UNIVERSIDAD DE GRANADA

DEPARTAMENTO DE MICROBIOLOGÍA. FACULTAD DE FARMACIA

PROGRAMA DE DOCTORADO EN BIOLOGÍA FUNDAMENTAL Y DE SISTEMAS

Procesos Microbianos en Biorreactores de Membrana con Lechos Fluidificados en Tratamientos de Aguas Residuales

Microbial Processes in Moving Bed Membrane Bioreactors for Wastewater Treatments

Patricia Reboleiro Rivas

Tesis Doctoral 2014

Editor: Editorial de la Universidad de Granada
Autor: Patricia Reboleiro Rivas
D.L.: GR 2027-2014
ISBN: 978-84-9083-217-2

UNIVERSIDAD DE GRANADA

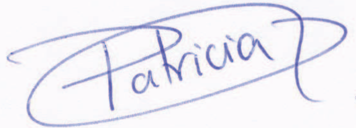
DEPARTAMENTO DE MICROBIOLOGÍA. FACULTAD DE FARMACIA

PROGRAMA DE DOCTORADO EN BIOLOGÍA FUNDAMENTAL Y DE SISTEMAS

Procesos Microbianos en Biorreactores de Membrana con Lechos Fluidificados en Tratamientos de Aguas Residuales

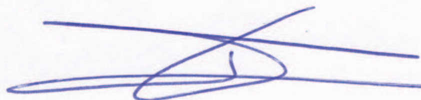
Microbial Processes in Moving Bed Membrane Bioreactors for Wastewater Treatments

Memoria para la obtención del grado de Doctor con mención internacional



Fdo. Patricia Reboleiro Rivas

***Directores*:**



Fdo.: D. **Jesús González López**
Catedrático en Microbiología
Facultad de Farmacia
Universidad de Granada

Fdo.: Dña. **María Victoria Martínez Toledo**
Catedrática en Microbiología
Facultad de Ciencias
Universidad de Granada

Fdo.: Dña. **María José Belén Juárez Jiménez**
Profesora Contratada Doctora
Facultad de Farmacia
Universidad de Granada

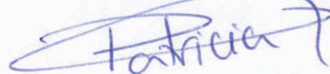
El doctorando *Patricia Reboleiro Rivas* y los directores de la tesis *Jesús González López*, *María Victoria Martínez Toledo*, *María José Belén Juárez Jiménez*, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, 10 de Junio de 2014

Director/es de la Tesis

Fdo.: **Jesús González López**

Doctorando



Fdo.: **Patricia Reboleiro Rivas**

Fdo.: **María Victoria Martínez Toledo**

Fdo.: **M. J. Belén Juárez Jiménez**

ÍNDICE

ABREVIATURAS / ABBREVIATIONS 21

RESUMEN / ABSTRACT 27

I. INTRODUCCIÓN GENERAL

1. Tecnologías de depuración biológica de las aguas residuales 37
1.1. Evolución de las tecnologías de depuración biológica 37
1.2. Biorreactores de membrana (MBRs, Membrane Biorreactors) 41
1.2.2. Ventajas de la tecnología MBR 44
1.2.3. Desventajas de la tecnología MBR 47
1.3. Biorreactores de Lecho Móvil (MBBRs, Moving Bed Biofilm Reactors) 51
1.4. Biorreactores de Membrana con Lecho Móvil (MBMBRs, Moving Bed Membrane Biorreactors) 54

2. Comunidades microbianas en las aguas residuales 57
2.1. Agregados Microbianos: *Biopelículas y Flóculos* 60
2.1.1. La matriz de sustancias poliméricas extracelulares (EPS) 64
2.1.2. Transporte de sustratos en los agregados 66
2.2. Implicaciones de los agregados microbianos en la depuración biológica 69
2.2.1. Eliminación de materia orgánica: Enzimas extracelulares 70
2.2.2. Eliminación de nutrientes: el nitrógeno 73

3. Estudio de la diversidad bacteriana de las aguas residuales 79
3.1. Estudios de la diversidad bacteriana basados en métodos independientes de cultivo 81
3.1.1. Generalidades 81
3.1.2. Técnicas moleculares: *DGGE/TGGE y qPCR* 84
3.1.3. Metagenómica: *Tecnologías de nueva generación* 87
3.1.3.1. Pirosecuenciación e Illumina 89

4. Diversidad bacteriana en las plantas de tratamiento de aguas Residuales 93

Referencias 95

II. OBJETIVOS 111

III. CHAPTER 1
Enzymatic activities in a Moving Bed Membrane Bioreactor for real urban wastewater treatment: effect of operational conditions

1. Introduction 115

2. Materials and Methods 117
2.1. Experimental set-up 117
2.1.1. Pilot-scale experimental plant 117
2.1.3. Operating conditions 119
2.2. Biofilm recovery 121
2.3. Physico-Chemical analysis 121
2.4. Evolution of microbial enzymatic activities 122
2.5. Statistical analysis 123

3. Results and Discussion 124

3.1. Operational parameters 124
3.2. Enzymatic activities 127
3.3. Statistical multivariate analysis: influence of physico-chemical parameters on enzymatic activities 133

References 139

IV. CHAPTER 2
Study of the structure and dynamic of total Bacteria and ammonia-oxidizing bacteria communities, involved in the depuration process of urban wastewater in moving bed membrane bioreactor: TGGE fingerprinting and 454-pyrosequencing methodologies.

1. Introduction 145

2. Materials and Methods 149
2.1. Experimental design 149

2.1.1. Pilot-scale experimental plant | 149
2.1.2. Bioreactor operating conditions | 149
2.2. Biofilm recovery | 151
2.3. Physical-chemical analysis | 152
2.4. Structure and dynamics of total bacteria and ammonia oxidizing bacteria by TGGE | 154
2.4.1. DNA extraction and PCR amplifications of partial bacterial and ammonia-oxidizing β-proteobacteria 16S rRNA genes | 154
2.4.2. TGGE gels | 156
2.4.3. Fingerprinting analysis | 156
2.4.4. DNA sequencing of TGGE-isolated bands and Phylogenetic analysis | 158
2.5. Analysis of total bacterial and ammonia oxidizing bacteria by 454-pyrosequencing | 159
2.6. Statistical analyses | 160

3. Results and Discussion | 161
3.1. Study of total Bacteria and AOB communities' structure by PCR- TGGE fingerprinting | 161
3.1.1. Cluster analysis of TGGE profiles | 162
3.1.2. Analysis of diversity, structure and functional organization of total Bacteria and AOB communities | 168
3.1.3. Phylogenetic study of the prevalent TGGEs bands of total Bacteria and AOB | 179
3.2. Study of total Bacteria community structure by 454 pyrosequencing | 187
3.2.1. Richness and Diversity indices | 188
3.2.2. Total Bacteria community composition | 192
3.2.3. Statistical multivariate analysis: Influence of physico-chemical parameters on total Bacteria community structure at the different taxonomic levels and at OTUs level | 205

References | 212

V. CHAPTER 3

Quantitative study of the nitrifying and denitrifying communities, involved in the depuration process of urban wastewater in moving bed membrane bioreactor: Effect of operational conditions

1. Introduction 229

2. Materials and Methods 233

2.1. Experimental design: description of the pilot-scale MBMBR experimental plant and operating conditions 233

2.2. Physico-chemical analysis 235

2.3. Quantitative investigation of N-cycle bacteria by Qpcr 237

2.3.1. DNA extraction and qPCR assays 238

2.4. Statistical analysis 242

3. Results and discussion 243

3.1. Absolute and relative abundances of total Bacteria, AOB, NOB (*Nitrospira*) and denitrifiers (*nosZ*) in both SB and AB fractions 243

3.2. Absolute abundance of total Bacteria, AOB, NOB (*Nitrospira*) and denitrifiers in the MBMBR and biofilm contribution. 251

3.3. Statistical multivariate analysis: influence of operational parameters on the abundance of total Bacteria, AOB, NOB and denitrifiers 253

References 261

VI. OVERALL DISCUSSION 275

VII. CONCLUSIONES / CONCLUSIONS 291

RELACIÓN DE TABLAS Y FIGURAS / TABLES AND FIGURES 297

ABREVIATURAS / ABBREVIATIONS

ABREVIATURAS / ABBREVIATIONS

AB BA	Attached Biofilm Biopelícula Adherida
ANOSIM	Analysis of similarities Análisis de similaridad
ANOVA	Analysis of variance Análisis de la varianza
AOB	Ammonia-oxidizing bacteria Bacterias oxidadoras de amonio
APS	Adenosin-5´-fosfosulfate Adenosina-5´-fosfosulfato
ARN RNA	Ribonucleid acid Ácido ribonucleico
ATP	Adenosine triphosphate Adenosina trifosfato
BLASTn	Nucleotide Basic Local Alignement Search Tool
BOD_5 DBO_5	Biological Oxygen Demand at 5 days Demanda Biológica de Oxígeno a los 5 días
bp pb	Base pairs Pares de bases
BTS STB	Biofilm Total Solids Sólidos Totales en la Biopelícula
BVS SVB	Biofilm Volatile Solids Sólidos Volátiles en la Biopelícula
CAS	Conventional Activated Sludge Sistema convencional de fangos activos

CFR(s)	Carrier Filling Ratio(s) Porcentaje(s) de relleno
COD DQO	Chemical Oxygen Demand Demanda Química de Oxígeno
DCA	Detrended Correspondence Analysis Análisis de correspondencia sin tendencia
DGGE	Denaturating Gradient Gel Electrophoresis Electroforesis en gel con gradiente desnaturalizante
DMSO	Dimethyl sulfoxide Dimetil sulfóxido
DNA ADN	Desoxiribonucleid acid Ácido desoxirribonucleico
DNRA	Dissimilatory Nitrate Reduction to Ammonium Reducción desasimilatoria del nitrato a amonio
dNTP	Desoxiribonucleotides triphosphate Desoxirribonucleótidos trifosforilados
Dy	Dynamics index Índice de dinámica
E	Experiment Experimento
EBI	European Bioinformatics Institute
EMBL	European Molecular Biology Laboratory
emPCR	Emultion PCR PCR en emulsion
EPSs	Extracellular Polimeric Sustances Sustancias poliméricas extracelulares
Fo	Functional organization index Índice de organización funcional

H'	Shannon-Wiener index Índice de Shannon-Wiener
HRT(s) TRH(s)	Hydraulic Retention Time(s) Tiempo(s) de Retención Hidráulico
IFAS	Integrated Fixed-Film Activated Sludge
LSD	Least Significant Difference Diferencia mínima significativa
MBBR(s)	Moving Bed Biofilm Reactor(s) Biorreactor(es) de lecho móvil
MBMBR(s)	Moving Bed Membrane Bioreactor(s) Biorreactor(es) de membrana con lecho móvil
MBR(s)	Membrane Bioreactor(s) Biorreactor(es) de membrana
MLTSS STLM	Mixed Liquor Total Suspended Solids Sólidos Totales en suspensión del Licor Mezcla
MLVSS SVLM	Mixed Liquor Volatile Suspended Solids Sólidos Volátiles en suspensión del Licor Mezcla
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing Secuenciación de nueva generación
NOB	Nitrite-oxidizing bacteria Bacterias oxidadoras de nitrito
OTU(s)	Operational taxonomic unit(s) Unidad(es) taxonómica(s) operacional(es)
PCR	Polymerase Chain Reaction Reacción en cadena de la polimerasa
pDNA ADNp	Plasmid DNA ADN plasmídico

