering of samples in stages II and III which received air from those supplied with pure O_2. A total of 60 band classes were identified in the bacterial fingerprints, and a RDA analysis was also performed to evaluate the relationship between the bacterial community profiles, based on the presence/absence of band classes, and the environmental variables. According to the results of the Monte Carlo permutation test, temperature, VSS concentration and the type of aeration (pure O_2 versus air) were the significant factors ($p<0.05$) explaining the variations of the bacterial fingerprints. An ordination plot was generated consisting of a triplot of the significant environmental parameters, samples and species (band classes absent/present in the samples) (Fig. 3). Strong species-environment correlation was found for the 1st ($r=0.954$) and 2nd ($r=0.860$) ordination axes. The 1st ordination RDA axis (horizontal) was mainly correlated to VSS concentration, and described a 32.0% of the total variance of species data and 62.4% of the variance of the species-environment relation. The 2nd ordination axis (vertical) was mostly associated with the aeration by pure O_2 and the temperature, and described a 10.1% of the total variance of species data and 19.8% of the variance of the species-environment relation.



Variable	F1(horizontal)	F2 (vertical)
Temperature	-0.76	+0.62
VSS	-0.90	-0.33
COD	+0.20	+0.43
pH	+0.54	-0.41
Cumulative % of total variation	46.9	54.6

Fig. 1. Redundancy analysis (RDA) ordination diagram (triplot) showing samples (numbered circles), operational parameters (straight arrows), and enzyme activity data (dotted arrows) during operation of the MBR in stages I, II and III. First axis is horizontal, second axis is vertical. T: temperature; COD: chemical oxygen demand in influent water; pH: pH of influent water; VSS: volatile suspended solids; P: protease; E: esterase; G: glucosidase; ALKP: alkaline phosphatase; AP: acid phosphatase; O_2: pure O_2 as aeration source; Air: air as aeration source. Gray circles: samples from stage I; black circles: samples from stage II and open circles: samples from stage III.

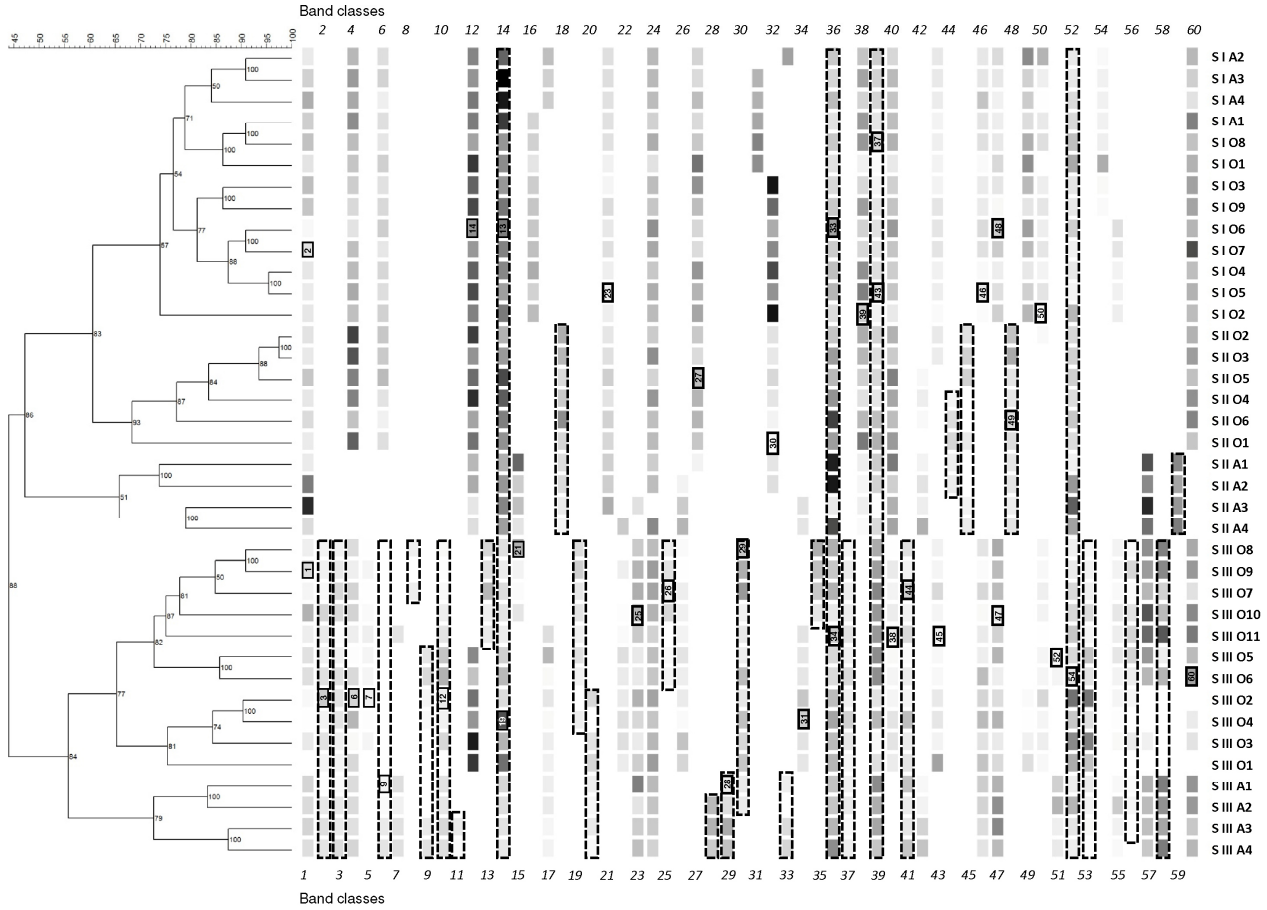


Fig. 2. Dendrogram generated by UPGMA clustering (Dice correlation coefficient) of 16S-rRNA based TGGE patterns from activated sludge samples taken during operation of the MBR in stages I, II and III, using Gel Compar II v. 4.601 (Applied Maths, Belgium). The scale bar indicates the percentage of similarity. Numbers in nodes represent the cophenetic correlation coefficient values. Band classes universal to all samples and exclusive of a same stage are highlighted and named with italic numbers *1* to *60*. Samples S I correspond to stage I; S II to stage II and S III to stage III, respectively. A: air as aeration source, and O: pure O_2 as aeration source.

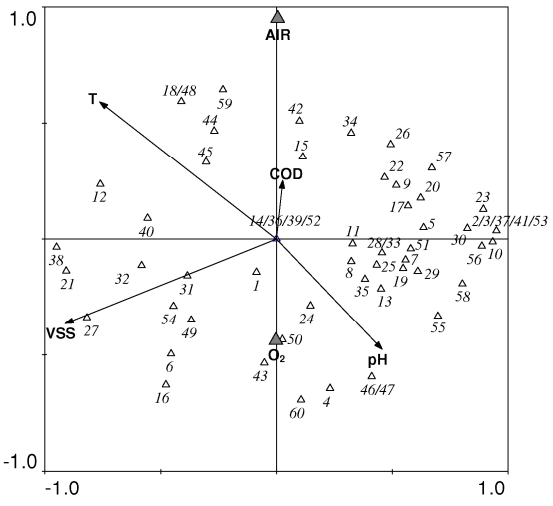
The band classes number *14, 36, 39* and *52* were found universal for all the experimental stages, meaning that none of the physico-chemical parameters affected the bacterial community, and on the triplot they are focused on the center of both axes. Band class *27* is mainly present in samples from stage I and from the phase of stage II sourced with O_2, and its relative abundance was found highly correlated to the VSS concentration. Several band classes (*18, 44, 45, 48* and *59*) were found exclusive of stage II and, according to RDA analysis, their presence was positively correlated with temperature. Band classes *46* and *47* were exclusive of stages I and III and their presence was negatively correlated to temperature, meaning that low temperatures favored their development and growth. Finally, nineteen band classes were exclusive of stage III (*2, 3, 5, 8, 9, 10, 11, 13, 19, 20, 25, 28, 29, 30, 33, 35, 37, 41, 53, 56* and *58*), thus, the prevalence of these populations was negatively correlated to both the VSS concentration and temperature.

The fingerprint analysis reveals that the bacterial community developed in the MBR was highly dynamic, as few populations were universally present in samples from all stages, or common to all samples from a same stage. These results are in agreement with previous work in a similar, conventionally aerated MBR (Molina-Muñoz et al., 2009), and in an MBR sourced exclusively with pure O_2 (Calderón et al., 2012a).

3.3. Analysis of the diversity and functional organization of the bacterial communities in the sludge.

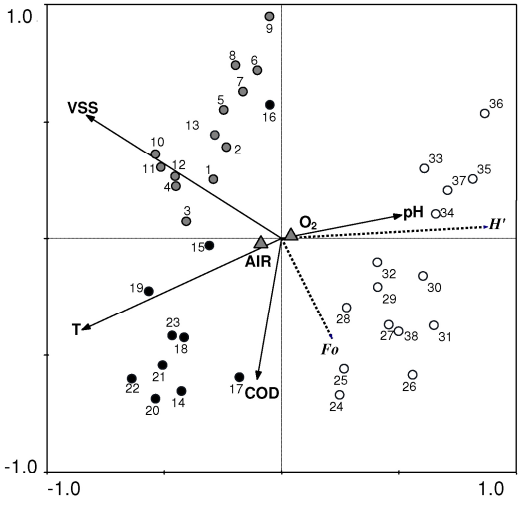
Studies based on the bacterial community profiles allowed the calculation of the Shannon-Wiener (*H'*) and *Fo* indices (Table 2). The values of *H'* ranged from 2.62 ± 0.04 to 3.17 ± 0.04. These values are similar to those described in other studies of the MBR communities based on fingerprinting techniques (Calderón et al., 2012a; Molina-Muñoz et al., 2009) and indicated a high level of population richness in the three experimental stages (Table 2). Samples from stage III displayed significantly higher values of the *H'* index, compared to stages I and II.

The values of the *Fo* index calculated for the communities analyzed in the MBR ranged from 43 ± 0.02 to 50 ± 0.01%. According to Marzorati et al. (2008), *Fo* index values around 45% represent a balanced community, potentially able to preserve its functionality under changing environmental conditions. Our results thus indicate that the communities developed in the MBR system were fully functional in all the stages, despite of the differences in aeration, temperature or biomass retention levels in the bioreactor.



Variable	F1(horizontal)	F2 (vertical)
Temperature	-0.76	+0.58
VSS	-0.92	-0.36
COD	+0.03	+0.25
pH	+0.45	-0.47
O_2	0.0	-0.40
Cumulative % of total variation	32.0	42.1

Fig. 3. Redundancy analysis (RDA) ordination diagram (triplot) showing band classes (open triangles), identified by italic letters, and operational parameters (straight arrows) during operation of the MBR in stages I, II and III. First axis is horizontal, second axis is vertical. T: temperature; COD: chemical oxygen demand in influent water; pH: pH of influent water and VSS: volatile suspended solids; O_2: pure O_2 as aeration source; Air: air as aeration source.



Variable	F1(horizontal)	F2 (vertical)
Temperature	-0.86	-0.40
VSS	-0.83	+0.52
COD	-0.10	-0.62
pH	+0.52	+0.09
Cumulative % of total variation	53.9	60.0

Fig. 4. Redundancy analysis (RDA) ordination diagram (triplot) showing samples (numbered circles), operational parameters (straight arrows), and Functional organization index (Fo) and Shannon-Wiener diversity index data (H') (dotted arrows) during operation of the MBR in stages I, II and III. First axis is horizontal, second axis is vertical. T: temperature; COD: chemical oxygen demand in influent water; pH: pH of influent water; VSS: volatile suspended solids; O_2: pure O_2 as aeration source; Air: air as aeration source. Gray circles: samples from stage I; black circles: samples from stage II, and open circles: samples from stage III.