PPi	Inorganic Pyrophosphate Pirofosfato inorgánico
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Real-Time Polymerase Chain Reaction Reacción en cadena de la polimerasa cuantitativa en tiempo real
RDA	Redundancy analysis Análisis de redundancia
Rr	Range weighted richness index Índice de rango ponderado de riqueza
rRNA ARNr	Ribosomal RNA ARN ribosómico
SB BS	Suspended Biomass Biomasa Suspendida
SND	Simultaneous Nitrification and Denitrification Nitrificación y desnitrificación simultánea
SRT	Solids Retention Time Tiempo de retención de sólidos
T	Temperature Temperatura
TGGE	Temperature Gradient Gel Electrophoresis Electroforesis en gel con gradiente de temperatura
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WWTP(s)	Wastewater Treatment Plant(s) Planta(s) de tratamiento de aguas residuales
WWTS(s)	Wastewater Treatment System(s) Sistema(s) de tratamiento de aguas residuales

RESUMEN / ABSTRACT

RESUMEN

Hoy en día la mejora del tratamiento biológico de las aguas residuales es un objetivo clave para conseguir una mejor calidad de los efluentes tratados. Con ello, se logra por un lado proteger el medio ambiente y por otro la reutilización de dichas aguas. En los últimos años, una alternativa emergente para mejorar el proceso biológico del tratamiento de aguas residuales se basa en la idea de combinar el crecimiento microbiano suspendido y el crecimiento adherido. En este sentido, se han propuesto diferentes combinaciones de tecnologías basadas en los procesos de biopelícula y membranas de filtración (biorreactores de membrana híbridos). Desde un punto de vista biológico, la incorporación de los procesos de biopelícula confiere diversas ventajas a los biorreactores de membrana (MBRs), tales como una mayor actividad de la biomasa, mayor resistencia de la biomasa a sustancias tóxicas o *socks* de carga orgánica y mejora de los procesos de nitrificación y desnitrificación. Uno de los MBRs híbridos más novedosos es el biorreactor de membrana con lecho fluidificado móvil (MBMBR), el cual se fundamenta en la adición de soportes al proceso convencional de los MBRs. El material de soporte se mueve libremente en el biorreactor y poco a poco es colonizado por la biomasa, dando lugar al desarrollo de una biopelícula.

El tratamiento biológico de las aguas residuales se nutre de la actividad metabólica de los microorganismos para la transformación de sustancias tóxicas, la degradación de contaminantes orgánicos y la eliminación de nutrientes de efluentes industriales y urbanos. En particular, el proceso de biodegradación comienza con la hidrólisis de macromoléculas, llevada a cabo por las enzimas extracelulares. Este proceso es considerado como un paso limitante en la degradación de la materia orgánica, ya que una importante fracción de la materia orgánica presente en el influente

debe ser hidrolizada por enzimas antes de que ésta pueda ser utilizada metabólicamente por las bacterias. En consecuencia, la investigación de las enzimas extracelulares así como el estudio de la estructura y dinámica de las comunidades microbianas son considerados esenciales para entender los factores ambientales/operacionales que afectan a la eficiencia y estabilidad del proceso de depuración biológica, así como para desarrollar estrategias para mejorar la eliminación de contaminantes orgánicos y nutrientes.

En este contexto, esta investigación se ha basado en la caracterización microbiológica de la biomasa suspendida (BS) y la biopelícula adherida (BA) desarrollada en un MBMBR, así como en el establecimiento de una adecuada correlación entre los parámetros operacionales y los datos biológicos. Los objetivos de la presente Tesis Doctoral fueron: el análisis de las actividades enzimáticas extracelulares (fosfatasa ácida, fosfatasa alcalina y α-glucosidasa), el estudio de la estructura y dinámica de la comunidad bacteriana total y las poblaciones de las bacterias oxidadoras de amonio (AOB), y el estudio cuantitativo de las bacterias degradadoras de compuestos nitrogenados. Con objeto de establecer una relación entre las condiciones operacionales y los datos biológicos, se realizaron cuatro fases experimentales con un porcentaje de relleno (CFR) del 20% (v/v) y otras cuatro con un 35% (v/v) de CFR, combinando para cada CFR dos concentraciones de sólidos totales en suspensión del licor mezcla (STLM) (2.500 y 4.500 mg/L) y dos tiempos de retención hidráulico (TRH) (10 y 24h).

Las actividades hidrolíticas fosfatasa ácida, fosfatasa alcalina y α-glucosidasa fueron mayores en la biomasa suspendida que en la biopelícula adherida, independientemente de las condiciones operacionales. Esto posiblemente fue debido a

una mayor difusión del sustrato en la biomasa suspendida. Con objeto de evaluar la relación entre las actividades enzimáticas y los parámetros operacionales se realizó un análisis de redundancia (RDA). Los niveles de actividad enzimática extracelular de la BS y la BA no fueron influenciados por los mismos parámetros operacionales. Las actividades enzimáticas de la BS fueron influenciadas por la concentración de sólidos volátiles del licor mezcla (SVLM), la demanda química de oxígeno (DQO), la temperatura (T) y el TRH, mientras que las actividades enzimáticas de la biopelícula fueron afectadas principalmente por la concentración de sólidos totales de la biopelícula (STB), el CFR y la DQO.

Para abordar el estudio de la estructura y dinámica de la comunidad bacteriana total y de las poblaciones bacterias oxidadoras de amonio, se utilizaron las metodologías de TGGE y pirosecuenciación 454. La estructura y dinámica de la comunidad bacteriana total y de las poblaciones de AOB, fueron muy similares tanto en la biomasa suspendida como en la biopelícula adherida. Esto podría estar relacionado con el movimiento libre y continuo de los soportes dentro del biorreactor, favoreciendo de este modo, las interacciones entre ambas fracciones (BS y BA). Otra de las posibles hipótesis podría estar basada en el tiempo de residencia de los soportes dentro del biorreactor. Para lograr una mayor comprensión de los principios ecológicos que caracterizan la comunidad bacteriana total y las poblaciones de AOB, se calcularon diferentes índices teóricos de riqueza, organización funcional, dinámica y diversidad. Estos índices describieron un alto grado de diversidad y una óptima organización funcional de la comunidad bacteriana total. Por el contrario, las poblaciones de AOB se caracterizaron como más especializadas y probablemente más frágiles frente a cambios en las condiciones ambientales/operacionales. Los filos predominantes en ambos tipos de muestras (BS y BA) y en todos los experimentos fueron *Proteobacteria* (clases α y β

Proteobacteria) y *Actinobacteria* (clases *Acidimicrobiia* y *Actinobacteria*), seguido de *Bacterioidetes, Chloroflexi* y *Firmicutes.* En cuanto a la composición taxonómica de las poblaciones bacterianas oxidadoras de amonio (clase β-*Proteobacterias),* las bandas secuenciadas de TGGE se relacionaron filogenéticamente con el género *Nitrosomonas* (*N. oligotropha/ureae*, *N. cryotolerans* y *N. europea*). De igual modo se detectaron mediante pirosecuenciación 454 diversos miembros relacionados con la familia *Nitrosomonadaceae* (del orden *Nitrosomonadales*) aunque no se pudieron clasificar a nivel de género. El análisis estadístico multivariante indicó que los cambios en la concentración de SVLM y STB, el CFR, la DBO_5 y la temperatura, afectaban directamente a la estructura y la dinámica de la comunidad bacteriana total y de las poblaciones de AOB en ambos tipos de muestra (BS y BA).

Por último, se evaluó mediante PCR cuantitativa a tiempo real (qPCR) la abundancia de los genes diana de bacterias totales (16s rRNA), bacterias oxidadoras de amonio (16s rRNA y *amoA* de AOB), bacterias oxidadoras de nitrito (16s rRNA de *Nitrospira*, NOB) y bacterias desnitrificantes (*nosZ*). Los resultados obtenidos mostraron que la abundancia y dinámica de bacterias totales, AOB, NOB y desnitrificantes fueron bastante similares en ambas fracciones (BS y BA), sugiriendo que la biomasa suspendida y la biopelícula adherida podrían jugar un papel igual de importante en el proceso de nitrificación y desnitrificación en el MBMBR. El análisis RDA y el test de permutación de Monte Carlo confirmaron un efecto significativo de la concentración de VSLM y STB, de la DQO, de la temperatura y del CFR en los valores de abundancia de bacterias totales, AOB, NOB y desnitrificantes en ambos tipos de muestras (BS y BA).

ABSTRACT

Nowadays, there is certainly no doubt that improving of biological wastewater treatments is a key goal to get better quality of the effluents, which not only contribute to protect the environment, but also to enable their reuse. In recent years, the idea of combining suspended and attached growth to improve biological process on well established wastewater treatment technologies represents an interesting solution. In this sense, different combinations of biofilm-based and membrane filtration technologies (hybrid membrane bioreactors) have been proposed. From a biological point of view, the incorporation of biofilm process confers several advantages to the membrane bioreactor system (MBR) such as higher biomass activity, higher biomass resistance to toxic or organics loads socks and improving of nitrification and denitrification processes, since the biofilm favours the development of slow growing bacteria, such as nitrifying bacteria. One of the most innovative hybrid MBRs systems is the moving bed membrane bioreactor (MBMBR) described as a combination of the moving bed biofilm rector (MBBR) and the membrane technology. In essence, MBMBRs systems are based on the addition of freely moving support material (carriers) to the biological reactor of MBRs. Biofilm development takes place in the carriers.

The biological wastewater treatments feed on the metabolic activity of microorganisms for the transformation of toxic substances, the degradation of organic pollutants and the removal of nutrients from urban and industrial effluents. In particular, the biodegradation process begins with the hydrolysis of macromolecules performed by extracellular microbial enzymes. This initial process is considered as the main rate-limiting step in organic matter degradation, since an important fraction of organic

matter, present in the influent, must be hydrolysed by enzymes before it can be utilised by bacterial metabolism. Consequently, it is generally accepted that the investigation of extracellular hydrolases, as well as the study of structure and dynamics of microbial communities are essential to understand the environmental or operational factors affecting efficiency and stability of the biological process, as well as to develop strategies to improve the performance of removing organic contaminants and nutrients.

In this context, this research has focussed on the microbiology characterization of the suspended biomass (SB) and attached biofilm (AB) developed in a moving bed membrane bioreactor (MBMBR) as well as on the establishment of an adequate link between operational parameters and biological data. Specifically, the analysis of microbial hydrolytic enzymatic activities (acid phosphatase, alkaline phosphatase and α-glucosidase), the study of structure and dynamic of total Bacteria and AOB (ammonia-oxidizing bacteria) communities, and the quantitative study of N-cycle bacteria were the goals of the research. In order to establish a rough link between operational conditions and biological data, four experimental phases with 20% and 35% (v/v) carrier filling ratios (CFRs) were conducted, which combined two mixed liquor total suspended solid (MLTSS) concentrations (c.a. 2,500 and 4,500 mg/L) and two hydraulic retention times (HRTs) (10 and 24 h).