Table 2. Multifactor ANOVA analysis of the Functional organization index (*Fo*) and Shannon index (*H'*). Values shown are average ± standard deviations. Values marked with the same lowercase letter are not significantly different (p>0.05).

		H'	*Fo*
Stage I	Oxygen	2.82 ± 0.03[a]	44% ± 0.02[a]
	Air	2.83 ± 0.04[a]	46% ± 0.02[b]
Stage II	Oxygen	2.73 ± 0.04[a]	43% ± 0.02[a]
	Air	2.62 ± 0.04[a]	49% ± 0.02[b]
Stage III	Oxygen	3.12 ± 0.02[b]	50% ± 0.01[b]
	Air	3.17 ± 0.04[b]	43% ± 0.02[a]

To further support these observations, an RDA analysis was performed, relating the values of the *H'* and the *Fo* indices calculated in all samples to the variations of physico-chemical parameters (VSS concentration, temperature, pH and COD) and the aeration source used (pure O_2 versus air). According to the results of the Monte Carlo permutation test, the significant factors explaining the variations of the *H'* and *Fo* indices were temperature ($p<0.05$) and VSS concentration ($p<0.05$). An ordination plot was generated, consisting of a triplot of the significant environmental variables, samples, and species (*H'* and *Fo*) (Fig. 4). The triplot showed a clear segregation of the samples in three different groups, belonging to each stage of operation. Strong species-environment correlation was found for the first ordination axe (r= 0.893, horizontal), which was mainly correlated to the temperature and VSS concentration and described a 53.9% of the total variance of species data and an 89.8% of the variance of the species-environment relation. The second ordination axis (vertical) is less significant (r= 0.435), and it only described a 6.1% of the total variance of species data and 10.2% of the variance of the species-environment relation. These results indicate that the diversity of the bacterial community was higher at lower temperatures and VSS concentrations, while *Fo* was negatively correlated to VSS.

The RDA analysis confirmed that temperature and VSS were the most influential factors regarding the observed changes on diversity and functional organization of the bacterial communities measured by *H'* and *Fo*, while the aeration source effect was negligible. These results are in agreement with previous work by Tocchi et al. (2012), who observed that different aeration rates and dissolved oxygen concentrations markedly influence the activity and composition of protozoan and bacterial communities, although the overall amount of bacterial diversity based on the calculation of indices such as *H'* remained stable.

3.4. Phylogenetic study of the DNA sequences of the prevalent TGGE bands.

A total of 35 TGGE bands were successfully reamplified and sequenced from TGGE gels, corresponding to the dominant populations in the mixed liquor community from all the stages, and representing 55.5% of the total band classes recognized (Fig. 5). All the partial 16S-rRNA gene sequences were deposited on the European Nucleotide Archive (EMBL/ENA) with the accession numbers HE978274–HE978308. A prevalence of Proteobacteria in the set of sequences analyzed was found for all experimental stages. The main group of identifiable TGGE bands was related to Alphaproteobacteria (17 sequences, 48.5%). The second group in order of abundance was Betaproteobacteria (14 sequences, 40%), followed by Firmicutes (3 sequences, 8.5%), and only one sequence was related to Actinobacteria (3%). While identified Alphaproteobacteria populations included members evolutionarily related to genera of 9 different families of the Rhizobiales, Sphingomonadales and Rhodobacterales, all

Betaproteobacteria-related sequences were affiliated to the Family Comamonadaceae (Burkhorderiales), indicating the dominance of members of the cosmopolitan fresh-water cluster β1 in the bacterial community. Similar trends of abundance of the orders of Alpha- and Betaproteobacteria in MBR systems were reported by other authors (Xia et al., 2010; Ye et al., 2011).

The analysis of the sequences of these isolated TGGE bands showed that most of the universal bands were related to Alphaproteobacteria (sequences 13 and 19; 33 and 34; 37 and 43, representative of band classes 14, 36 and 39, respectively, see Fig. 2), except class 52 (represented by sequence 54) which was related to Firmicutes.

Band class *27*, whose presence is highly correlated to VSS concentration, was found phylogenetically close to the Betaproteobacteria *Comamonas badia*. *Comamonas badia* was described by Tago and Yokota (2004) as a species isolated from activated sludge, being the type species characterized by its ability to degrade phenol and induce floc formation under laboratory conditions, either in single culture or as part of a consortium.

Fig. 5. Neighbor-Joining phylogenetic tree showing the positions of 35 Bacteria sequences from reamplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on ca. 150 nt length of sequences. The scale bar indicates a 5% divergence. Bootstrap values over 50% are shown in nodes.

Representatives of bands classes *46* (sequences 46) and *47* (sequences 47 and 48), were also successfully reamplified and sequenced, showing high identity to members of the Alphaproteobacteria (*Agrobacterium* sp., and *Rhodobacter* sp., respectively). Among the band classes found exclusive of stage II, only band class *48* (sequence 49) was identified by DNA sequencing, being also closely related to a cultivated strain of the alphaproteobacteria *Agrobacterium* sp.

The results presented in this paper are in agreement to some of the current available knowledge on the average composition of the bacterial community in urban WWT plants, including CAS- and MBR-based systems. Several authors have recently explored the bacterial diversity in full-scale urban WWT plants by means of phylogenetic microarrays (Xia et al., 2010) and high-throughput DNA sequencing (Hu et al., 2012; Sanapareddy et al. 2009; Ye et al., 2011; Zhang et al., 2011), and all of them supported the notion of the universal prevalence of Proteobacteria in WWT plants, a fact already well-established by earlier work based on molecular methods such as clone libraries, FISH or fingerprinting (Calderón et al., 2012a, 2012b; Molina-Muñoz et al., 2009). In particular, Yu and Zhang (2012) analyzed both the microbial community structure and gene expression of activated sludge samples by metagenomic and metatranscriptomic approaches, and the order of abundance found was Proteobacteria, followed by Actinobacteria, Bacteroidetes and Firmicutes, from both DNA- and cDNA-based approaches. Nevertheless, significant differences were observed by several authors regarding the distribution of the most abundant genera among the sludge samples, depending on the geographical area, the temperature, the salinity, the type of WWT technology, the organic load, the pH, the SRT applied to the aeration tank, and the season, among other influencing factors (Xia et al., 2010; Ye et al., 2012; Zhang et al., 2011).

Many of the available previous studies specifically focused on MBRs and based on molecular approaches such as FISH, TGGE or massive parallel sequencing conclude that Betaproteobacteria dominate the bacterial communities in WWT bioreactors (Molina-Muñoz et al., 2009; Sannaparedy et al., 2009; Zhang et al., 2011), while the prevalence of Alphaproteobacteria was considered restricted to WWT plants receiving a high load of salinity (Ye and Zhang, 2012; Zhang et al., 2011). Nevertheless, recent work documented the prevalence of Alphaproteobacteria in plants based on the MBR technology treating low-salinity wastewater (Hu et al., 2012; Xia et al., 2010), demonstrating that the dominance of members of this bacterial class is also attributable to factors other than salinity.

The two following groups that were found in order of abundance in this work were Firmicutes and Actinobacteria. Although Firmicutes are abundant in wastewater (Xia et al., 2010; Ye et al., 2011), their low prevalence in the bioreactor compared to members of the Alpha- and Betaproteobacteria classes has been attributed to the weakness of the Firmicutes microcolonies, which do not resist the strong shear forces imposed in the MBR (Hu et al., 2012).

4. Conclusions

The use of pure O_2 showed no differences compared to air regarding the performance of hydrolytic activities in an MBR treating urban wastewater, indicating that the previously described enhancement of organic load removal by applying pure O_2 to WWTs is unrelated to the improvement of the depolymerization of particulate matter. While the aeration source significantly influenced bacterial community structure, it did not affect its diversity (*H'* index) nor functional organization (*Fo* index). VSS and temperature significantly influenced the performance of all the hydrolytic enzymes, as well as on the diversity and structure of the bacterial communities, under all conditions tested.

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Appendix A. Supplementary data:

Table S1. Name of samples and aeration source.

Sample Name	Stage	Aeration source	Oxygen	Air
1	I	oxygen	1	0
2	I	oxygen	1	0
3	I	oxygen	1	0
4	I	oxygen	1	0
5	I	oxygen	1	0
6	I	oxygen	1	0
7	I	oxygen	1	0
8	I	oxygen	1	0
9	I	oxygen	1	0
10	I	air	0	1
11	I	air	0	1
12	I	air	0	1
13	I	air	0	1
14	II	oxygen	1	0
15	II	oxygen	1	0
16	II	oxygen	1	0
17	II	oxygen	1	0
18	II	oxygen	1	0
19	II	oxygen	1	0
20	II	air	0	1
21	II	air	0	1
22	II	air	0	1
23	II	air	0	1
24	III	oxygen	1	0
25	III	oxygen	1	0
26	III	oxygen	1	0
27	III	oxygen	1	0
28	III	oxygen	1	0
29	III	oxygen	1	0
30	III	oxygen	1	0
31	III	oxygen	1	0
32	III	oxygen	1	0
33	III	oxygen	1	0
34	III	oxygen	1	0
35	III	air	0	1
36	III	air	0	1
37	III	air	0	1
38	III	air	0	1

CAPÍTULO III

Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions

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Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions

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HIGHLIGHTS

- Carrier FR is the major operational parameter on the biofilm formation in a lab-scale MBBR.
- HRT and carrier type not influence statistically on the bacterial diversity of the biofilm.
- Carrier FR is the critical parameter in the start-up of MBBR-based WWTP.

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ABSTRACT

Different types of carriers were tested as support material in a lab-scale moving bed biofilm reactor (MBBR) used to treat urban wastewater under three different conditions of hydraulic retention time (HRT) and carrier filling ratios (FR). The bacterial diversity developed on the biofilms responsible of the treatment was studied using a cultivation-independent approach based on the polymerase chain reaction-temperature gradient gel electrophoresis technique (PCR-TGGE). Cluster analysis of TGGE fingerprints showed significant differences of community structure dependent upon the different operational conditions applied. Redundancy analysis (RDA) was used to determine the relationship between the operational conditions (type of carrier, HRT, FR) and bacterial biofilm diversity, demonstrating a significant effect of FR = 50%. Phylogenetic analysis of PCR-reamplified and sequenced TGGE bands revealed that the prevalent Bacteria populations in the biofilm were related to Betaproteobacteria (46%), Firmicutes (34%),Alphaproteobacteria (14%) and Gammaproteobacteria (9%).

1. Introduction

Urban and industrial wastewater reclamation is nowadays one of the major research topics resourced by people and governments. Untreated or insufficiently treated wastewater discharged to the environment causes several problems, such as eutrophication (Luostarinen et al., 2006; Plattes et al., 2007). In order to improve the quality of treated wastewater and meet the demands of environmental regulations, implementation of advanced technologies for treatment is required (Trapani et al., 2010). Biological processes based on biofilms have been proved to offer satisfactory solutions for the removal of organic components and nitrogen from wastewater, avoiding some of the problems associated with activated sludge process, such as large reactor size, need for settling tanks, and biomass recycling (Luostarinen et al., 2006; McQuarrie and Boltz, 2011).

Moving bed biofilm reactors (MBBRs) have been widely applied to treat both urban and industrial wastewaters. This technology allows BOD_5 and N removal rates similar to those of activated sludge-based processes, with the advantage of a smaller tank volume (Andreottola et al., 2000). MBBRs can be operated as anoxic or aerobic phases with freely moving buoyant plastic biofilm carriers. The systems include a submerged biofilm reactor and a liquid-solid separation unit. The objective of the MBBR systems is to achieve the growth of the biomass as a biofilm on small carriers, which have lower density than water, are continuously kept in the tank and are able to move freely in the reactor without sludge recycling (Ødegaard, 2008). Biofilm carriers made out of different materials and designs have been developed and are commercially accessible.

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Also, studies based on its mechanical and technical removal rates are available (McQuarrie and Boltz, 2011).

Knowledge on the microbiota composition involved in biofilm processes and the mechanisms by which operational variations may influence their structure is regarded as crucially important for the optimization of nutrient removal rates on MBBR systems and to implement control strategies (Boltz et al., 2011; Ciesielski et al., 2010). However, to date, little research is available related to the characteristics of the microbial biofilms grown on MBBRs, in particular, information concerning their community composition (McQuarrie and Boltz, 2011). The aim of this work was to find the relationships between different operational conditions (type of carrier, carrier filling ratio, and hydraulic retention time) and the community structure of bacterial biofilms developed on MBBRs. For this purpose, scanning electron microscopy (SEM) and molecular fingerprinting tools such as polymerase chain reaction (PCR) coupled to temperature gradient gel electrophoresis (TGGE) were used. Identification of the dominant bacteria inhabiting the biofilm was achieved by DNA sequencing of the prevalent TGGE bands.

2. Methods

2.1. Description of the lab-scale MBBRs and operating conditions

The lab-scale plant consisted of three reactors (each one with a 3-L operating volume), operated in parallel and fed from a common feed tank by a multichannel peristaltic pump. The feed tank was filled periodically with water from the outlet of the primary settler from the wastewater treatment plant (WWTP) of Estación depuradora Puente de Los Vados, (EMASAGRA, Granada, Spain).

Special sieve arrangements were adopted to retain the carriers inside the aerobic reactors. The necessary aeration system is supplied by a compressor, from which three lines are derived for each of the reactors. To ensure adequate air diffusion and the homogeneity of the mixed liquor in the bioreactor, each reactor was equipped with a porous plate and a stirring system. A system for the purge of excess sludge was also provided in each reactor. Dissolved oxygen (DO) was measured daily using an oximeter to ensure aerobic conditions. Each of the three reactors was operated for three weeks with each of the carrier filling ratios, in three consecutive phases lasting 7 days each, with influent flows of 0.6, 0.3 and 0.2 L/h, corresponding to hydraulic retention times (HRT) of 5, 10 and 15 h, respectively. Table 1 shows a summary of the operational parameters of the reactors and the names of all the analyzed samples of biofilms corresponding to their respective HRT, FR and type of carrier. Carrier inoculation was done by recycling mixed liquor as described by Jahren et al. (2002) with halfload activated sludge from the WWTP which had a medium concentration of suspended solids equal to 3 ± 0.27 g/l.

Each reactor was filled with a different carrier material (Aqwise ABC5 in Reactor 1, K1 in Reactor 2, and BIOCONS in Reactor 3). The Aqwise ABC5 and K1 carriers were made from high density polyethylene and the BIOCONS carrier was made from polyurethane sprayed with activated carbon. Characteristics of the reactors and carriers are described in detail elsewhere (Martín-Pascual et al., 2012). The three carriers were tested at filling ratios (FR) of 50%, 35% and 20%, as previously described by Martín-Pascual et al. (2012).

2.2. Scanning electron microscopy analysis

The small pieces of carrier material with adhered biofilm were sampled from the reactors to observe and analyze the structure of the biofilm with a LEO 1430-VP SEM, coupled to an Oxford ISIS 400 EDX system. The samples were fixed, dehydrated and gold-coated as previously described by Calderón et al. (2011).

2.3. DNA extraction and PCR amplification of partial bacterial 16S rRNA genes

DNA was extracted from biofilms sampled from the three bioreactors operated under all the tested conditions. A volume of ca. 60 ml of biofilm-colonized carriers were collected from the bioreactors with a sieve sampling device, in order to separate the carriers from the mixed liquor. The collected carriers were placed in sterile containers, added with 20 ml of sterile saline solution and vortexed for 1 min. The suspended biofilm material was collected by centrifugation at 3000*g* for 10 min. DNA was immediately extracted from the biofilm samples (ca. 200 mg), using the FastDNA Spin Kit for Soil and the FastPrep24 apparatus (MP-BIO, Germany).

Two-step approaches were used for PCR amplification, as previously described by other authors for TGGE or DGGE fingerprinting (Calderón et al., 2011; Molina-Muñoz et al., 2009). Reaction mixtures (50 μl final volume) contained 1 × Gold PCR buffer (150 mM Tris–HCl, pH 8.0, 500 mM KCl, Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 1.5 mM $MgCl_2$ (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 5% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA), 200 μM of dNTPs (Roche Molecular Biochemicals, Germany), 20 pM of each primer, 1 U of AmpliTaq Gold polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), and 100 μg bovine serum albumine (New England Biolabs, UK). One microliter of the DNA extracted (5 ng) was used as a template. HPLC-purified oligonucleotides were purchased from Sigma (St. Louis, MO, USA), and were used for all PCRs, performed in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). The temperature profile for the first PCR reaction was as follows: initial denaturation at 95 °C for 7 min; 25 cycles of denaturation at 94 °C for 1 min 10 s, annealing at 56 °C for 40 s, extension at 72 °C for 2 min; and final extension at 72 °C for 6 min 10 s. A modified form of the touchdown thermal profile technique (Calderón et al., 2011) was used for the second PCR; this technique involved 7 min of activation of the polymerase at 94 °C before two cycles consisting of 1 min at 94 °C, 1 min at 65 °C, and 2 min at 72 °C. The annealing temperature was subsequently decreased by 1 °C for every second cycle until it reached 55 °C, at which point 10 additional cycles were carried out; finally, a 10-min extension step at 72 °C was performed. The final PCR products were cleaned and concentrated using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). 80–100 ng of DNA were loaded into each well for TGGE.

2.4. TGGE

The denaturing gels (6% polyacrylamide (37.5:1 acrylamide:bis-acrylamide), 20% deionized formamide, 2% glycerol and 8 M urea) were made and run with a 2 × Tris–acetate–EDTA buffer. TGGE was performed using a TGGE Maxi system (Whatman-Biometra, Goettingen, Germany). All chemicals required were purchased from Sigma Aldrich (St. Louis, MO, USA). The temperature gradient was 43–63 °C, as previously optimized by Molina-Muñoz et al. (2009). Gels were run at 125 V for 18 h. The bands were made visible with silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) (Calderón et al., 2012). Different PCR reactions were tested and different TGGE gels were run to check the reproducibility of the results.

2.5. Fingerprint analysis

The band patterns generated by TGGE were normalized, compared and clustered using the Gel Compar II v. 5.101 software

Table 1
Summary of operational parameters in the reactors used in the study, and names given to samples of biofilms used for the fingerprinting studies. FR: filling ratio; HRT: hydraulic retention time; sCOD, soluble chemical oxygen demand; MLSSt: total suspended solids; MLSSv: volatile suspended solids.

Carrier type	Carrier FR (%)	HRT (h)	sCOD influent (mg O_2/l)	sCOD removed (%)	$MLSS_t$ (mg/l)	$MLSS_v$ (mg/l)	Sample name
AQWISE ABC5	20	5	146 ± 16	28.52 ± 5.19	2033 ± 84	1855 ± 106	16
		10	137 ± 30	34.54 ± 5.10	2019 ± 88	1848 ± 102	15
		15	116 ± 28	40.40 ± 8.08	2052 ± 103	1907 ± 98	9
	35	5	99 ± 23	39.36 ± 7.46	2026 ± 70	1840 ± 84	22
		10	133 ± 19	48.71 ± 9.28	2005 ± 118	1901 ± 127	25
		15	128 ± 17	50.20 ± 5.78	1903 ± 79	1724 ± 84	17
	50	5	129 ± 42	40.49 ± 7.13	2101 ± 116	1890 ± 185	18
		10	140 ± 40	50.37 ± 9.11	2081 ± 70	1900 ± 89	2
		15	166 ± 32	56.97 ± 5.95	1918 ± 147	1769 ± 148	6
K1	20	5	146 ± 16	29.83 ± 3.79	2110 ± 37	1995 ± 55	13
		10	137 ± 30	30.06 ± 7.16	1952 ± 100	1808 ± 104	10
		15	116 ± 28	43.21 ± 4.65	2070 ± 120	1981 ± 148	19
	35	5	99 ± 23	38.87 ± 6.26	2130 ± 101	2011 ± 109	23
		10	133 ± 19	50.13 ± 4.26	2105 ± 127	1948 ± 187	26
		15	128 ± 17	53.37 ± 4.25	1987 ± 109	1804 ± 126	20
	50	5	129 ± 42	39.92 ± 6.92	2216 ± 126	2026 ± 177	4
		10	140 ± 40	57.25 ± 8.38	2177 ± 117	1984 ± 117	3
		15	166 ± 32	58.92 ± 7.38	2069 ± 213	1938 ± 204	7
BIOCONS	20	5	146 ± 16	5.38 ± 0.96	2138 ± 73	2048 ± 72	14
		10	137 ± 30	16.28 ± 5.58	2067 ± 94	1992 ± 103	12
		15	116 ± 28	25.50 ± 4.43	2129 ± 108	2047 ± 109	11
	35	5	99 ± 23	10.66 ± 2.11	2067 ± 45	1986 ± 56	24
		10	133 ± 19	24.50 ± 10.77	2057 ± 133	1966 ± 129	27
		15	128 ± 17	32.60 ± 5.28	1975 ± 64	1887 ± 62	21
	50	5	129 ± 42	24.57 ± 9.67	2037 ± 48	1935 ± 43	5
		10	140 ± 40	43.24 ± 5.25	2014 ± 69	1937 ± 72	1
		15	166 ± 32	46.13 ± 4.89	2021 ± 51	1935 ± 50	8

(Applied Maths, Belgium). Cluster analyses of the TGGE profiles were done using a band assignment independent method (Pearson product–moment correlation coefficient). A method based on band presence/absence (Dice coefficient) was also used and compared. For band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied (Calderón et al., 2011). The dendrograms relating band pattern similarities were automatically calculated with the unweighted pair group method with arithmetic mean algorithm (UPGMA). The significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients. Based on the TGGE fingerprints, the Shannon–Wiener index of diversity, *H'* (Shannon and Weaver, 1963), was calculated for each TGGE lane using the function:

$$H' = -\sum_{i=1}^{S} pi \ln pi$$

where *S* represents the total number of bands in a given TGGE lane, and *pi* represents the relative intensity of a given band in the whole densitometric curve of the corresponding lane. Relative intensities of bands were calculated using Gel Compar II (Calderón et al., 2012).

The calculation of the *Fo* index, which allows for the evaluation of the functional redundancy of the microbial communities analyzed by fingerprinting methods (Marzorati et al., 2008), was done as previously described (Calderón et al., 2012).

2.6. DNA reamplification and sequencing

As a result of TGGE profiles, portions of silver-stained individual bands were picked up with sterile pipette tips and placed in 10 μL of filtered and autoclaved water. Three microlitre of the resulting DNA suspensions were used to re-amplify the separated bands using the appropriate primers. The PCR products were electrophoresed in agarose gels to be checked, and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The recovered DNA was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, CA, USA).