Regardless of the operational conditions, hydrolytic activities (acid phosphatase, alkaline phosphatase and α-glucosidase) in the mixed liquor (suspended biomass) were higher than in the biofilm under all the experimental conditions tested, it was possibly due to better substrate diffusion in the mixed liquor. A redundancy analysis (RDA) was performed to evaluate the relationship between enzymatic activities and operational parameters. The levels of extracellular enzymatic activities were not influenced by the

same variables in the SB and AB samples. Hydrolytic activities in the SB fraction were more sensitive to changes in mixed liquor volatile solids concentration (MLVSS), chemical oxygen demand (COD), temperature (T) and HRT, while they were mainly affected by biofilm total solids (BTS), CFR and COD in the AB fraction.

Fingerprinting and next generation sequencing methodologies (TGGE and 454-pyrosequencing, respectively), were used to approach the study of the structure and dynamic of total Bacteria and ammonia-oxidizing bacteria (AOB) communities. These molecular techniques revealed that the structure of both total Bacteria and AOB communities was greatly similar in both SB and AB samples under all the experimental conditions tested. This fact could be connected with the free and continuous movement of carriers inside bioreactor, which could favour interactions between both SB and AB fractions. Another hypothesis was based on the residence time of carriers inside bioreactor.

For better understanding about population richness and evenness in the samples, several theoretical indices (richness, functional organization, dynamics and diversity) were calculated for total Bactria and AOB communities. These indices described a total Bacteria community characterized by a high degree of diversity and optimal functional organization. By contrast, AOB community was characterized as more specialized and probably more fragile to environmental/operational conditions changes. TGGE bands sequencing and 454-pyrosequencing, showed that *Proteobacteria* and *Actinobacteria* were the predominant phyla in both sample types (SB and AB), followed by *Bacterioidetes*, *Chloroflexi* and *Firmicutes*. Within the phylum *Proteobacteria*, α- and β- *proteobacteria* were the most predominant classes. *Acidimicrobiia* and *Actinobacteria* were the predominant classes related to phylum *Actinobacteria*. As for

taxonomic composition of AOB community, all the TGGE-bands sequenced and belong to ammonium oxidizing β-*proteobacteria,* were related to genus *Nitrosomonas* (*N. oligotropha/ureae*, *N. Cryotolerans* and *N. europaea)*. The order *Nitrosomonadales* and the family *Nitrosomonadaceae* were detected by 454-pyrosequencing, but the members related to this family were unclassified at genus level. Multivariate analysis indicated that the structure and dynamic of total Bacteria and AOB communities in both SB and AB samples were mainly affected by changes in MLVSS, BTS, temperature, CFR and BOD_5.

The abundance of target genes of total Bacteria (16S rRNA), ammonia-oxidizing bacteria (16S rRNA and *amoA* of AOB), nitrite-oxidizing bacteria (16S rRNA of *Nitrospira*, NOB) and denitrifying bacteria (*nosZ*) was evaluated by quantitative real-time PCR (qPCR). The results obtained showed that the abundance and dynamics of total Bacteria, AOB, NOB and denitrifiers were fairly similar in both SB and AB fractions, suggesting that SB and AB could play equally important roles in the nitrification-denitrification process in the MBMBR. RDA analysis and Monte Carlo permutation test confirmed a significant effect of MLVSS, BTS, COD, temperature and CFR on the abundance values of total Bacteria, AOB, NOB and denitrifiers in both samples (SB and AB).

I. INTRODUCCIÓN GENERAL

"Yo soy yo y mi circunstancia, y si no la salvo a ella no me salvo yo"….Ortega y Gasset. Considerando el medio ambiente como un conjunto de circunstancias (físicas, sociales, económicas, etc.) inherentes a la vida del ser humano, podemos entender el origen de todo proceso y tratamiento que tenga como objetivo mejorar la calidad medioambiental.

1. Tecnologías de depuración biológica de las aguas residuales

1.1. Evolución de las tecnologías de depuración biológica

En el siglo XIX, la revolución industrial trajo consigo grandes problemas medioambientales, la migración rural hacia las grandes ciudades y la creciente industria, incrementaron la producción y acumulación de aguas residuales industriales y urbanas, las cuales eran vertidas directamente a los ríos. Estas aguas, vehículo de sustancias contaminantes y microorganismos patógenos causantes de diversas enfermedades, se convirtieron en uno de los principales riesgos para la sociedad y para los seres vivos que habitaban en estos ecosistemas.

Figura I.1. Caricatura de la travesía en barco por el río Támesis del científico Michael Faraday y su carta publicada en el diario *The Times* en 1855, describiendo el mal estado del mismo (Strange, 2010).

Como consecuencia de esta nueva circunstancia, nació la necesidad de buscar y desarrollar diferentes tecnologías para el tratamiento de las mismas. Si bien, en los orígenes de esta búsqueda, los procesos de depuración desarrollados (precipitación química, filtración e irrigación) no estaban directamente relacionados con la actividad biológica, es en 1865 cuando el Dr. Alexander Mueller (1) demostró que las aguas

residuales podían ser purificadas por organismos vivos (Alleman, 1983; Tiyasha *et al*., 2013).

A finales del siglo XIX, coincidiendo con la "Edad de Oro de la Microbiología" comienza a cobrar importancia el papel de los microorganismos en la depuración de las aguas residuales. Los primeros tratamientos biológicos se basaron en la formación y utilización de una biopelícula microbiana como filtro biológico. Es por ello que, a principios de la década de 1880, los sistemas basados en los procesos de biopelícula, tales como *filtros percoladores* (trickling filter), *filtros intermitentes* (intermittent filter) y *lechos de contacto* (contact bed), se convirtieron en la tecnología más empleada en los procesos de tratamiento biológico de aguas residuales (Alleman, 1983; Rodgers *and* Zhan, 2003; Rosso *et al*., 2011). Con posterioridad, en 1914, Edward Arden *and* William T. Lockett diseñaron el *sistema convencional de fangos activos* (CAS, Conventional Activated Sludge); estos autores observaron que cuando se almacenaba el agua residual bajo condiciones aeróbicas, transcurrido un tiempo se producía la sedimentación del fango, y la parte líquida libre de sólidos podía ser retirada para añadir de nuevo agua residual, aumentando, de este modo, la concentración de bacterias aeróbicas responsables de la depuración del agua residual. Es así como emerge un nuevo concepto en la depuración biológica, dado que, en este proceso aerobio, es la biomasa bacteriana suspendida la responsable del consumo y oxidación de la materia orgánica disuelta (Gernaey *et al.*, 2004).

(1) En 1878, Mueller obtuvo una patente para la realización de un proceso de desinfección, depuración y utilización de las aguas residuales por el cultivo científico de levaduras (Bruch, 1899). Por otro lado, en 1877 los científicos Schloesing y Muntz demostraron que la purificación de aguas residuales se debe a bacterias, ya que la nitrificación no era posible en suelos esterilizados por calor o tratados con cloroformo. *Información obtenida del libro "Investigations on the purification of Boston sewage, history of the sewage-disposal problem, de los C.E.A. Winslow and Earle B. Phelps, y editado en Washington (1906) por el Departamento Geológico del Ministerio del Interior de los Estados Unidos de América.*

Desde entonces y hasta nuestros días, el *sistema convencional de fangos activos* se ha convertido en uno de los tratamientos biológicos más empleados en la depuración de efluentes industriales y urbanos. Es de destacar que el empleo de las tecnologías basadas en los procesos de biopelícula fue limitado durante la primera mitad del siglo XX; no obstante es, a partir de la segunda mitad del siglo XX, cuando se comienzan a desarrollar nuevas configuraciones de los *Reactores de Biopelícula*, con el consiguiente resurgimiento de las tecnologías basadas en los procesos de biopelícula (Rodgers *and* Zhan, 2003; Gernaey *et al*., 2004; Rosso *et al*., 2011).

Durante la primera mitad del siglo XX, los ríos donde se vertían las aguas residuales tratadas, eran considerados parte del proceso de depuración; Como consecuencia de esta práctica, surge el concepto y la medida de la *Demanda Biológica de Oxígeno* (DBO_5), ya que 5 días era el tiempo estimado que tardaban los ríos en el Reino Unido en verter sus aguas al mar. A partir de la segunda mitad del siglo XX, y debido al continuo incremento de la población, los ríos ya no podían amortiguar las altas concentraciones de materia orgánica y nutrientes (N y P) de los efluentes tratados, advirtiéndose la necesidad de eliminar o reducir estos contaminantes en los efluentes ya que eran responsables de la eutrofización de los ríos. Esta problemática inspiró una línea de investigación en el campo de la Bacteriología en la década de los años 60, para al tratamiento de las aguas residuales; Se observó que la nitrificación dependía de la *tasa máxima de crecimiento específico* de los microorganismos autótrofos implicados en el proceso de nitrificación, siendo ésta, además, más lenta en comparación con la de los microorganismos heterótrofos; es por ello, que se ideó incrementar la edad del fango en las plantas de tratamiento con objeto de reducir la concentración de amonio en los efluentes. (Henze *et al*., 2008). Otro importante avance de esta década fue el desarrollo de los *sistemas de nitrificación-desnitrificación* de fangos activos mediante la

incorporación de zonas anóxicas en los biorreactores; esta práctica surgió tras conocer que los nitratos producidos en el proceso de nitrificación podían ser usados por algunas bacterias heterótrofas en condiciones anóxicas o anaeróbias. (Henze *et al.*, 2008).

No obstante, y a pesar de estos avances, la escasez de los recursos hídricos y la necesidad de preservar su calidad, contribuyeron al inicio de políticas orientadas al control de la contaminación de los mismos (Cumbre de Río de Janeiro de 1992) (2). Desde entonces y hasta nuestros días, la legislación medioambiental viene siendo cada vez más estricta en referencia a la concentración de contaminantes orgánicos y nutrientes (C, N y P) en los efluentes de las aguas residuales (Onnis-Hayden *et al.*, 2011; Pal *et al.*, 2012). Hoy en día, los tratamientos de aguas residuales tienen que abordar las exigencias de la legislación nacional e internacional con el objetivo de contribuir a una mejora de la calidad de vida y evitar la degradación del medio ambiente, reduciendo el consumo de recursos y el gasto energético. Para ello, durante las últimas décadas se han desarrollado y estudiado diversas tecnologías, enfocadas principalmente en la mejora de los tratamientos biológicos, los cuales representan una

(2) La Declaración de Río sobre el Medio Ambiente y el Desarrollo se adoptó en la Conferencia de Naciones Unidas sobre Medio Ambiente y Desarrollo, llevada a cabo en Río de Janeiro, en junio de 1992. Como un conjunto de principios (27 en total) sin fuerza jurídicamente vinculante, la Declaración busca reafirmar y desarrollar la Declaración de la Conferencia de las Naciones Unidas sobre el Medio Humano (Estocolmo, 1972). En el **Principio 7 se expone** que existen responsabilidades comunes pero diferenciadas, frente a la salud y la integridad de ecosistemas, y se fundamenta en las distintas contribuciones a la degradación ambiental global: *Los Estados deberán cooperar con espíritu de solidaridad mundial para conservar, proteger y restablecer la salud y la integridad del ecosistema de la Tierra. En vista de que han contribuido en distinta medida a la degradación del medio ambiente mundial, los Estados tienen responsabilidades comunes pero diferenciadas. Los países desarrollados reconocen la responsabilidad que les cabe en la búsqueda internacional del desarrollo sostenible, en vista de las presiones que sus sociedades ejercen en el medio ambiente mundial y de las tecnologías y los recursos financieros de que disponen.*

alternativa económica y respetuosa con el medioambiente (Kermani *et al*., 2008; Khan *et al*., 2011).

1.2. Biorreactores de membrana (MBRs, Membrane Biorreactors)

Entre las nuevas tecnologías, los biorreactores de membrana (*MBRs, Membrane Bioreactors*) combinan el tratamiento biológico de fango activo con la separación directa sólido-líquido a través de membranas de micro o ultrafiltración (Yang *et al*., 2009; Chang *et al*., 2011).

A pesar de que la tecnología MBR se presenta como un novedoso sistema de depuración de aguas residuales, la investigación e introducción de los mismos tuvo lugar a finales de la década de 1960, y aunque la idea parecía prometedora en sus inicios, la justificación del alto coste de las membranas, así como la pérdida rápida de su viabilidad, principalmente debida al ensuciamiento de las mismas, retrasó su aplicación a escala real.

Sin embargo, es en 1989 cuando se introduce un cambio en el diseño del proceso consistente en sumergir las membranas en el biorreactor aplicando, además, un sistema de aireación con objeto de disminuir el ensuciamiento de las mismas; Esta nueva idea consiguió reducir considerablemente el gasto energético, y los tratamientos convencionales comenzaron a ser desbancados por esta nueva y prometedora tecnología (Le-Clech *et al*., 2006; Leiknes *and* Odegaard, 2007).

No obstante, aunque los costes de instalación y operacionales de los MBRs exceden los costes de los sistemas convencionales, el incremento del valor y precio del

agua, junto con la necesidad de su reutilización, unido a la obligatoriedad de cumplir la nueva legislación internacional en cuanto a la calidad de los efluentes, hace que los costes tiendan a estar compensados (Radjenović *et al*., 2008).

Es por ello que, el uso de esta tecnología ha experimentado un crecimiento exponencial, de tal manera que actualmente está siendo ampliamente utilizada a escala real en el tratamiento de aguas residuales industriales y urbanas a nivel mundial (Kraemer *et al*., 2012; Malaeb *et al*., 2013).

Figura I.2. Evolución del mercado global de la tecnología MBR y su estimación para el año 2015 (BCCResearch, 2011).

Figura I.3. Nube de palabras que representa las principales "*Keywords*" utilizadas en los estudios realizados sobre la tecnología MBR (Judd, 2010).

En función de la configuración de las membranas, los sistemas MBRs se clasifican en dos tipos:

a) ***Biorreactores de membrana externa o de recirculación:*** En estos sistemas, la unidad de membrana está ubicada en un módulo externo al reactor biológico; el fango está sometido a un continuo flujo de recirculación mediante bombeo, localizándose entre el módulo de membranas y el biorreactor. El empleo de este tipo de sistemas a escala real es bastante escaso, debido al alto consumo energético de la bomba de recirculación (Radjenović *et al*., 2008; Le-Clech, 2010). En la **Figura I.4. (A)**, se muestra un esquema de este tipo de MBR.

b) ***Biorreactores de membrana integrada o sumergida:*** En estos sistemas, el módulo de membrana se encuentra inmerso en el reactor biológico. La filtración a través de las membranas se consigue bien presurizando el biorreactor, o bien creando una presión negativa en el lado permeado de la membrana (Rosenberger *et al*., 2002; Radjenović *et al*., 2008). **Figura I.4. (B).**

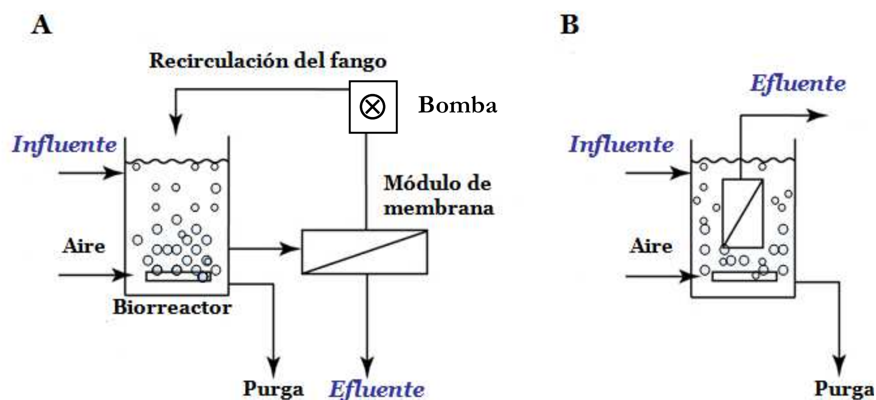


Figura I.4. Representación de las dos configuraciones de la tecnología MBR. **A)** Biorreactores de membrana externa o de recirculación**. B)** Biorreactores de membrana integrada o sumergida. Esquema adaptado de Judd, 2008.

Por otra parte, los MBRs pueden subclasificarse como aerobios (MBR) o anaerobios (AnMBR). Estos últimos, se definen como una combinación de un proceso biológico que tiene lugar en ausencia de oxígeno y la tecnología de membranas (Visvanathan *and* Abeynayaka, 2012). Los AnMBR fueron introducidos a principios de la década de 1980. Sin embargo, son pocas las investigaciones realizadas en estos sistemas, y principalmente enfocadas en el tratamiento de efluentes agrícolas, de la industria textil y papelera, así como de la industria alimentaria, cervecera, vinícola y de los efluentes de las destilerías (Liao *et al.,* 2006). Las principales ventajas de los AnMBR son los bajos requerimientos nutricionales, la baja producción de lodos, la reducción del gasto energético (ya que no es necesario el proceso de aireación), y todo ello unido a la posibilidad de producir una fuente de energía renovable, gracias al biogas generado (metano) (Liao *et al.,* 2006; Futselaar et al., 2013). No obstante, la baja tasa de crecimiento de los microorganismos metanogénicos y la complejidad microbiana en estos sistemas dificultan la explotación de los mismos, siendo la retención de biomasa el *tendón de Aquiles* de estos sistemas anaerobios (Visvanathan *and* Abeynayaka, 2012).

1.2.2 Ventajas de la tecnología MBR

Hemos de advertir que en los s*istemas convencionales de fangos activos*, la separación del agua residual tratada y el fango, tiene lugar mediante sedimentación en el proceso de clarificación (Judd, 2008); en consecuencia, la concentración de biomasa en el licor mezcla está limitada al volumen del clarificador, siendo la capacidad de sedimentación del fango una importante variable del proceso (Clara *et al*., 2005). Estos parámetros no tienen tanta relevancia en los *sistemas MBRs* dado que las membranas de filtración reemplazan el proceso de clarificación; comparando con los sistemas

convencionales de fangos activos, estas membranas confieren a los sistemas MBRs una serie de ventajas (Yang *et al*., 2009; Molina-Muñoz *et al*., 2010; Calderón *et al*., 2012a) de las cuales cabe destacar:

1. ***La posibilidad de trabajar con alta concentración de biomasa suspendida,*** lo cual conlleva otra serie de ventajas añadidas (Leiknes *and* Odegaar, 2007; Judd, 2008), tales como:

   - La Reducción del tamaño de las instalaciones.
   - El Permitir trabajar con mayores tiempos de retención del fango (SRT, *Sludge Retention Time*), lo cual favorece la disminución de la producción de fangos, así como una mayor mineralización de los mismos (Le-Clech, 2010). A su vez, un mayor SRT unido a la retención completa de la biomasa gracias al proceso de filtración favorece el desarrollo de microorganismos de crecimiento lento, tales como las bacterias nitrificantes, las cuales pueden ser eliminadas en un sistema CAS (Pauwels *et al*., 2006; Mannina *et al*., 2009; Radjenovic *et al*., 2009; Khan *et al*., 2011).

2. ***Control independiente del SRT y el tiempo de retención hidráulico (HRT, Hidraulic Retention Time).*** En los sistemas CAS, para que tenga lugar la sedimentación del fango, son necesarios HRTs prolongados, ya que los flóculos del licor mezcla deben alcanzar un tamaño suficientemente elevado (>50µm) que permita su sedimentación. Es de destacar que en un sistema MBR sólo se requiere un tamaño de flóculos superior al tamaño de poro de la membrana (Judd, 2008).

3. ***Alta calidad del efluente,*** en términos de eliminación de nutrientes, de capacidad de desinfección, de eliminación de microcontaminantes, metales pesados y disruptores hormonales (Pauwels *et al*., 2006; Le-Minh *et al*., 2010a; Le-Minh *et al*., 2010b; Khan *et al*., 2011; Cosenza *et al*., 2013).

La alta calidad del efluente, no sólo deriva de las ventajas anteriormente descritas, las cuales claramente contribuyen a una mayor biodegradación de los contaminantes orgánicos, sino también al reducido tamaño de poro de las membranas de filtración el cuál varía en función del tipo de membrana elegida para el proceso (Schäfer *et al*., 2000):

- *Membranas de Microfiltración*; con tamaños de poro ≥ 0,1 µm; estas membranas proporcionan una elevada eliminación de sólidos suspendidos, incluyendo un gran número de bacterias, así como la eliminación parcial de ciertos virus (3) y macromoléculas.

- *Membranas de Ultrafiltración*; con tamaños de poro comprendidos entre 0,1 µm y 0,01 µm, donde pueden ser retenidos la totalidad de virus y bacterias.

(3): El tamaño medio (expresado como diámetro x longitud) de los virus de las familias *Parvoviridae* y *Filoviridae* está comprendido entre 18-26 nm y 80-1000 nm respectivamente. Los virus que pueden ser retenidos por este tipo de membranas corresponden a los clasificados dentro de las siguientes familias: *Herpesviridae, Poxviridae, Coronaviridae, Retroviridae, Orthomyxoviridae, Bunyaviridae, Bornaviridae, Rhabdoviridae, Paramyxoviridae y Filoviridae.* Información obtenida del libro "Microbiología Médica" (Lange, 25ª edición).

4. Ausencia de problemas derivados de los procesos de bulking y formación de espumas del fango (Rosenberger *et al*., 2002; Leiknes *and* Odegaard, 2007). Como se ha comentado anteriormente, la capacidad de sedimentación del fango representa una de las principales limitaciones de los sistemas CAS. En este sentido, los procesos de bulking y la formación de espumas, constituyen dos de los principales fenómenos que reducen considerablemente la capacidad de decantación del fango biológico (Radjenović *et al*., 2008). Por el contrario, en los sistemas MBRs esta limitación se reduce gracias al empleo de la tecnología de membranas de filtración, ya que, debido al empleo de las mismas no es necesaria la decantación del fango.

1.2.3. Desventajas de la tecnología MBR

A pesar de las ventajas que ofrece la tecnología de membranas al sistema MBR, también existen una serie de desventajas. La colmatación de las membranas, más conocido como "*fouling de membrana*" continúa siendo hoy día la principal limitación del sistema, ya que -inevitablemente- la capacidad de filtración debido a este fenómeno disminuye con el tiempo; por tanto, el estudio de la mejora de la capacidad de filtración de las membranas sigue siendo el objetivo primordial de la última década (Le-Clech *et al*., 2006; Delrue *et al*., 2011).