2.7. Phylogenetic analysis

The DNA sequences were analyzed and compared using the biocomputing tools provided online by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The BLASTn program (Altschul et al., 1997) was used for sequence similarity analysis. The ClustalX v. 2.0.3 software (Jeanmougin et al., 1998) was used for the alignment of the DNA sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). A *p*-distance based evolutionary tree was inferred using the Neighbor-Joining algorithm. The bootstrap test was conducted to infer the reliability of branch order, with a round of 1000 reassemblings. Bootstrap values below 50% are not shown in the tree.

2.8. Statistical analysis

To calculate the analyses of variance (ANOVA), STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) was used. A significance level of 95% ($p < 0.05$) was selected.

Redundancy analysis (RDA), an ordination method of direct gradient analysis (Lepš and Šmilauer, 1999), was performed to search for patterns in the set of operational conditions (HRT, FR, and type of carrier) and to assess their relationship with the composition of the bacterial communities developed on the different carriers. TGGE banding patterns generated by Gel Compar II were converted to a binary matrix by scoring bands (=species) as present (1) or absent (0), and this matrix was used for RDA without transformation. RDA was chosen as the ordination method after initial analysis by detrended correspondence analysis (DCA) revealed that the operational parameters data exhibited a linear, rather than unimodal, response to the bacterial community (Lepš and Šmilauer, 1999). The Monte Carlo permutation test was used to assess the statistical significance of the canonical axes. All the multivariate statistics were computed using the Canoco for Windows v. 4.5 software (ScientiaPro, Budapest, Hungary).

3. Results and discussion

3.1. Analysis of biofilm communities by SEM and EDX

SEM images of the three carrier types with and without biofilms developed on their surfaces are shown in Figs. S1–S3 (supplementary material). Biofilms formed on the three different carriers when the reactors were operated at an HRT of 5 h are shown in Fig. S1. Some differences in the structure of the biofilms were visible, as a more mature biofilm and better colonized carrier surface were observable for 50% FR, compared to 35% and 20% FR (Fig. S1). Different microorganisms of diverse morphologies were observed, including rod-shaped and filamentous cells (Fig. S2). The EDX analysis detected only organic material and no inorganic deposits were found.

3.2. Analysis of the structure of bacterial communities by TGGE fingerprinting.

3.2.1. Influence of operational conditions applied on the composition of the biofilm bacterial community

To compare and evaluate differences in the composition of the Bacteria community in biofilms generated on the three reactors operating under different conditions, PCR-TGGE fingerprinting was applied as a molecular tool. Cluster analysis based on the Pearson coefficient showed that samples taken from the reactors grouped in three separated clusters corresponding to the different filling ratio percentages (Fig. 1). Samples taken from reactors operated at 20% and 35% FR retained more similarity (clustering at 50%), while those retrieved from the reactors operated at 50% FR clearly clustered away (36%). Grouping was not influenced by the type of carrier or the HRT. Cluster analysis based on the Dice coefficient showed equivalent results to the Pearson- based clustering. A total of 93 band classes were detected. Band classes A, B, C, D, E, F, G and H were present in all samples (Fig. 1).

The average values of the Shannon index (H') calculated for the different bacterial communities developed under all the operation conditions tested were compared by ANOVA analysis, showing that the only parameter that displayed a significant influence ($p < 0.01$) on bacterial diversity was the carrier filling ratio (FR) (Table 2A). The Student's T test showed that the biofilms developed in the reactors operated at 50% FR had a significantly higher value of H', indicative of a higher bacterial diversity (Table 2B).

The significant influence of FR on the diversity of the biofilm bacterial community was also confirmed by RDA, based on TGGE fingerprinting data as described in Material and Methods. According to the results of the Monte Carlo permutation test, FR is a significant factor ($p < 0.01$) explaining the variations of the composition of the biofilm bacterial community analyzed by TGGE profiling, while HRT and carrier type had no significant influence. The biplot diagram generated is displayed in Fig. 2. Strong species–environment correlation was found for the 1st ($r = 0.97$) and 2nd ($r = 0.95$) ordination axes. The first ordination RDA axis (horizontal) was mainly correlated to 50% FR and 20% FR, and described a 16.6% of the total variance of species data (40% of variance of the species–environment relation). The second ordination axis (vertical), which was strongly associated to 35% FR, describes a 12.6% of the total variance of species data (70.85% of variance of the species–environment relation). The biplot shows a clear segregation of samples, corresponding to three groups determined by the carrier filling ratio.

Regarding the functionality of the Bacteria communities, there were not significant differences ($p > 0.05$) among the average

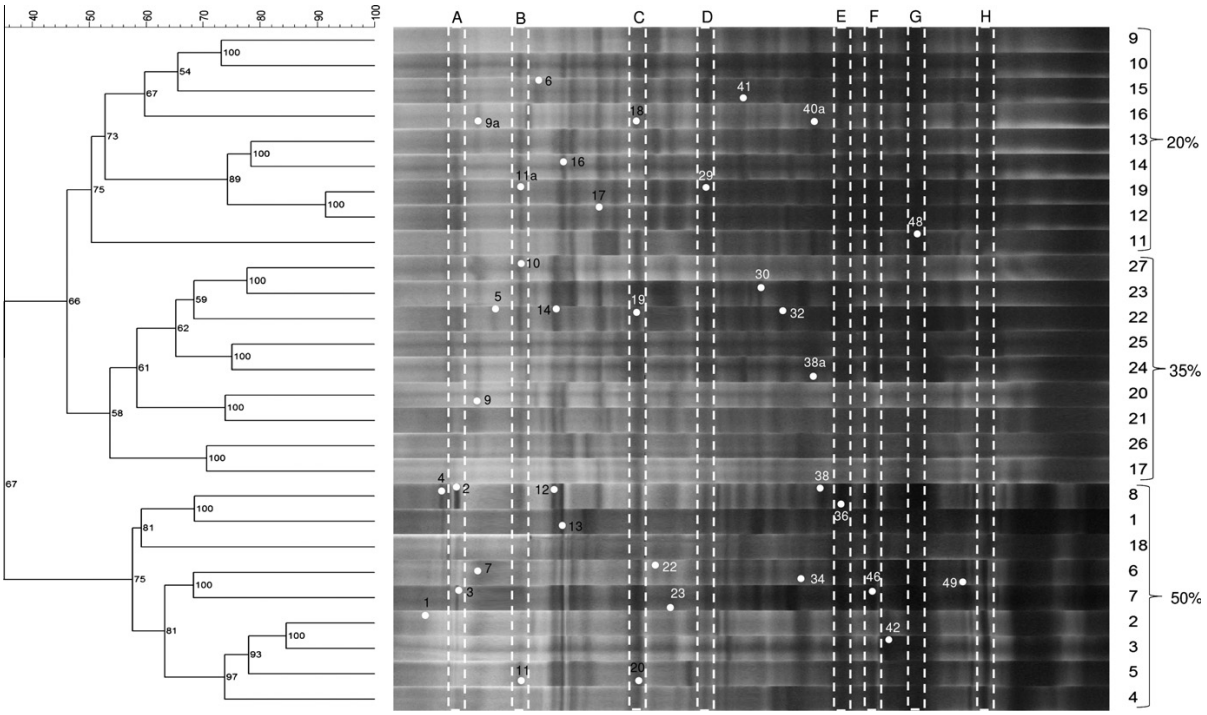
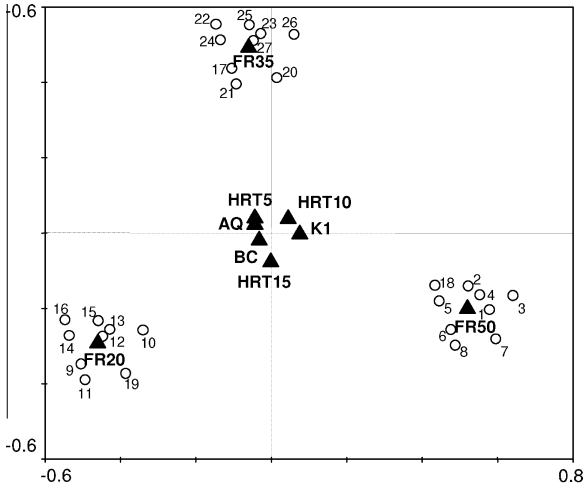


Fig. 1. Dendrogram generated by UPGMA clustering (Pearson correlation coefficient) of 16S-rRNA based TGGE patterns from biofilm samples taken during operation of the MBBR, using Gel Compar II v. 4.601 (Applied Maths, Belgium). Profiles are based on the amplification and separation of the V3 hypervariable region of the 16S rRNA gene. Bands which were successfully amplified and sequenced for the phylogenetic analysis shown in Fig. 3 are numbered and marked with an arrowhead. The scale bar indicates the percentage of similarity. Numbers in nodes represent the cophenetic correlation coefficient values. Band classes universal to all samples are highlighted and named with letters A–H.

Table 2
Effect of the operational parameters (carrier type, hydraulic retention time and carrier filling ratio) on the Shannon's diversity index (*H'*) of the biofilm communities developed in the MBBR. (A) Results of ANOVA analysis. (B) Results of the Student's *t* test for carrier filling ratio. LSD: least significant difference ($p < 0.05$).

(A) ANOVA results Factor	*p*-Value*
Carrier type	0.9438
Hydraulic retention time	0.2242
Carrier filling ratio	0.0009
(B) Student's t test for carrier filling ratio: Carrier filling ratio:	Average H'
20%	3.25
35%	3.21
50%	3.38*
LSD ($p < 0.05$)	0.08

* Significantly different ($p < 0.05$).



Relationships between RDA ordination axes and operational conditions:

Variable	F1(horizontal)	F2(vertical)
FR20	-0,46	-0,28
FR35	-0,06	0,52
FR50	0,52	-0,18
HRT 5	-0,04	0,04
HRT 10	0,05	0,04
HRT 15	0	-0,08
AQ	-0,04	0,02
K1	0,08	0
BC	-0,03	-0,02
Cumulative % of variance of species data	16.6%	29.2%
Cumulative % of variance of species-environment relation	40.0%	70.4%

Fig. 2. Redundancy analysis (RDA) ordination diagram (biplot) showing samples (numbered circles) and operational parameters (black triangles) during operation of the MBBR under all conditions tested. First axis is horizontal, second axis is vertical. HRT5, HRT10, HRT15: hydraulic retention times of 5, 10 and 15 h, respectively; AQ, K1, BC, carriers AQWISE ABC5, K1 and BIOCONS, respectively; FR20, FR35, FR50, carrier filling ratios of 20%, 35% and 50%, respectively.

values of the *Fo* indices calculated for the biofilms formed on each of the reactors, under any operation conditions tested. From the average values of *Fo* obtained (16 ± 2%), it could be inferred that the bacterial communities for each reactor had high evenness. In ecological terms, these communities may result from a lack of selective pressure and do not present a well-defined internal structure with regards to species dominance. Therefore, the communities are assumed to have an 'on average' low functional organization (Marzorati et al., 2008).

Research devoted to the biology of MBBR systems has increased in the last years. Several studies compare the influence of different type of carriers, based on the material, shape and size, on the removal of C and N by bacterial communities in these systems (Chu and Wang, 2011; Gong et al., 2011; Levstek and Platz, 2009). Regarding the study of the biofilm community composition and diversity, available previous work was specifically aimed at analyzing the nitrifying communities (Bernet et al., 2004), as MBBR systems are particularly efficient for the elimination of nitrogen compounds (Ødegaard, 2006).