El fenómeno de colmatación, es debido a la deposición de partículas orgánicas e inorgánicas, así como a la formación de una biopelícula en la superficie de la misma, y/o a la deposición de partículas coloidales en el interior de los poros de la membrana (Cosenza *et al*., 2013). Como consecuencia directa de estos depósitos de sedimentos sobre y en el interior de las membranas, se hace necesario acudir a las limpiezas químicas de las mismas así como a continuos flujos de retrolavado (limpiezas físicas),

mediante aireación a contracorriente; todo ello contribuye a un aumento del gasto energético, ocasionando altos costes operacionales, lo que hace pensar que esta tecnología no sea del todo eficiente (Drews *et al*., 2010; Cosenza *et al*., 2013).

Desde un punto de vista práctico, el *fouling de membrana*, puede ser reversible, irreversible o irrecuperable (**Figura I.5**). Se considera *fouling, reversible o temporal*, cuando puede ser eliminado de la membrana mediante limpieza física (retrolavado); *irreversible o permanente*, cuando puede ser eliminado mediante limpieza química; el fouling es *irrecuperable o absoluto* cuando no puede ser eliminado mediante ningún tipo de limpieza (Judd *et al*., 2008; Radjenovíc *et al*., 2008).

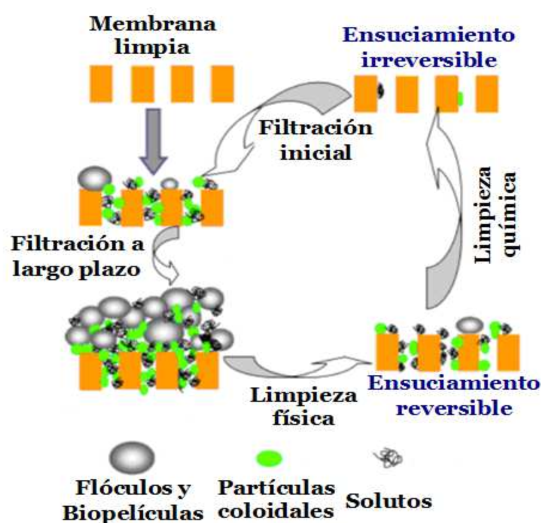


Figura I.5. Proceso de colmatación reversible e irreversible y limpieza de membranas (Meng *et al.,* 2009).

De acuerdo con Delrue *et al*. (2011), las causas de colmatación de membranas se clasifican en tres grupos:

1) Debido a las características de las membranas.

En este sentido, las propiedades del material de las membranas, tales como, hidrofobicidad y tamaño de poro, así como el tipo de configuración de las mismas, parecen tener una influencia directa en el proceso de colmatación (Drews *et al*., 2010; Delrue *et al*., 2011). El material del que están fabricadas las membranas puede ser orgánico o inorgánico. Los materiales orgánicos incluyen; polímeros hidrófilos (ej. poliamidas, poliacrilonitrilo, polisulfonas) o hidrófobos (ej. polietileno, poliolefina, fluoropolímeros) (4). Por otro lado, las membranas de carácter inorgánico, consisten en una capa semi-permeable hecha de material inorgánico, tales como, metales, óxidos metálicos, cerámica y vidrio poroso de zeolita (5) (Gao *et al*., 2009). Los polímeros orgánicos son los más utilizados, principalmente debido a su bajo coste y mayor facilidad de manejo (Gao *et al*., 2009).

(4): **Poliamidas**: polímeros de cadena larga que contienen unidades amida (N-H-C=O). Estos polímeros se obtienen por polimerización de un ácido con una amida. Los más utilizados son la poliamida 6 o nailon 6 (PA6) desarrollada por primera vez en 1952, y la poliamida 66 o nailon 66 (PA66). (http://www.ensinger.es). **Poliacrilonitrilo** (PAN) (-CH2-CH-CN)n: el químico francés Charles Maureu describió por primera vez el Acrilonitrilo en 1893; este polímero se utilizó en la manufactura del caucho sintético durante la Primera Guerra Mundial. Actualmente se utiliza para la fabricación de múltiples materiales: Fibras acrílicas (PAN 85%); Fibras modacrílicas; Fibras de grafito; Caucho nitrílico; y en la fabricación de copolímeros ABS y SAN (http://poliacrilonitrilo-pan.blogspot.com.es). **Polisulfonas**: Son los polímeros termoplásticos, rígidos, resistentes y transparentes. Estos polímeros son conocidos por su alta resistencia y estabilidad a altas temperaturas. Constituyen, con frecuencia, un sustituto superior de los policarbonatos. Mantienen sus propiedades en un rango de temperatura desde –100 °C hasta 150 °C. (http://www.entecpolymers.com/es). **Poliolefinas**: termoplásticos parcialmente cristalinos del grupo de los plásticos estándar. Dentro de este grupo se incluye el polietileno (PE) $(CH_2\text{-}CH_2)_n$ y el polipropileno (PP), juntos representan aproximadamente la mitad de todo el volumen de producción de los plásticos. (http://www.ensinger.es). **Fluoropolímeros**: Polímeros descrito por primera vez a finales de los años 40, y donde los átomos de hidrógeno han sido sustituidos por fluor totalmente (PFA/MFA y FEP) o parcialmente (PVDF,ETFE y el ECTFE). (http://www.plasticseurope.es).

En referencia a la configuración de las membranas, los cinco tipos de filtros más utilizados en la tecnología MBR son: 1) de fibra hueca (*Hollow-fiber*), 2) de placa lisa (*Flat plate*), 3) de forma espiral (*Spiral-wound*), 4) de tipo tubular y 5) de tipo bastidor (*Frame*) (Gao *et al*., 2009). Siendo los filtros de membrana de fibra hueca uno de las más ampliamente utilizados a escala real en los sistemas MBR (Gao *et al*., 2009). No obstante, en bibliografía existen discrepancias entre el comportamiento de los materiales hidrófobos e hidrófilos, así como en el diseño de filtros de fibra hueca y de placa lisa frente al fenómeno de colmatación de las membranas (Radjenovíc *et al*., 2008; Gao *et al*., 2009; Drews *et al*., 2010).

2) ***Debido a las condiciones operacionales del sistema***

Los parámetros operacionales descritos como influyentes en el *fouling de membrana*, incluyen; la concentración de sólidos suspendidos, el tiempo de retención de sólidos (SRT), el tiempo de retención hidráulico (HRT), el ratio Nutrientes / Densidad microbiana y la concentración de oxígeno disuelto (Gao *et al*., 2009; Delrue *et al*., 2011).

Es importante resaltar que, a diferencia de las características que presenta la membrana, estas condiciones operacionales no afectan de forma directa el rendimiento de filtración. Sin embargo, estas impactan de forma directa sobre las características del licor mezcla (Delrue *et al*., 2011).

(5): Zeolita: mineral microporoso miembro del grupo de los aluminosilicatos, que se usa comercialmente como absorbente. Aplicaciones: uso como pastillas antidiarréicas, alimento para animales de granjas, etc. El término "zeolita" fue acuñado en 1756 por el geólogo sueco *Axel Fredrik Cronstedt*, que observó como a medida que se calentaba una muestra de estilbita, se producía vapor; basándose en este fenómeno, lo denominó "zeolita", que se deriva de la unión de las palabras griegas "zeō" que significa "hervir", y "lithos", que significa "piedra"; o sea "piedra que hierve". (http://www.ecured.cu)

3) ***Debido a las características del licor mezcla***

La viscosidad, la cantidad de bacterias filamentosas y las sustancias poliméricas extracelulares (*EPSs, Extracellular Polimeric Sustances*) representan las principales características del fango que pueden repercutir en el proceso de ensuciamiento (Zhang *and* Cao, 2010; Meng *et al*., 2006; Cosenza *et al*., 2013). Como consecuencia directa de la ventaja de poder trabajar con mayores concentraciones de sólidos en los MBRs, la viscosidad del fango parece incrementar exponencialmente (Radjenovíc *et al*., 2008). Este aumento de la viscosidad limita la transferencia de oxígeno y sustratos, afectando al proceso de degradación aerobia de materia orgánica y nutrientes, así como al proceso de colmatación de las membranas (Judd, 2008; Delrue *et al*., 2011).

Por lo tanto, para conseguir un eficiente proceso de depuración y aumentar la vida útil de las membranas, se requiere una mayor tasa de aireación en estos sistemas, lo cual repercute de forma directa sobre los costes de operación. En referencia a las bacterias filamentosas, cabe destacar que algunos autores (Li *et al*., 2008 y Wang *et al*., 2010) no encontraron una relación directa entre estas bacterias y el *fouling de membrana*. Sin embargo, sí se pudo demostrar una correlación directa entre el contenido de bacterias filamentosas y la producción de EPSs (Wang et al., 2010) siendo además responsables del proceso de colmatación de las membranas. Actualmente, los EPSs son considerados como los principales causantes del *fouling de membrana* (Drews *et al*., 2006; Wang *et al*., 2009; Wang *et al*., 2013).

1.3. Biorreactores de Lecho Móvil (MBBRs, Moving Bed Biofilm Reactors)

A finales de la década de los años 80 y principios de los 90, la idea de mejorar las características de los fangos activos y de los procesos de biopelícula desemboca en

el desarrollo de una nueva tecnología denominada *biorreactores de lecho móvil* (MBBRs, Moving Bed Biofilm Reactors) (Ødegaard, 2006). Estos nuevos sistemas están emergiendo como una eficaz alternativa en las últimas décadas, debido a su eficiencia en la eliminación de carbono orgánico, amonio, nitratos y nitritos (Ødegaard, 2006; Di Trapani *et al*., 2010).

Esta tecnología se basa en la adición de soportes al sistema de fangos activos capaces de moverse libremente por todo el tanque del biorreactor gracias a la aplicación de una corriente de aire (MBBRs aerobios) o mediante agitación mecánica (MBBRs anóxicos) (**Figura I.6**). Sobre estos soportes se produce la colonización gradual de la biomasa, es decir, los procesos de crecimiento adherido y suspendido que tienen lugar conjuntamente en el reactor biológico (Wang *et al*., 2005; Chu *and* Wang, 2011; Martín-Pascual *et al*., 2011; Pal *et al*., 2012).

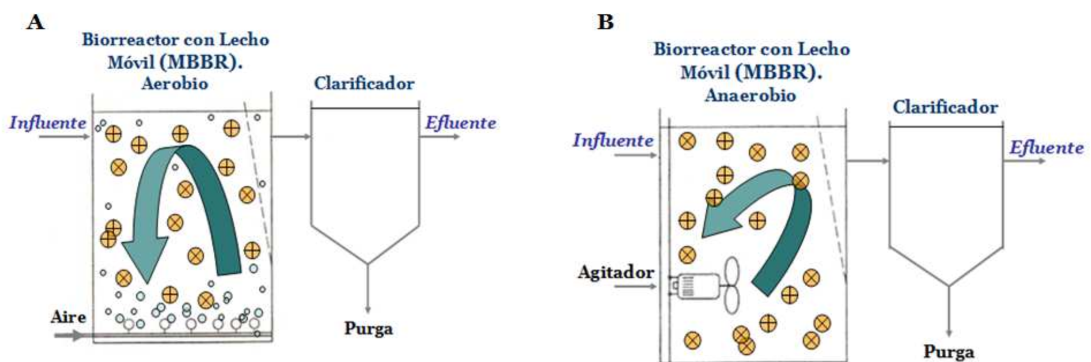


Figura I.6. Representación de la configuración aerobia (A) y anaerobia (B) de un sistema MBBR. Esquema adaptado de Ødegaard, 1999.