The results of the present study pointed out that the carrier filling ratio is the major parameter influencing the bacterial community structure, while significant differences due to the type of carrier tested were not observed, under the conditions of the study. Carrier filling ratio was previously reported as an important parameter influencing the performance of MBBR systems. In this sense, Wang et al. (2005) evaluated the influence of different carrier filling ratios (10–75%) on the pollutant removal rates, biomass and biofilm activity of a MBBR, using a polyvinyl chloride carrier and synthetic wastewater. These authors demonstrated that COD removal rates increased for carrier filling ratios 10–50%, but did not improve over this value. Similar results were obtained by Martín-Pascual et al. (2011), with higher COD removal rates in reactors that worked with 50% FR, regardless of the carrier type tested.

Wang et al. (2005) reported that differences in the morphology and composition of biofilms were observable by SEM analysis when the carrier concentration was changed in the reactor. These differences were explained by the higher overall amount of biomass and the restricted movement of the carriers in the bioreactor at higher carrier filling ratios, which increases collisions and abrasion forces among the carrier particles, leading to the selection of the bacteria able to grow on the carrier under these conditions (Martín-Pascual et al., 2012; Wang et al., 2005). As the carrier concentration increases, the total surface area of carrier increases accordingly and more positions are available for bacteria to attach. However, when the FR is more than the 50%, the shear stresses on biofilm becomes greater and detachment of the biofilm enhanced (Gjaltema et al., 1995).

Bacterial communities in MBBRs or analogous moving bed biofilm systems have seldom been monitored by molecular methods, but available data also demonstrates that different concentrations of carrier material influence community composition of the biofilms. Bernet et al. (2004) analyzed the nitrifying communities of inverse turbulent bed reactors by single strand conformation polymorphism (SSCP) targeting PCR-amplified partial 16S rRNA genes, comparing 10% and 30% FR in separated reactors and concluding that the biofilms formed under a 30% FR showed higher diversity of nitrifying bacteria, in correspondence to higher ratios of nitrogen removal.

3.2.2. Phylogenetic study of the DNA sequences of the prevalent TGGE bands

A total of 35 TGGE bands were successfully reamplified and sequenced from TGGE gels, corresponding to the dominant populations in the biofilms communities and representing 60% of the total band classes recognized. All the partial 16S-rRNA gene sequences were deposited on the European Nucleotide Archive (EMBL/ENA) with the accession numbers HE863673–HE863707.

A prevalence of Proteobacteria in the set of sequences analyzed was found for all stages. The main group of identifiable TGGE bands was related to Betaproteobacteria (15 of 35 sequences, 43%) (Fig. 3). The second group in order of abundance was Firmicutes

(34%) followed by Alphaproteobacteria (14%), while only two sequences were related to an uncultured Gammaproteobacteria and one sequence was related to an uncultured Actinobacteria clone. These results are in agreement with previous reports derived from both laboratory and full-scale wastewater treatment plants based on technologies other than MBBRs, which conclude that Proteobacteria dominated the composition of the bacterial communities, Betaproteobacteria being the major component in most of the analyzed systems (Molina-Muñoz et al., 2009; Rosenberg et al., 2000; Wagner and Loy, 2002). Recent data regarding the bacterial diversity in full-scale urban WWTPs investigated by means of phylogenetic microarrays and high-throughput DNA sequencing

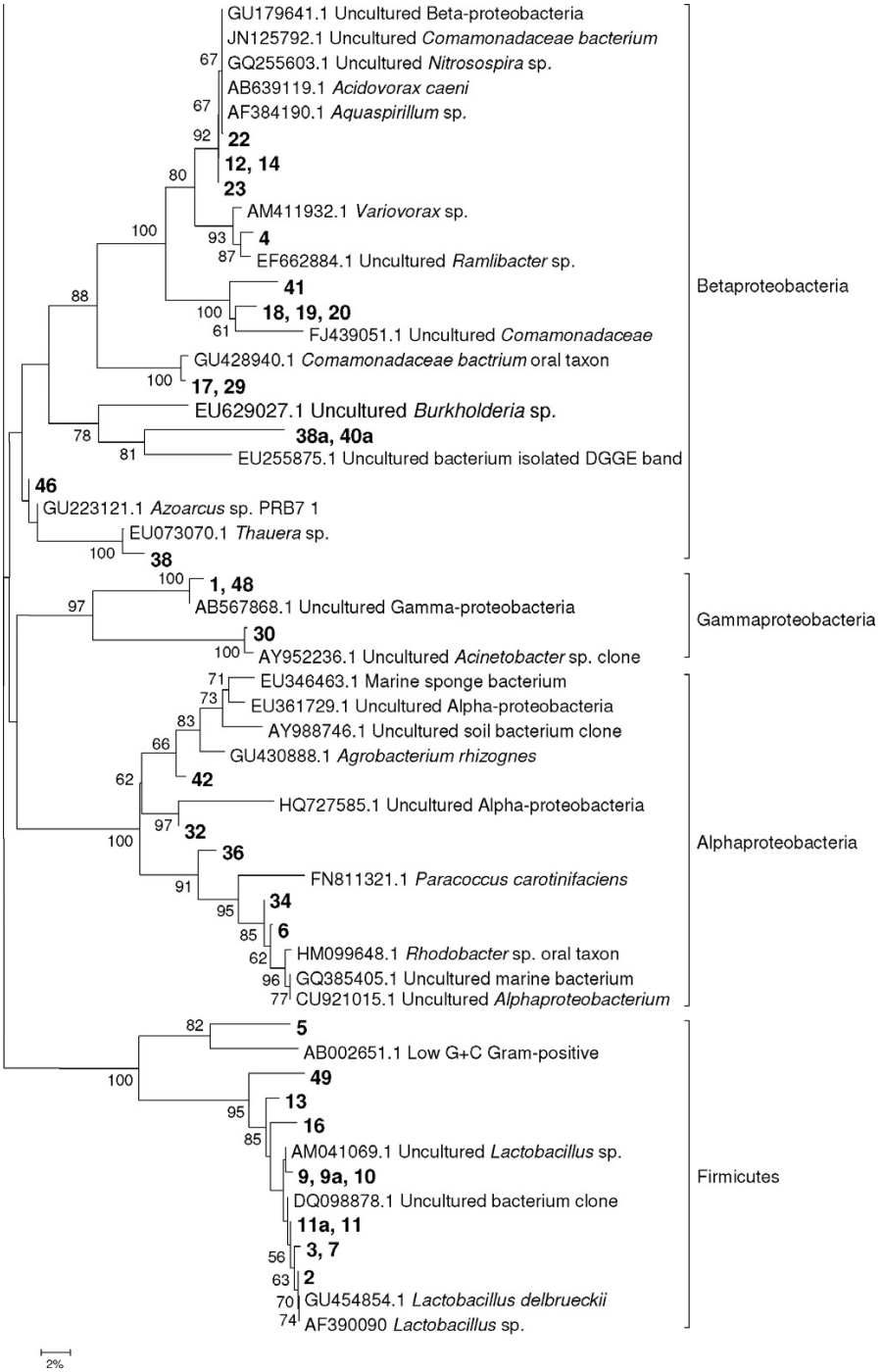


Fig. 3. Neighbor-Joining phylogenetic tree showing the positions of 35 bacteria sequences from re-amplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on ca. 200 nt length of sequences. The scale bar indicates a 1% divergence. Bootstrap values over 50% are shown in nodes.

also support the universal prevalence of Proteobacteria in WWTPs (Xia et al., 2010; Ye et al., 2011). Little is known about the composition of the biofilm bacterial communities in MBBR systems. Bernet et al. (2004) detailed the composition of nitrifying bacteria in biofilm reactors fed with synthetic mineral wastewater containing a high ammonium concentration, demonstrating that *Nitrosomonas* sp. and *Nitrospira* played an important role in the removal of nitrogen from these systems, as also reported for most wastewater treatment technologies (Wagner and Loy, 2002).

Interestingly, the results of the community analysis described here showed that 34% of the TGGE bands identified by DNA sequencing were related to the genus *Lactobacillus*. Lactobacilli are obligate fermentative bacteria, however, they are often abundant in biofilms from aerobic wastewater treatment systems (Eusébio et al., 2011; Soondong et al. 2010). Anaerobic and microaerophilic bacteria inhabit complex biofilms exposed to air due to the depletion of the oxygen levels as the biofilm depth increases, which creates anoxic zones (Santegoeds et al., 1998). A large number of populations related to the genus *Lactobacillus* in wastewater is often linked to the presence of lactose sugar, a common feature of wastewaters originating from the food industry (Acharya et al., 2011). In this sense, the wastewater used in the study was taken from the primary settling tank of an urban wastewater facility, which receives effluents from a local dairy industry (Poyatos et al., 2007).

4. Conclusions

MBBR-systems are becoming widespread for wastewater treatment, and studies regarding the impact of operation conditions on bacterial community composition and performance are gaining importance in the improvement of reactor design and optimization. The presented results show that carrier FR is the major operational factor influencing the bacterial community structure of the biofilms in a lab-scale urban WWTP. These results are in accordance with previous work linking carrier FR to MBBR performance in terms of biological removal of COD and nitrogen. Hence, available data point to the optimization of carrier FR as a critical step in the start-up of MBBR-based WWTPs.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.06.078.

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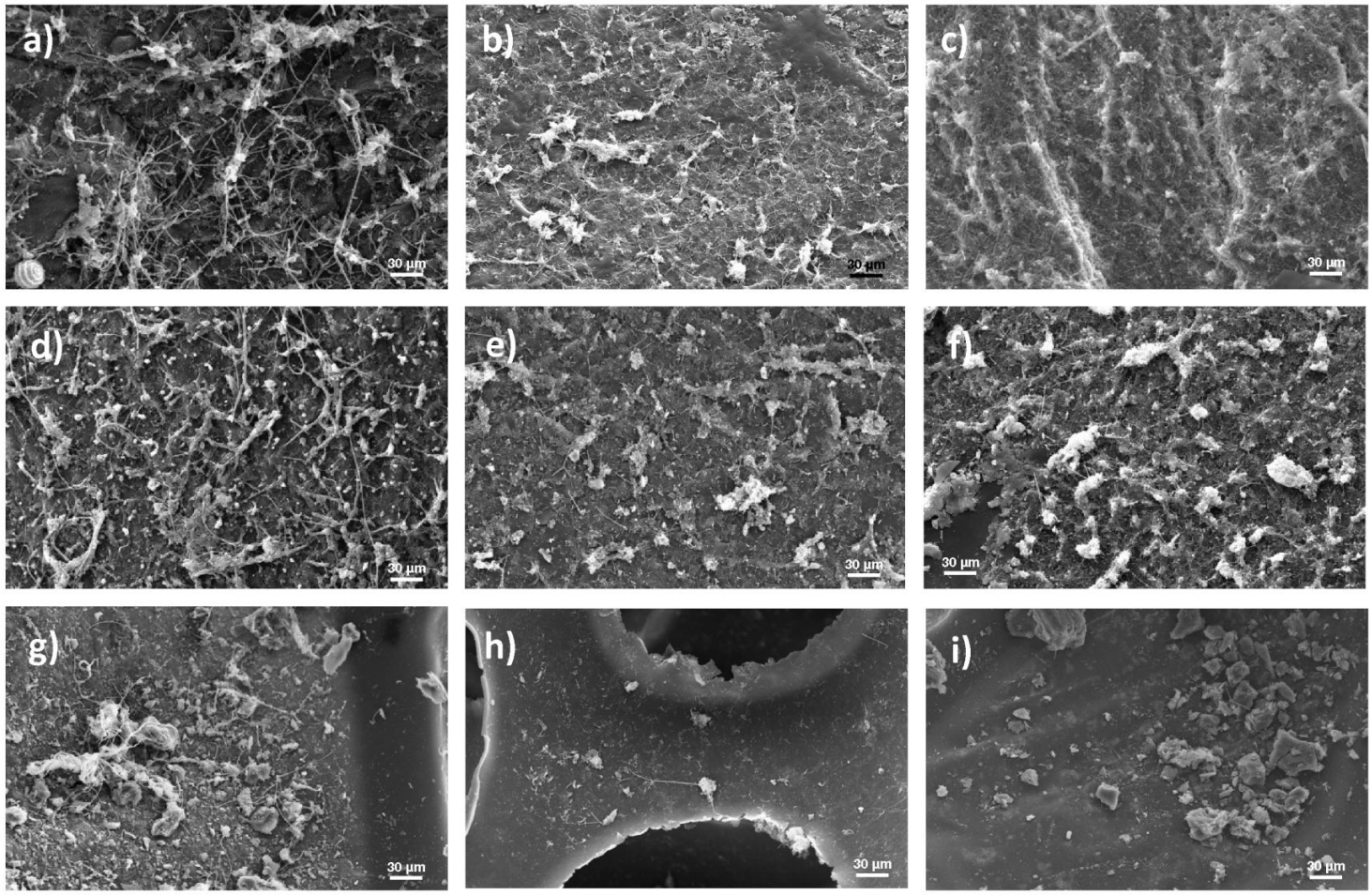
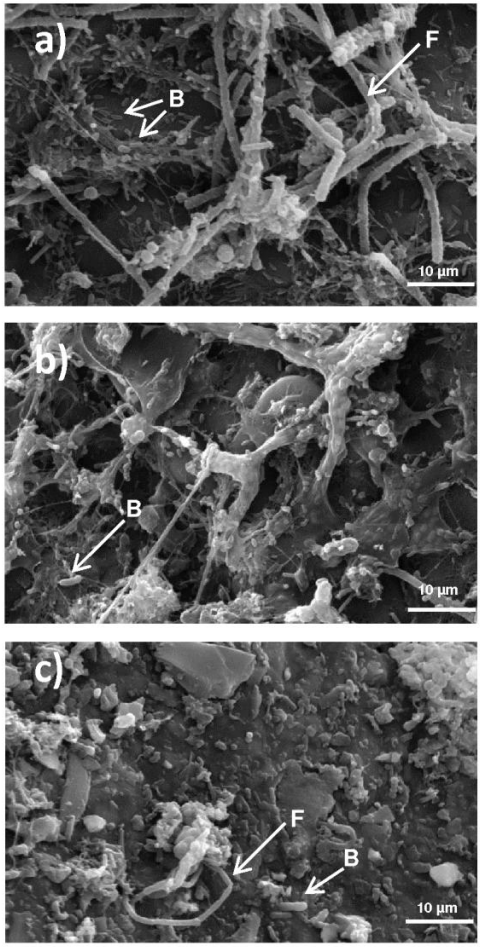
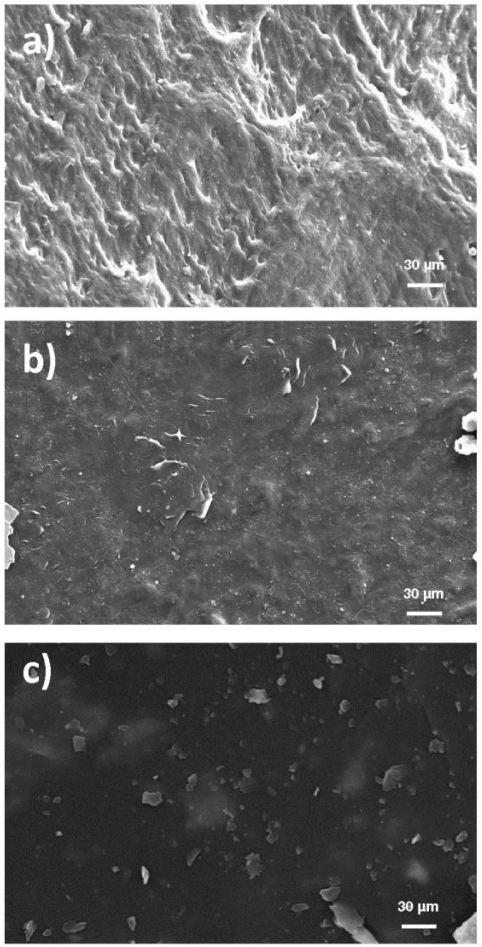


Supplementary Figure 1. SEM images of biofilms formed on the three different carriers at 50%, 35% and 20% filling ratios (FR). Figures a, b and c correspond to the AQWISE ABC5 carrier at 50%, 35% and 20 % FR respectively. Images d, e and f correspond to the K1 carrier at 50%, 35% and 20% FR and images g, h and i correspond to the BIOCONS carrier at 50%, 35% and 20% FR respectively.



Supplementary Figure 2. SEM images of biofilms formed on the three different carriers at 50% filling ratio (FR) and HRT of 5 h. Figure a, b and c corresponds to the AQWISE ABC5, K1 and BIOCONS carriers, respectively. B: rod-shaped cells, F: filamentous forms.



Supplementary Figure 3. SEM images of the three different carriers at zero time, before biofilms were developed on their surfaces. Figure a, b and c corresponds to the AQWISE ABC5, K1 and BIOCONS carriers, respectively.

4. DISCUSIÓN GENERAL

Como se ha descrito en la Introducción, los procesos biológicos son ampliamente utilizados para el tratamiento de aguas residuales tanto municipales como industriales, por su alta eficiencia en la remoción de materia/nutrientes orgánicos y sus relativamente bajos costos de operación. Dependiendo del objetivo de remoción que se quiera alcanzar, el tipo de influente, la naturaleza de los contaminantes asociados, o bien el espacio y requerimientos energéticos de los que se dispongan, se puede elegir el sistema de tratamiento a utilizar.

Desde un punto de vista meramente microbiológico, es importante considerar e incluso reconsiderar que son los microorganismos asociados al tratamiento los que realizan y determinan la eficiencia en el proceso de depuración. A lo largo de los trabajos efectuados que conforman esta tesis, se ha intentado dar especial énfasis a la investigación basada en la composición de la microbiota involucrada en sistemas basados en biorreactores de membrana (MBR) desde un acercamiento al fenómeno de taponamiento de las membranas, seguido de lo que sucede en el fango y considerando finalmente la posible integración del sistema de biopelículas a la tecnología de membranas (MBBR-MBR). En este sentido, los conocimientos basados en la ecología microbiana de las comunidades involucradas y sus mecanismos, por los cuales el funcionamiento de la planta puede influir en su estructura y dinámica, se consideran de crucial importancia para la optimización de la tecnología utilizada así como para el planteamiento de nuevas estrategias de control.

Acerca de los estudios desarrollados sobre la tecnología MBR a lo largo del capítulo I en un sistema AnMBR, se puede destacar que para el tratamiento efectuado con baja carga en el reactor anaerobio tipo UASB (624 mg/l), la remoción efectuada es considerable (71% de COD) y es mayoritariamente efectuada por el sistema de membranas (92% de COD). Aunado a esto es inminente que el fenómeno de taponamiento en las membranas se hace presente, y ha sido el foco de nuestro estudio, pues el “fouling” es el factor limitante del tratamiento (Meng et al., 2009). Así podemos destacar varias cosas; respecto a la comunidad bacteriana encontrada puede resaltarse que bajo las condiciones de limpieza efectuada se deriva una comunidad altamente especializada, es decir, que las poblaciones encontradas son diversas y presentan diferentes grados de susceptibilidad a los compuestos antimicrobianos como el NaClO. De este modo, las poblaciones microbianas más resistentes al hipoclorito de sodio son las que brindan la estructura base de la biopelícula para el taponamiento, y los grupos específicos microbianos son seleccionados para unirse o completar la biopelícula preexistente cuando se reanuda la operación después de la limpieza química sin importar la naturaleza del influente (agua residual cruda o bien efluente UASB). Así, los grupos predominantes fueron Firmicutes (46.1%), seguido de Proteobacteria de las clases Alpha (30.8 %) y Beta (11.5 %). Es interesante resaltar que el grupo predominante (Firmicutes) se reduce en abundancia cuando hay una limpieza química y éste es posteriormente remplazado por otras poblaciones con mejor capacidad para retenerse a la membrana y continuar el taponamiento. En este sentido, posterior a nuestra publicación, un estudio efectuado por Yu y colaboradores (2012) estudió la comunidad microbiana en un AnMBR en muestras del fango y de la capa taponante de las membranas, con y sin un tratamiento acoplado de ultrasonido con el objetivo de evitar el taponamiento. Los resultados obtenidos por secuenciación masiva de 454 no difieren mucho de lo que hemos

encontrado nosotros, siendo el grupo predominante Firmicutes. En este sentido, los clostridios son responsables del proceso de hidrólisis y fermentación de la materia orgánica durante la digestión anaerobia en los reactores UASB para el tratamiento de aguas, y tienen la capacidad de formar parte de biopelículas heterogéneas bajo dichas condiciones (Fernández et al., 2007; Gao et al., 2010; Patil et al., 2010).

Respecto a la comunidad de arqueas que forman parte de la capa taponante estudiada, se puede resaltar que no existen diferencias significativas en los índices de riqueza (*Rr*) y organización funcional (*Fo*) aplicados, lo que indica que este dominio no sufre alteraciones importantes cuando se provoca una limpieza química para remover el taponamiento, es decir, que la comunidad de arqueas aunque sea menos diversa que la de bacterias está bien instaurada en la membrana y no se ve afectada pese a los cambios químicos que pueda sufrir. Los grupos encontrados por huella genética fueron relativos a arqueas metanógenas de la clase *Methanospirillaceae* y *Methanosaeta.* La composición de la comunidad metanógena en el fango anaerobio del reactor UASB ha demostrado ser crucial en la estructura e integridad de los gránulos y estructura de los flóculos (McKeown et al., 2009). Hasta la fecha, incluyendo los estudios de secuenciación masiva, no se hace referencia a la presencia ni abundancia de ese dominio involucrado en el fouling de membranas, sólo existen estudios relacionados con la diversidad y presencia de este dominio en muestras de fango activo. Fredriksson y colaboradores (2012) describieron la comunidad de arqueas presente en una planta de tratamiento de agua residual tanto industrial como urbana a escala real en Suecia, mediante análisis de librerías de clones (16S rRNA) y análisis de TRFLP. Los resultados obtenidos demostraron que la comunidad de arqueas estimada era del 1.6% respecto al número total de células en el tanque de aireación, que este porcentaje aumentó considerablemente hasta un 75% en el digestor, y que las especies dominantes eran las relacionadas con *Methanosaeta.* En otro estudio muy reciente realizado por Yu y Zhang (2012), basado en el análisis metagenómico y metatranscriptómico de la microbiota involucrada en el fango activo de una planta de tratamiento en Hong Kong, se demostró que la comunidad dominante fue la de bacterias y que las arqueas presentes tuvieron una abundancia en términos de DNA de 1.10% respecto a la comunidad total, y del 0.34% si el estudio se basada en cDNA. Es decir, que la abundancia de este grupo es menor respecto al de Bacteria, que representa el 92.16 %, y en términos de comunidad activa es aún menor pues Bacteria representa un 68.42% cDNA y el otro 31.23% se refiere a la comunidad eucariota, virus y otras. El artículo incluido en el capítulo I de esta tesis concuerda con los resultados publicados posteriormente por otros autores, destacando así que nuestro estudio es pionero en términos de biofouling para esta comunidad.