Estos sistemas operan de forma similar a los sistemas CAS, no obstante, los procesos de formación de biopelícula les confieren a los MBBRs una serie de ventajas (Odegaard *et al*., 1994; Andreottola *et al.*, 2000; Wang *et al*., 2005; Ødegaard, 2006; Plattes *et al*., 2006; Kermani *et al*., 2008; Chan *et al*., 2009; Martín-Pascual *et al*., 2011; Qiqi *et al.*, 2012), respecto a los CAS, tales como:

* Mayor simpleza de operación.
* Mejor transferencia del oxígeno.
* Mayor estabilidad del proceso biológico.
* Ausencia de problemas de bulking.
* Mayor acumulación y actividad de la biomasa, favoreciendo el desarrollo de microorganismos de crecimiento lento.

A diferencia de la mayoría de reactores de biopelícula estos sistemas utilizan todo el volumen del tanque para el crecimiento de la biomasa, ya que el crecimiento adherido tiene lugar en soportes que se mueven libremente en el volumen de agua del biorreactor (Ødegaard, 2006). Además, esta tecnología carece de los principales inconvenientes presentes en los sistemas de biopelícula fija, como son el riesgo de atascamiento de los filtros, así como la necesidad de un contralavado para la limpieza de los mismos (Rodgers *and* Zhan, 2003; Rodges *et al*., 2006, Ivanovic *and* Leiknes, 2012).

Sin embargo, los MBBRs al igual que los CAS, dependen del proceso de sedimentación para separar el efluente tratado de la biomasa. En comparación con los CAS, en los MBBRs se produce una menor capacidad de sedimentación de la biomasa, por lo que se hace necesaria una coagulación o filtración directa para lograr una calidad aceptable del efluente (Ødegaard, 1999; Leiknes *and* Ødegaard, 2001). Esto representa

la principal limitación de los sistemas MBBRs, dado que de este proceso depende en gran medida la eficiencia del tratamiento biológico (Lee *et al*., 2006).

1.4. Biorreactores de Membrana con Lecho Móvil (MBMBRs, Moving Bed Membrane Biorreactors)

Es a principios del siglo XXI cuando se publican los primeros trabajos donde se describe el uso de la tecnología de membranas de filtración para el tratamiento de los efluentes generados en los sistemas MBBRs. Estos primeros estudios surgen con el objetivo de sustituir el proceso de sedimentación y mejorar la calidad de los efluentes de los sistemas MBBRs (Leiknes *and* Knudsen, 2000; Leiknes *and* Ødegaard, 2001). Los resultados obtenidos no sólo demostraron una alta calidad del efluente sino que también pusieron de manifiesto importantes ventajas del sistema frente a los MBRs (Leiknes *and* Ødegaard, 2001).

Desde entonces y con objeto de superar algunas de las limitaciones de los sistemas MBRs, en los últimos años está emergiendo una nueva tecnología capaz de combinar los procesos de biopelícula y la tecnología de membranas; estos nuevos sistemas se denominan *Biorreactores de Membrana con Lecho Móvil* (MBMBRs, Moving Bed Membrane Bioreactors), y constituyen una alternativa altamente viable y eficaz en el tratamiento de aguas residuales industriales y urbanas (Leiknes *and* Ødegaard, 2007; Ivanovik *and* Leiknes, 2008; Ivanovik *and* Leiknes, 2012).

Este sistema híbrido, resultado de la adición de soportes al proceso convencional de los MBRs, consta de dos reactores, un MBBR donde tiene lugar el proceso biológico, seguido por un reactor de membranas (Leiknes *and* Ødegaard, 2007;

Ivanovic *and* Leiknes, 2012). Como consecuencia de la combinación de estas dos tecnologías, el sistema MBMBR presenta las ventajas de ambos procesos y consigue amortiguar la principal desventaja de los MBBRs y algunas de las desventajas de los MBRs. En la **Figura I.7,** se muestra el esquema de configuración de el sistema híbrido MBMBR, propuesto por Leiknes *and* Ødegaard (2007).

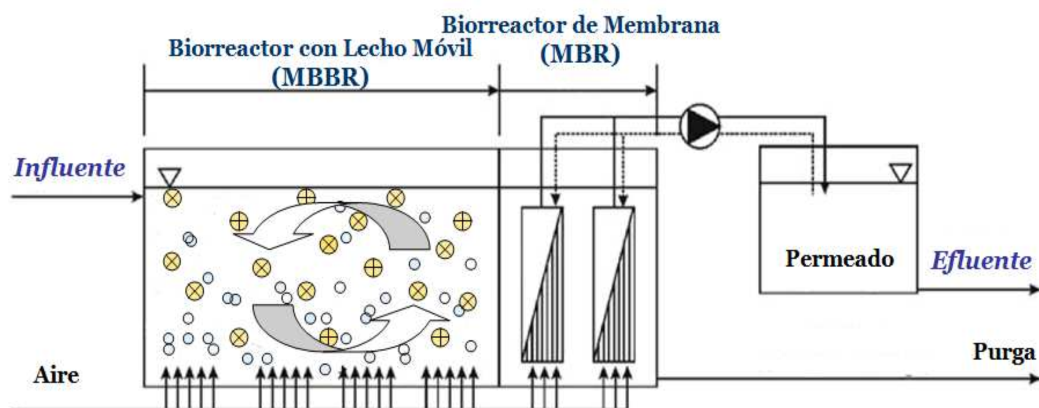


Figura I.7. Representación de la configuración de un sistema MBMBR. Esquema adaptado de Leiknes and Ødegaard, 2007.

Una de las ventajas de los MBMBRs es la formación de la biopelícula sobre los soportes libres, consiguiendo de este modo reducir la concentración de sólidos suspendidos en el bioreactor (Phattaranawik *and* Leiknes, 2009; Ivanovic *and* Leiknes, 2011; Rahimi *et al*., 2011). Las ventajas adicionales que se consiguen gracias a esta combinación son:

1. Menor viscosidad del licor mezcla, permitiendo reducir el gasto energético de aireación en el sistema.

2. Menor formación de biopelícula en las membranas, es decir reducción del "*fouling" de membrana*.

3. Mejora de los procesos de nitrificación-desnitrificación, y eliminación de fósforo debido al gradiente de oxígeno generado en la biopelícula.

Soportes y Porcentaje de relleno (CFR, Carrier Filling Ratio)

El tipo de soporte y el porcentaje de los mismos (CFR, Carrier Filling Ratio, v/v) constituyen dos de las principales variables operacionales en los sistemas MBBRs y MBMBRs (Rodgers *and* Zhan, 2003). En los últimos años, algunos autores han puesto de manifiesto la influencia de estas variables en la eliminación de la materia orgánica, en la eficiencia del proceso de nitrificación, en la morfología y composición de la biopelícula, así como en la estructura de la comunidad bacteriana de la biopelícula (Wang *et al*., 2005; Levstek *and* Plazl, 2009; Martín-Pascual *et al*., 2011; Calderón *et al.,* 2012b). Existe una gran variedad de soportes que se diferencian en función del tamaño, diseño, tipo de material, área superficial específica y capacidad de tratamiento; uno de los más utilizados en la tecnología MBBR a escala real es el soporte de tipo Kaldnes K1, desarrollado por AnoxKaldnes (Noruega) (6) (**Figura I.8**). (Levstek *and* Plazl, 2009; Ivanovic *and* Leiknes, 2012).

(6): AnoxKaldnes ha desarrollado diversos tipos de material de soporte con diferente forma, tamaño y superficie en función de las características de las aguas residuales, el tratamiento previo, las normas de descarga y los volúmenes disponibles. Actualmente existen cinco tipos diferentes de soportes: K1, K3, K5, BiofilmChip ™ M, BiofilmChip ™ P y F3. (http://www.veoliawaterst.com/mbbr/en/carriers.htm)

En este tipo de soporte la biomasa se desarrolla principalmente en las capas internas (Ødegaard, 2006).

Figura I.8. Soporte Kaldnes

En referencia al porcentaje de relleno, éste puede estar sujeto a preferencias, sin embargo, para permitir que los soportes se muevan libremente en el biorreactor es recomendable que este no supere el 70% (v/v), siendo un 67% (v/v) el porcentaje de relleno estándar (Ødegaard, 2006).

2. Comunidades microbianas en las aguas residuales

La idea de que los microorganismos viven en comunidades o en un ensamblaje formado por más de una especie ha estado presente desde los inicios de la Microbiología (Handelsman, 2007).

La primera evidencia documentada de la existencia de una comunidad de microorganismos, se remonta al siglo XVII, cuando Antonie van Leuwenhoek (7) observó a través de su microscopio monocular lo que describió como agregados "*animalcules*" aislados de la superficie interdental (Costerton, 1999; Cheng *et al.*,

2010). Con posterioridad, la publicación del libro "*The Structure and Function of Bacteria*" en 1900, pone de manifiesto la elevada frecuencia con la que las células bacterianas se encuentran reunidas en masas, unidas por una sustancia de aspecto gelatinosa. Estas estructuras eran consideradas como "agregados sociales de individuos", y podían encontrarse suspendidas en líquidos o bien adheridas a superficies sólidas (Fischer, 1990).

Aunque a finales del siglo XIX, era ampliamente conocida la existencia de este tipo de comunidades microbianas, su estudio quedó reducido a unos pocos investigadores, dado que la tendencia dominante en Microbiología hasta las últimas décadas del siglo XX, consistió en el estudio de los microorganismos como cultivos puros aislados en laboratorio. En consecuencia, los microorganismos eran estudiados principalmente como células individuales y descritos en los medios de cultivo en base de sus propiedades morfológicas y fisiológicas. En este sentido, cabe resaltar el papel de Winogradsky (8), quien consideró que las condiciones de cultivo en laboratorio distorsionaban las propiedades microbianas en comparación con los hábitats naturales.

(7): La ciencia de la Microbiología nació con su carta a la *Philosophical Transactions of the Royal Society of London*, en 1677. Mercader de paños en Delf (Holanda), la mayor parte de su tiempo libre lo dedicaba a pulir pequeñas lentes de gran aumento, con las cuales pudo construir microscopios simples monoculares. Con estos microscopios descubrió estructuras microscópicas no descritas hasta ese momento. Libro: "Tratado de Microbiología", 2ª ed. Salvat. 1983.

(8): *Winogradsky* en Rusia y *Beijerinck* en Holanda, hacia las postrimerías de la era pasteuriana, iniciaron la exploración de la microbiología del suelo y descubrieron una sorprendente variedad de vías metabólicas, mediante las cuales los distintos tipos de bacterias adaptados a diferentes nichos ecológicos mantenían su propia vida. "Tratado de Microbiología", 2ª ed. Salvat. 1983.

De acuerdo con esta idea, orientó sus investigaciones hacia nuevas estrategias de investigación microbiológica en suelos, contribuyendo de este modo al estudio de los microorganismos adheridos a superficies (Panikov, 1995; Lappin-Scott, 1999).

Otro autor destacable en el estudio microbiano de las biopelículas, fue el microbiólogo marino Claude Zobell (9), considerado como uno de los pioneros; Este autor propuso el concepto de biopelícula en 1936, definiéndola como una fina capa de células microbianas adheridas a las paredes de una botella donde se observaba una mayor actividad biológica en comparación con las células en suspensión. En 1943, Zobell aportó nuevos conocimientos sobre las biopelículas, tras observar que el número de bacterias adheridas a una superficie era extremadamente mayor a la cantidad bacterias suspendidas en un volumen de agua (Costerton *and* Lewandowski, 1995; Cheng *et al*., 2010; Percival *et al*., 2011).

Por otra lado, en la década de 1930, se comienza a estudiar la formación de flóculos microbianos en el fango activo. Cabe resaltar que inicialmente se pensaba que la formación de tales agregados en suspensión era debida únicamente a una sola especie bacteriana "*Zoogloea ramigera*"; Posteriormente, en la década de 1950, se pudo demostrar que todas las especies bacterianas tenían la habilidad de formar flóculos bajo determinadas condiciones ambientales. Por otro lado, también en esta década se pudo establecer la relación existente entre la formación de flóculos y la acumulación de biopolímeros extracelulares (Salehizadeh *and* Shojaosadati, 2001).

(9): Originario de Dinamarca, (1904-1989), estudió en la Universidad Estatal de Utah y en California, donde trabajó a tiempo parcial en el departamento de bacteriología para poder financiar sus estudios. Se licenció en Bacteriología en 1927 y recibió su Maestría en 1929. Tras trabajar durante algunos años en Microbiología Médica (*Brucella*), trabajó en Microbiología Marina en el *Instituto Scripps de Oceanografía* desde 1932.

En 1964, Atkinson introduce el término de película biológica o microbiana para explicar el manto de células adheridas a las paredes de un biorreactor (Cheng *et al*., 2010). Pocos años después, a finales de la década de 1960, los avances en microscopía, concretamente la incorporación del microscopio electrónico de barrido (SEM), marcaron el inicio de los estudios físicos y químicos de los agregados microbianos. Cabe citar el trabajo realizado en 1969 por Jones y colaboradores, quienes examinaron la biopelícula formada en un filtro percolador empleado en el tratamiento de aguas residuales, observando que ésta estaba compuesta por una gran variedad de microorganismos y que el componente principal de la matriz eran polisacáridos (Percival *et al.*, 2011). Otro avance para la comprensión de la importancia de estas comunidades, fue la incorporación del concepto de "ubiquidad" de la biopelícula, descrito por primera vez en 1978 (Costerton *et al.*, 1995).

A finales del siglo XX y principios del XXI, los continuos avances en Microscopía (microscopio de fluorescencia, microscopio de barrido con láser confocal) y en técnicas moleculares han permitido una amplia comprensión de los procesos biológicos, estructura y función de los mismos. Este conocimiento, ha contribuido a un mejor desarrollo de la biotecnología y concretamente de los tratamientos de las aguas residuales, lo que ha supuesto el uso de estos agregados en los procesos biológicos de depuración desde hace más de un siglo (Davey *and* O´Toole, 2000; Singh *et al.*, 2006; Percival *et al.*, 2011).

2.1. Agregados Microbianos: *Biopelículas y Flóculos*

Hoy día, gracias a los avances en Microbiología comentados anteriormente, podemos afirmar que, al igual que sucede en la Naturaleza, en las plantas de tratamiento

de aguas residuales, los microorganismos no suelen vivir como cultivos puros de células individuales dispersas, sino que tienden a agruparse formando agregados

polimicrobianos y generando comunidades heterogéneas donde los microorganismos mantienen una relación de interdependencia entre ellos y llevan a cabo, además, sus propias funciones metabólicas (Davey *and* O´Toole, 2000; Handelsman, 2007; Sheng *et al.*, 2010). Estos agregados pueden desarrollarse sobre una gran variedad de superficies bióticas o abióticas, originando un manto o película microbiana denominada "biopelícula". Además, los microorganismos también forman ensamblajes en las interfases aire-agua y en suspensión, dando lugar a la formación de unas estructuras ligeramente esféricas denominadas *flóculos* o *gránulos* que, en ocasiones, son considerados como una biopelícula en suspensión (Davey *and* O´toole, 2000; McSwain *et al.*, 2005; Flemming *and* Wingender, 2010).

Existen controversias en referencia a la consideración de los diferentes agregados bajo el término de biopelícula, debido principalmente a la creencia de que es necesaria una superficie o interfase para el desarrollo de la misma (concepto inherente en la definición de biopelícula), ya que el primer paso para la formación de la biopelícula consiste en la adhesión de un pequeño número de células bacterianas a una superficie (Costerton, 1999). Sin embargo, también ha sido aceptado que cualquier tipo de ensamblaje microbiano (fijado a una superficie o en suspensión) forma una biopelícula, dado que las diferencias entre los agregados (principalmente en estructura y composición) son menores en comparación con las características comunes que presentan. No obstante, es importante resaltar que el término "*agregados*" ha sido elegido y ampliamente utilizado para expresar estas asociaciones de microorganismos, que consisten principalmente en una biomasa microbiana y material polimérico

extracelular producido por los microorganismos que lo componen (Lewandowski, 1995; Costerton *and* Allison *et al.*, 2000; Davey *and* O´toole, 2000; Lui *and* Tay, 2002; McSwain *et al.*, 2005; Weber *et al.*, 2007; Flemming, 2009).

El término "*biopelícula*" se define como una comunidad compleja, espacial y temporalmente heterogénea, en la cual los microorganismos se encuentran integrados en una matriz de sustancias poliméricas extracelulares (*EPSs, extracellular polymeric substances*), y cuyo desarrollo tiene lugar de forma adherida sobre una superficie o soporte de carácter orgánico o inorgánico (Allison *et al.*, 2000; Nicolella *et al.*, 2000; Cheng *and* Demirci, 2010).

El proceso de formación de la biopelícula (**Figura I.9**), se divide en cuatro etapas (Stoodley *et al.*, 2002; Cheng *et al.*, 2010):

1. Etapa inicial de ***adherencia***, en la cual los microorganismos suspendidos en el líquido son transportados a la superficie por difusión, convección o motilidad, creando una débil y reversible adherencia con la superficie sólida.

2. Etapa de ***colonización***, en la cual se produce la adhesión irreversible, debido a la producción de EPSs por los microorganismos.

3. Etapa de ***crecimiento y maduración***, las cuales dependen principalmente de la disponibilidad de nutrientes, por lo que la concentración y la difusión de los mismos juega un papel fundamental en la formación de la biopelícula.

4. Etapa de ***dispersión***, en la cual parte de las células que conforman la biopelícula son desprendidas al medio circundante. Este desprendimiento de células de la biopelícula puede estar causado por depredación o por diferentes factores ambientales, siendo las fuerzas hidrodinámicas una de las principales causas. Sin embargo, la dispersión también puede estar provocada por determinadas bacterias en respuesta a la limitación de sustratos en la biopelícula (por ejemplo la limitación de oxígeno). No obstante, la formación de la biopelícula está considerada como un proceso activo, donde tiene lugar un balance entre los procesos de adhesión y dispersión (Nadell *et al.*, 2009).

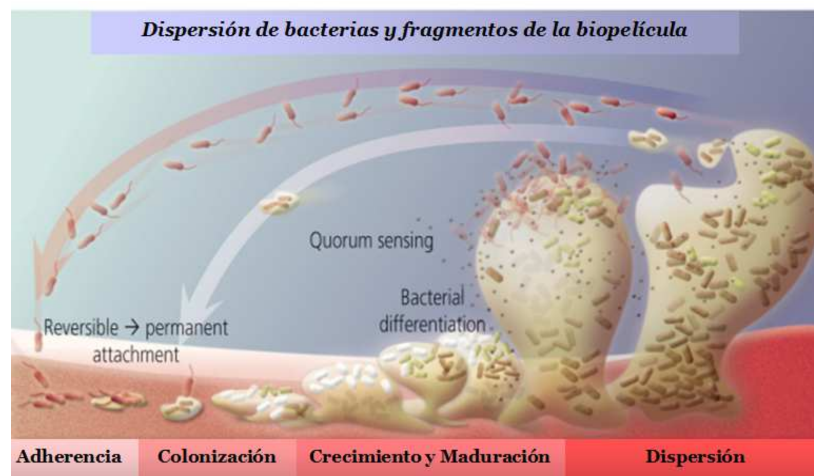


Figura I.9. Etapas de desarrollo de la biopelícula (Phillips *et al.*, 2011).

La estructura de la biopelícula es heterogénea, desigual en el espacio y cambiante en el tiempo, y puede verse afectada por diferentes factores, tales como, la disponibilidad y difusión de nutrientes, las fuerzas de atracción entre los microorganismos y la superficie, la producción de EPSs, la adhesión intercelular, el crecimiento de los microorganismos, la temperatura y el pH (Sheng *et al.*, 2010).

El término "*flóculo*", está asociado principalmente al tratamiento de efluentes de aguas residuales, y se define como una comunidad mixta de células individuales, sólidos suspendidos y EPSs ligeramente ensamblados, capaz de desarrollarse de forma suspendida, con una simetría radial irregular altamente porosa, y cuya estructura es temporal y espacialmente heterogénea. Su formación tiene lugar bajo determinadas condiciones del reactor; puede originarse de forma natural mediante el proceso denominado *Biofloculación*, o generarse tras la adición de diversos agentes al medio que favorezcan la coagulación de la materia orgánica para su posterior floculación (Allison *et al.*, 2000; Nicolella *et al.*, 2000; Jarvis *et al.*, 2005; Han *et al.*, 2012). La formación de los flóculos, al igual que la biopelícula, surge como consecuencia del balance existente entre el proceso de agregación y el de dispersión, aceptándose que el crecimiento del flóculo está controlado por la rotura del mismo (Jarvis *et al.*, 2005). Los flóculos, presentan una estructura débil, compuesta por una doble capa de EPSs; una fina capa ligeramente ensamblada, denominada LB-EPS (*loosely bound EPS*), y una capa fuertemente ensamblada que rodea y mantiene unidas las células, denominada TB-EPS (*tightly bound EPS*) (Yu *et al.*, 2008; Wang *et al.*, 2013).

2.1.1 La matriz de sustancias poliméricas extracelulares (EPS)

La característica principal que comparten ambos tipos de agregados (flóculos y biopelículas), es que los microorganismos que los conforman viven en una matriz altamente hidratada de sustancias poliméricas extracelulares (EPSs), las cuales representan un papel esencial en la formación y estructura de los agregados, y concretamente en el caso de la biopelícula también en el proceso de adhesión a superficies (McSwain *et al.*, 2005; Flemming, 2008). Aunque determinadas estructuras bacterianas, tales como, flagelos, pilis y fimbrias, así como determinados

microorganismos (bacterias filamentosas) contribuyen a la consolidación de los agregados, los EPSs constituyen la principal fuerza de cohesión entre los microorganismos, permitiendo el desarrollo de estas comunidades microbianas (Flemming *and* Wingender, 2010).