Con el objetivo de aumentar las estrategias antitaponamiento, es necesario implementar herramientas que retarden la colonización microbiana, especialmente de los microorganismos que tienen una fuerte adhesión inicial y que prevalecen pese a los métodos químicos de limpieza. Por ello se han propuesto varias opciones. En primer lugar, una solución relativamente sencilla y de base técnica podría ser modificar las condiciones hidrodinámicas en el biorreactor, como la aireación. Una aireación adecuada puede determinar el tamaño del

flóculo del fango, pero hay que tener en cuenta que la excesiva aireación puede provocar una fragmentación de los floculos y consecuentemente la producción y liberación de productos microbianos solubles (SMP) que a su vez pueden convertirse en los mayores agentes taponantes de las membranas (Meng et al., 2009).

Otra posible solución química para el taponamiento sería la adición de adsorbentes o coagulantes en la suspensión de fango, que puedan disminuir el nivel de solutos o coloides, o bien, potenciar la floculación. La adición de carbón activado, "powered activated carbón" (PAC), es un procedimiento muy sencillo que conlleva a la formación de carbono biológicamente activado (BAC) que no solo forma los bioflóculos, sino que también adsorbe los biopolímeros en suspensión del fango (Ng et al., 2006). El éxito de la adición de un adsorbente radica en que la concentración adicionada sea la adecuada, de lo contrario la acción puede ser contradictoria (Meng et al., 2009). Los coagulantes son capaces de remover los SMP por neutralización de carga o puenteo. La adición de una concentración óptima de calcio, quitosán, cloruro férrico o sulfato de aluminio puede inducir una menor concentración de SMP, disminuir la hidrofobicidad, disminuir la concentración de bacterias filamentosas y mejorar la floculación, lo que deriva en la reducción de la resistencia de la capa taponante y resistencia en el bloqueo de poros de la membrana (Meng et al., 2009; Herrera-Robledo et al., 2011). Sin embargo, el potencial impacto de los coagulantes o adsorbentes en la comunidad de la biomasa o su metabolismo debe ser controlada, pues la descarga de los compuestos químicos utilizados puede generar un posible riesgo ambiental (Iversen et al., 2009; Meng et al., 2009).

Por otra parte, consideramos que una solución más efectiva en el tema de fouling pero quizá más costosa que las soluciones antes mencionadas, sería la aplicación de estrategias biotecnológicas como el quórum quenching (QQ) o bien, la utilización de nanopartículas y nuevos materiales en la fabricación de las membranas, pues la formación de la biopelícula es como tal un proceso biológico inevitable. Por ello y sin lugar a dudas, el paso previo a la aplicación de estas posibles soluciones es el conocimiento profundo de cómo está constituida la capa taponante sobre las membranas (Calderón et al., 2011).

El quórum sensing (QS) o la comunicación activa entre microorganismos usando moléculas señal, da lugar a la formación de biopelículas en los sistemas MBR (Kim et al., 2012). Por ello, se han iniciado estudios en este sentido como los realizados por Kim et al. (2012); Xiong et al. (2010) y Yeon et al. (2009), donde la solución consiste en el sabotaje de la comunicación bacteriana por la disrupción de moléculas señal como las AHL (*N*-acetil homoserina lactonas) en el caso de las bacterias Gram negativas, por lo que se paraliza el sistema de QS. De esta manera, al bloquear el mecanismo de QS disminuye la formación de EPS y, el aumento de la presión transmembrana (TMP) máxima que se genera al presentarse el fenómeno de taponamiento, se ve aplazada sustancialmente (Fig. 4.1).

Por otra parte, la modificación de las membranas desde su fabricación con nanomateriales se basa en la adición de TiO_2, Al_2O_3 o bien en modificar la rugosidad de la misma. En nuestro estudio, las muestras pertenecientes al tratamiento III referentes a las membranas alimentadas con efluente UASB bajo 2400 horas de operación, reflejaron por análisis de

microscopía electrónica acoplada a difracción de rayos X la presencia de carbonatos de calcio y magnesio así como sulfatos y óxidos de cobre. En este sentido, se debe tener especial cautela cuando las membranas sean modificadas con compuestos inorgánicos, pues se puede inducir el aumento del taponamiento inorgánico, que de manera natural ocurre en las membranas (Rivadeneyra-Torres et al., 2012). Por lo tanto, se sugiere que las membranas inorgánicas sólo se usen en aplicaciones especiales como el tratamiento de agua residual a altas temperaturas (Meng et al., 2009; Rana y Matsuura, 2010).

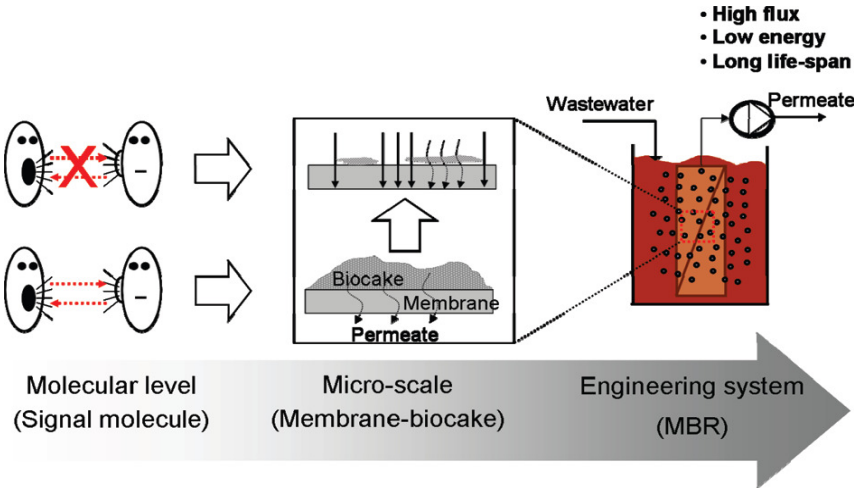


Fig. 4.1 Concepto de QS basado en el control de biofouling en sistemas MBR (Yeon et al., 2009)

Con objeto de seguir investigando la comunidad bacteriana en los sistemas MBR, el capítulo II de esta Tesis engloba estudios relacionados con esta tecnología de depuración, enfocados en el fango del biorreactor de un sistema aerobio de membranas sumergidas, con el objetivo de comprobar si al reemplazar el suministro convencional con aire por el de oxígeno puro se ejerce alguna influencia sobre la actividad biológica y diversidad de las comunidades bacterianas. Se realizaron primeramente una serie de ensayos en un MBR a escala piloto con suministro de oxígeno puro, consistentes en dos ciclos de operación bajo distintas condiciones de temperatura y concentración de VSS en el biorreactor, cuyos resultados se exponen en el subcapítulo II.1. Posteriormente, se ampliaron los estudios en la misma planta piloto, realizando otra serie de experimentos consistentes en tres nuevos ciclos de operación que en este caso se subdividieron en dos fases, la primera empleando oxígeno puro como fuente de aireación y la segunda empleando aire, al objeto de comparar más detalladamente la influencia de la fuente de aireación sobre las características biológicas del sistema.

Para el estudio de la actividad biológica en la planta piloto MBR, se incorporó en todos los estudios la medida de las actividades enzimáticas hidrolíticas como parámetro indicador, dado que éstas están directamente relacionadas con la remoción por mineralización de compuestos orgánicos que en su mayoría son carbohidratos, proteínas y lípidos (Burgess y Pletschke, 2008). El análisis estadístico multivariante tipo RDA fue aplicado para correlacionar el set de variables ambientales (parámetros fisicoquímicos del sistema MBR) y el set de datos biológicos (actividades enzimáticas) en las muestras analizadas. En la figura 1 de cada uno de los sub-

capítulos II.1 y II.2 se muestra claramente que, según el test de Monte Carlo, la concentración de sólidos suspendidos volátiles (VSS) y la temperatura fueron los parámetros físico-químicos que marcaron una diferencia estadísticamente significativa ($p<0.05$) entre los ciclos de operación analizados referentes a diferentes épocas del año. Destaca la importante influencia en todos los casos de la concentración de VSS en el biorreactor, que en los dos análisis RDA muestra la máxima correlación con el eje principal de ordenación (>90%), y su correlación negativa con los niveles de actividades enzimáticas en todos los ciclos de operación estudiados. Por lo tanto, es importante recalcar que en el sistema analizado en nuestro estudio la fuente de aireación no afecta de manera significativa al funcionamiento y metabolismo de las actividades enzimáticas hidrolíticas.

Molina-Muñoz et al. (2007, 2009) presentaron previamente estudios acerca de las actividades hidrolíticas en sistemas MBR, empleando una planta piloto análoga a la utilizada en nuestros estudios pero alimentada con aire, destacando igualmente la correlación negativa entre las concentraciones elevadas de VSS en el biorreactor y las actividades de esterasas, fosfatasas, proteasas y gluclosidasas. La similitud encontrada en los resultados puede explicarse por la inhibición de las actividades enzimáticas dadas las concentraciones de VSS, incluyendo la inhibición por retroalimentación de los productos finales y la acumulación de sustancias lentamente biodegradables con actividades tóxicas (Burgess y Pletschke, 2008). Otro factor a considerar es la retención de biomasa que se produce en los MBR, conduciendo a niveles de sólidos en suspensión mucho mayores que los que es posible alcanzar en sistemas de fango activo convencional, lo que puede limitar la disponibilidad de sustratos. De este modo, la mayor concentración de biomasa no es indicativa de una mayor actividad biológica. De hecho, Molina-Muñoz et al. (2007) encontraron una correlación negativa entre la concentración de biomasa retenida en un MBR y el recuento de células bacterianas viables.

La temperatura es un factor ambiental con una importante influencia en las actividades enzimáticas y en la solubilidad del fango en un biorreactor (Burgess y Pletschke, 2008). En nuestros estudios, se observan algunas diferencias en la ordenación de las actividades enzimáticas respecto a su correlación con la temperatura, pues en el primer grupo de experimentos (subcapítulo II.1) la mayoría de las actividades tienen un cierto grado de correlación positiva con este parámetro, mientras que en el segundo grupo de experimentos (subcapítulo II.2) hay una clara correlación negativa de las actividades fosfatasas y glucosidasa con la temperatura. Esta diferencia no es atribuible a la inclusión de la fuente de aireación como factor de variación en el análisis RDA de los experimentos realizados con dos fases distintas de aireación (subcapítulo II.2), puesto que el test de Monte Carlo indica que éste no es un factor que explique significativamente la ordenación de los datos de actividad enzimática, como muestra su posición relativa respecto a los ejes de ordenación (subcapítulo II.2, Fig. 1). Además, en los triplots mostrados en las figuras 1 de ambos subcapítulos se denota una clara segregación de las muestras analizadas según el estadío evaluado (círculos blancos, grises y negros), sin importar la fuente de aireación.

Existen algunas diferencias notorias entre los experimentos analizados en los subcapítulos II.1 y II.2. En primer lugar, destaca la mayor variabilidad de la concentración de VSS en el biorreactor experimentada en los ciclos de operación analizados en el subcapítulo II.1, en comparación con los analizados en el subcapítulo II.2 (Tabla 1A, subcapítulo II.1; Tabla 1A, subcapítulo II.2). Igualmente, es destacable que la concentración de VSS y la temperatura se correlacionen entre si negativamente en los ciclos de operación analizados en el subcapítulo II.1, mientras que esta correlación es positiva en los ciclos analizados en el subcapítulo II.2 (Fig. 1, subcapítulo II.1; Fig. 1, subcapítulo II.2). En todos los casos, el factor más explicativo de la distribución de las actividades enzimáticas en el conjunto de muestras analizadas es la concentración de VSS, por tanto es posible que su mayor o menor fluctuación y su correlación positiva o negativa con la temperatura determinen la tendencia que muestran las actividades respecto a esta última. En este sentido, un trabajo reciente publicado por Gómez-Silván y colaboradores (2012) demuestra que las variables que influyen sobre un sistema MBR, incluyendo la temperatura y la acumulación de biomasa en el biorreactor, muestran correlaciones altas y consistentes con las actividades enzimáticas hidrolíticas, pero las tendencias de correlación son variables dependiendo de la estación del año, como indicación de la existencia de diferentes estrategias de adaptación de las comunidades ante los cambios simultáneos de las variables del entorno.

Hasta el momento, no tenemos referencias de estudios previos realizados que contemplen la comparación de las actividades enzimáticas en la misma planta de agua residual confrontando la aireación con oxígeno puro contra aire. De acuerdo con nuestros resultados, el incremento en la eliminación de la materia orgánica cuando el oxígeno puro es usado en vez de aire reportado por otros autores (Esparza-Soto et al., 2006; Shammas y Wang, 2009) no está relacionado con un incremento de la despolimerización de las macromoléculas en el biorreactor.

Respecto al estudio de la diversidad y estructura de la comunidad bacteriana en el MBR, los resultados de los ensayos realizados en el capítulo II son similares en cuanto a la diversidad de la comunidad de Bacteria. En la primera tanda de ciclos de operación, empleando oxígeno puro como fuente de aireación (subcapítulo II.1), no se apreciaron diferencias con respecto a trabajos anteriores en cuanto a la diversidad (*H'*) y organización funcional (*Fo*) de la comunidad bacteriana estudiada, remarcando que dicha comunidad es versátil y capaz de adaptarse fácilmente a los cambios suscitados en parámetros como la temperatura y la acumulación de biomasa (VSS). En los experimentos analizados en el subcapítulo II.2, se corroboran las conclusiones anteriores, y además gracias a la operación del MBR en dos fases empleando O_2 y aire de manera consecutiva ha sido posible determinar que la estructura de la comunidad bacteriana sí es influenciada por la fuente de aireación empleada, aunque no repercute negativamente en el funcionamiento del sistema.

En todos los experimentos realizados, el dominio en la comunidad bacteriana estuvo determinado por Proteobacteria de la clase Alphaproteobacteria, seguida de Betaproteobacteria y en ambos casos, los siguientes Phyla menos abundantes fueron

Firmicutes y Actinobacterias (Fig. 4 subcapítulo II.1 y Fig. 5 subcapítulo II.2, respectivamente). En estudios realizados anteriormente en sistemas MBR análogos usando sólo aire como fuente de aireación por Molina-Muñoz et al. (2009) y Rosenberg et al. (2000), se observó que la clase predominante a lo largo de los cuatro estaciones del año fue Betaproteobacteria. Sin embargo, los resultados presentados en el capítulo II respecto a la alta abundancia de Alphaproteobacteria están de acuerdo con estudios más recientes realizados por otros autores empleando métodos de alta sensibilidad para caracterizar la composición de las comunidades bacterianas, tales como la secuenciación masiva en paralelo o la hibridación con microarrays filogenéticos, que muestran la abundancia de Alfaproteobacteria en diferentes sistemas de tratamiento de aguas residuales (Hu et al., 2012; Xia et al, 2010). Por tanto, ésta puede ser una característica del MBR analizado en nuestro estudio, debida a otros factores ajenos al uso del O_2 puro como fuente de aireación.

La baja abundancia de los grupos Firmicutes y Actinobacterias esta de acuerdo con lo encontrado por Yu y Zhang (2012) cuando realizaron un análisis exhaustivo con un enfoque metagenómico y metatranscriptómico para evaluar la composición microbiana de muestras de fango activo. La baja abundancia del grupo Firmicutes en estos estudios se atribuye por sus autores a la fragilidad de las microcolonias que no son capaces de persistir bajo las altas fuerzas de corte aplicadas por la aireación administrada al MBR (Hu et al. 2012). A lo largo de nuestros estudios encontramos sin embargo diferencias en la abundancia de poblaciones de Firmicutes, ya que los experimentos realizados en el subcapítulo II.1 revelaron la presencia de un 17 % de poblaciones relacionadas con este grupo en el fango del biorreactor, mientras que en los experimentos realizados en el subcapitulo II.2 este porcentaje fue del 8%. Estos datos sugieren que las diferencias en la abundancia de este grupo particular se deben a una variedad de factores y que serían necesarios experimentos adicionales para determinar si el uso de O_2 puro ejerce alguna influencia.

Los sistemas MBBR son cada vez más utilizados para el tratamiento de agua residual municipal e industrial como alternativa al sistema de fangos activos, y su aplicación está enfocada a la mayor eficiencia en la eliminación de nitrógeno, basada en el sistema de biopelículas (Andreottola et al., 2000). Con el objetivo de incrementar la eliminación de los contaminantes del agua residual por los sistemas MBR, se ha pensado incorporar la tecnología MBBR como parte de un lecho móvil en el fango presente en el MBR. De este modo, la finalidad del capítulo III estuvo basada en realizar estudios preliminares centrados sobre la microbiota presente y responsable de dicha remoción, pues hasta la fecha existen un gran número de estudios enfocados en aspectos ingenieriles pero sólo constan dos estudios basados en la comunidad microbiana responsable de la actividad biológica; el reportado por Biswas y Turner (2011) y el presentado en el capítulo III.

Los procesos biológicos basados en sistemas de biopelículas han demostrado ofrecer satisfactoriamente la remoción de compuestos orgánicos y nitrogenados provenientes del agua residual, evitando problemas como los asociados a los sistemas de fangos por la necesidad de reactores de gran tamaño, tanques de sedimentación y reciclaje de la biomasa

(McQuarrie y Boltz, 2011). Estos sistemas ofrecen una remoción similar a la de los fangos activos, con la ventaja de necesitar tanques de operación más pequeños (Andreottola et al., 2000). Los resultados presentados en el capítulo III ofrecieron nuevas aportaciones al estudio de MBBR. Al elucidar sobre el parámetro operacional con mayor influencia sobre la comunidad bacteriana instaurada en las biopelículas, el porcentaje de relleno (FR) fue el único que demostró tener una diferencia estadísticamente significativa, siendo la comunidad desarrollada con un 50% de FR la que presentó un mayor índice de biodiversidad *(H'*) (3.38). En el análisis descrito por Biswas y Turner (2011), las dos plantas de tratamiento evaluadas con diferentes tipos de agua residual no demostraron tener una diversidad tan alta (0.93 a 1.18) cuando ésta fue analizada por biblioteca de clones de 16S rRNA. Respecto a la organización funcional analizada en nuestro estudio, para todos los casos, se encontró que la comunidad bacteriana tuvo una alta uniformidad (16% ± 2 Pareto Lorenz), que en términos ecológicos significa que estas comunidades pueden resultar de una falta de presión selectiva y no presentan una estructura interna bien definida en cuanto a la dominancia de las especies. (Marzorati et al., 2008). El estudio filogenético demostró que la comunidad bacteriana de la biopelícula fue dominada por Betaproteobacteria seguida de Firmicutes, y con menor abundancia, Alpha- y Gammaproteobacteria (Fig. 3 capítulo III), a diferencia del estudio realizado por Biswas y Turner (2011), donde las comunidades bacterianas fueron dominadas por *Clostridia* y Deltaproteobacteria del grupo sulfatoreductores. En el capítulo III, además del porcentaje de relleno (FR) se evalúa además el tipo de relleno y el tiempo de retención hidráulica empleado (HRT). Se podría sugerir que es fundamental para el arranque y buen funcionamiento de los sistemas MBBR controlar el FR, pues es el factor limitante en la diversidad generada.

A lo largo de diferentes estudios referentes a muestras de fangos activos y la distribución en la abundancia de los grupos bacterianos, podemos recalcar que todos coinciden en atribuir los cambios relativos en la comunidad microbiana a factores operacionales, ambientales o bien geográficos como la temperatura, la salinidad, el tipo de tratamiento, la carga orgánica, el pH, el tiempo de retención celular, la aireación del tanque o biorreactor y el periodo estacional, entre otros (Xia et al., 2010; Zhang et al., 2011; Ye y Zhang, 2012; Calderón et al., 2012c). Por otra parte, cabe mencionar que la herramienta de TGGE es semi-cuantitativa y que existe un sesgo limitado por el alineamiento del cebador, ya que si éste es adecuado, se podrá observar una banda más intensa en el perfil, pero es importante resaltar que todos los métodos dependientes de PCR presentan estas limitaciones. Debe considerarse también la amplificación de regiones sin sentido (nonsense regions) y artefactos quiméricos producidos con frecuencia en la amplificación del gen ARNr. Así mismo, los perfiles determinados por fingerprinting pueden dar información estadística errónea debido a la cantidad de bandas de artefactos o regiones sin sentido producidas (Cowell et al., 2010). Sin embargo, el sistema de TGGE es una herramienta útil cuando lo que se quiere detectar es una huella de las comunidades más abundantes y que predominan numéricamente en la comunidad bajo algún tratamiento específico.

Por otro lado, si lo que se busca es determinar la diversidad microbiana encontrada en una biopelícula o bien en el fango para sistemas de tratamiento de aguas de manera completa, se recomienda optar por los análisis de secuenciación masiva (high throughput sequencing), aunque no hay que dejar de lado que también son métodos dependientes de PCR (Wang et al., 2012; Zhang et al., 2011). Sin embargo, parece que los resultados encontrados por técnicas independientes de cultivo no difieren mucho de los reportados por pirosecuenciación. En este sentido, y con el fin de dar un paso más, los análisis de secuencias multilocus (MLSA), basados en el análisis filogenético de múltiples genes codificadores de genes constitutivos (house-keeping genes), se han sugerido para limitar los criterios que definen las especies microbianas (Hanage et al., 2006). Por ello, las tendencias futuras de este campo de investigación deben estar enfocadas en definir los grupos funcionales microbianos basados en proteómica. Así, la funcionalidad del tratamiento basada en la comunidad microbiana será la clave del éxito de la biotecnología microbiana (Kim et al., 2012; Lacerda y Reardon, 2009).

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5. CONCLUSIONES

En base a los resultados obtenidos se presentan las siguientes conclusiones.

1. Las biopelículas taponantes formadas en un sistema AnMBR a escala piloto acoplado a membranas son de estructura compleja, estando la comunidad procariota compuesta por bacterias y arqueas, y presentando acumulación de cristales inorgánicos. Las poblaciones bacterianas muestran variabilidad en su respuesta a los procesos de limpieza química, seleccionándose una comunidad resistente diferenciada tras el empleo de NaClO, a diferencia de la comunidad de arqueas cuya estructura permanece muy estable ante este tratamiento químico.

2. El oxígeno puro aplicado como fuente de aireación en un sistema MBR sumergido convencional a escala piloto para el tratamiento de agua residual municipal, no tiene influencia sobre la diversidad bacteriana (*H'*), organización funcional (*Fo*) y grupos bacterianos predominantes en la comunidad del fango del biorreactor. En el global de los experimentos, se revela la presencia prevalente de poblaciones evolutivamente relacionadas con Alphaproteobacteria en el fango del biorreactor.

3. La evolución de las actividades enzimáticas hidrolíticas extracelulares en el sistema MBR sumergido convencional está determinada principalmente por el efecto de la concentración de sólidos suspendidos volátiles (VSS), seguida de la temperatura, y no está influenciada significativamente por la aplicación de oxígeno puro al sistema de tratamiento.

4. La diversidad bacteriana y su eficiencia en el proceso depurador de un sistema de biopelícula de lecho móvil (MBBR) para el tratamiento de agua residual municipal a escala piloto están directamente influenciadas por el porcentaje de relleno (FR) empleado, por lo que es el parámetro operacional clave a tener en cuenta para su posible integración en sistemas MBR.