Estas sustancias (EPSs), conforman una matriz de biopolímeros producidos por los propios microorganismos, cuya composición incluye, polisacáridos, proteínas, glicoproteínas, ácidos nucleicos, fosfolípidos y ácidos húmicos. En las plantas de tratamiento de aguas residuales, los polisacáridos y las proteínas suelen ser los principales constituyentes (Bin *et al.*, 2008; Ying *et al.*, 2010). Los EPSs representan el principal componente de los agregados, siendo la composición y concentración de los mismos los parámetros que determinan las características de porosidad, densidad, transporte de sustratos, contenido de agua, cargas iónicas, propiedades de sorción, hidrofobicidad y estabilidad mecánica de la matriz (Flemming, 2008; Percival *et al.*, 2011). Sin embargo, la composición y concentración de EPSs a su vez, depende en gran medida de las condiciones ambientales u operacionales y el tipo de microorganismos presentes. En este sentido, el tipo de microorganismos, el estado fisiológico de los mismos, la temperatura, las condiciones aeróbicas o anaeróbicas, el tipo de substrato, la disponibilidad de nutrientes, el pH, el SRT, la presencia de sustancias tóxicas y las condiciones hidrodinámicas han sido descritas como factores que condicionan la estructura de las comunidades microbianas formadas tanto en flóculos como en las biopelículas. (Lazarova *and* Manem., 1995; Sutherlann, 2001; Sponza, 2002; Raszka *et al.*, 2006; Flemming *and* Wingender, 2010; Sheng *et al.*, 2010; Percival *et al.*, 2011;).

La matriz de EPSs, no sólo favorece la cohesión entre células, permitiendo así un modo vida en comunidad, sino que además proporciona copiosos beneficios a los

microorganismos que la integran. En este sentido, los EPSs confieren una gran protección frente a agentes externos y cambios ambientales u operacionales, proporcionando un ambiente idóneo para establecer un gradiente de nutrientes, para la adsorción de metales pesados y materia orgánica, para la retención de enzimas extracelulares, para el intercambio genético y para la comunicación célula-célula denominada *Quorum sensing* (Dolan, 2000; Johnson, 2008).

El estudio de los EPSs en los sistemas de tratamiento de aguas residuales, ha suscitado un gran interés, debido al importante papel que juegan en la eficiencia del proceso. En los tratamientos convencionales de fangos activos, los EPSs representan uno de los principales constituyentes del fango, los cuales determinan las propiedades físico-químicas y biológicas del mismo, jugando un papel fundamental en los procesos de floculación y sedimentación del fango, así como en la habilidad de los microorganismos para eliminar contaminantes (Yu *et al.*, 2006; Liu *and* Fang, 2010). Por otro lado, en los sistemas MBR, parece existir una correlación directa entre la concentración de EPSs y la colmatación de las membranas, siendo los polisacáridos el componente clave en el proceso de acumulación de sedimentos (Jang *et al.*, 2007; Bin *et al.*, 2008; Wang *et al.*, 2009). Una mayor concentración de EPSs ha sido descrita en MBMBRs en comparación con los MBRs (Yang *et al.*, 2009).

2.1.2. Transporte de sustratos en los agregados

En el interior de los agregados, los sustratos pueden ser transportados mediante un flujo de convección o difusión a través de los poros y canales, o mediante difusión a través de la matriz de EPSs, existiendo por tanto una correlación directa entre las características de la matriz y el transporte de sustratos (Schramm *et al.*, 1999;

Flemming, 2008). Es por ello que, la penetración de sustratos depende principalmente de la densidad y porosidad del agregado, así como de la concentración de sustrato en el medio circundante (Nicolella *et al*., 2000). Estos tipos de transporte originan un gradiente de concentración de sustratos (principalmente de nutrientes y oxígeno) a lo largo de los agregados, dando lugar a diferentes microambientes que coexisten en estas comunidades, y favoreciendo la estratificación de los diferentes grupos microbianos a lo largo de los mismos, en función de sus actividades metabólicas. Esto sugiere que en estos consorcios, los microorganismos se organizan de tal forma que permite maximizar la productividad de cada uno, siendo el comensalismo un fenómeno generalizado en este tipo de comunidades (Jefferson, 2004; Singh *et al.*, 2006; Nadell *et al.*, 2009).

En este sentido, los microorganismos de crecimiento rápido se desarrollan en las capas más superficiales de los agregados, mientras que, los microorganismos de crecimiento lento se localizan en las capas más internas (Nicolella *et al.*, 2000). Otra consecuencia derivada de la difusión del sustrato, es el desarrollo de zonas anóxicas o anaerobias en el interior de los agregados, permitiendo de este modo el desarrollo de microorganismos anaerobios capaces de llevar a cabo importantes procesos de degradación de nutrientes, tales como, reducción de sulfatos, desnitrificación, eliminación de fósforo y metanogénesis (Biswas *et al.*, 2013).

En este contexto, cabe resaltar, que el tamaño de poro y la densidad de los agregados, representan algunas de las principales diferencias entre flóculos y biopelículas. Generalmente, los flóculos presentan un mayor tamaño de poro y una menor densidad en comparación con la biopelícula. Los flóculos presentan un grosor comprendido entre 10 y 500µm, en comparación con la biopelícula, cuyo grosor oscila entre micrómetros y centímetros (Nicolella *et al*., 2000; Cheng *and* Demirci, 2010;

Makowska *et al*., 2013). Estas diferencias, generalmente contribuyen a una rápida y mayor difusión del sustrato dentro de los flóculos en comparación con la biopelícula, y por tanto una mayor estratificación de microorganismos en la biopelícula en comparación con los flóculos (Morgenroth *et al.*, 2002). Sin embargo, en relación a la accesibilidad de la biomasa al sustrato en la biopelícula, también se debe tener en cuenta el área de superficie efectiva (Makowska *et al*., 2013).

En función de la tecnología empleada en la depuración de efluentes, principalmente debido a diferencias operacionales, el tamaño de los flóculos puede variar considerablemente. En este sentido, se ha descrito un menor tamaño de flóculos en los sistemas MBR en comparación con los CAS; en estos últimos, como consecuencia directa del tamaño y estructura de los mismos, el transporte de sustratos parece jugar un papel importante (Manser *et al.*, 2005). En los sistemas híbridos de depuración que combinan ambos tipos de agregados, el tamaño de los flóculos parece asemejarse al de los agregados formados en los sistemas CAS (diámetro comprendido entre 150-500 µm) (Makowska *et al*., 2013).

Por otro lado, en referencia a las biopelículas desarrolladas sobre soportes en los sistemas MBBRs y MBMBRs, el grosor y la porosidad de estos agregados, y por tanto la difusión de sustratos a través de los mismos, parece estar bastante condicionada por el tipo de relleno, así como por el porcentaje de relleno utilizado (Wang *et al.*, 2005).

Además, en ambos sistemas (MBBRs y MBMBRs) la convivencia de los microorganismos presentes en ambos tipos de agregados, parece favorecer múltiples interacciones entre ellos, así como la migración de microorganismos entre ambos tipos de agregados; el continuo movimiento de los soportes, permite el desprendimiento de

biomasa de la biopelícula al medio líquido, favoreciendo la formación de flóculos y viceversa. Sin embargo, la información acerca de este tipo de migraciones, actualmente, es escasa (Makowska *et al*., 2013).

2.2. Implicaciones de los agregados microbianos en la depuración biológica

Las habilidades que presentan los microorganismos para degradar diversas sustancias orgánicas y el papel que ejercen en los ciclos biogeoquímicos (nitrógeno, carbono y fósforo), los convierte en los protagonistas de un sin fin de procesos biotecnológicos (Daims *et al.*, 2006). En este sentido, el tratamiento biológico de las aguas residuales se fundamenta en la eliminación de los contaminantes biodegradables llevada a cabo por una biocenosis o comunidad de organismos vivos, principalmente microorganismos tales como, bacterias, arqueas, hongos, microalgas y protozoos. Mediante los procesos metabólicos de respiración o fermentación, estos microorganismos son capaces de transformar sustancias complejas en compuestos simples, consiguiendo la total o parcial eliminación de contaminantes orgánicos e inorgánicos, o la transformación de los mismos en compuestos no tóxicos (Liu *and* Tay, 2002).

Los microorganismos procariotas (especialmente las bacterias) han sido descritos como dominantes y principales responsables de la eliminación de contaminantes orgánicos y nutrientes en las plantas de tratamiento de aguas residuales (Valentín-Vargas *et al.*, 2012). Asimismo, una elevada proliferación de determinados microorganismos (por ejemplo de bacterias filamentosas) puede contribuir a los procesos de *bulking* y espumas, los cuales representan dos de los principales problemas de las plantas de tratamiento (Wagner *et al.*, 2002; Gilbride *et al.*, 2006). Por tanto, la

eficiencia y solidez del proceso de depuración biológica de las aguas residuales depende principalmente de la composición y actividad metabólica de las comunidades microbianas presentes, por lo que el estudio de las mismas se ha convertido en objetivo principal para mejorar la eficiencia del tratamiento biológico (Wagner *et al.*, 2002; McSwain *et al.*, 2005).

2.2.1. Eliminación de materia orgánica: Enzimas extracelulares

En los tratamientos biológicos de las aguas residuales, la degradación de la materia orgánica mediante oxidación biológica, depende principalmente de la actividad metabólica de las bacterias heterótrofas presentes, las cuales utilizan el oxígeno (bajo aireación) o el nitrato (bajo condiciones anóxicas) como aceptor final de electrones en sus reacciones metabólicas (Burgess *and* Pletschke, 2008). Aunque existen diferencias en la estructura y composición química de la pared celular de las bacterias *Gram positivo* y *Gram negativo*, el transporte de moléculas a través de las mismas se ve limitado a compuestos simples de pequeño tamaño (Cunha *et al.*, 2010). Sin embargo, en las aguas residuales una importante fracción de la materia orgánica (del 35% al 80%) está constituida por compuestos de un tamaño superior a 0,1µm, los cuales no pueden ser asimilados por los microorganismos (Cadoret *et al.*, 2002). Es por ello, que el proceso de biodegradación, comienza con la hidrólisis extracelular de macromoléculas, llevada a cabo por enzimas hidrolíticas extracelulares secretadas por los microorganismos o liberadas al medio tras la lisis celular (Cunha *et al*., 2010). Por lo tanto, la degradación biológica de la materia orgánica en las aguas residuales, no sólo está relacionada con diferentes grupos de microorganismos, sino también con las enzimas (10) extracelulares asociadas a los mismos (Burgess *and* Pletschke, 2008).

Las enzimas extracelulares pueden encontrarse unidas a la superficie celular (ectoenzimas), en forma libre en el volumen líquido o absorbidas en la matriz de sustancias poliméricas extracelulares (exoenzimas) (Burgess *and* Pletschke, 2008). Estas enzimas, responsables de la despolimerización de moléculas orgánicas, comprenden un amplio abanico de hidrolasas y oxidoreductasas (Gianfreda *et al*., 2004). No obstante, las hidrolasas han sido descritas como las principales responsables del proceso de despolimerización en las aguas residuales (Morgenroth *et al*., 2002). Dentro de este grupo, las exoenzimas (fosfatasas, glucosidasas, proteasas y lipasas) son las de mayor importancia en los tratamientos de las aguas residuales (Burgess *and* Pletschke, 2008).

En concordancia con lo comentado, las enzimas extracelulares parecen jugar un papel esencial en los procesos biológicos, siendo la concentración y la localización de los mismos, así como los mecanismos de transporte, los principales parámetros que determinan la velocidad y efectividad de reacción del proceso biológico (Guanghui *et al*., 2008).

(10): Las primeras observaciones de actividades promotoras de reacciones químicas estaban asociadas a fenómenos digestivos, siendo *Berzelius* quien introdujo el concepto de catálisis (1837). No obstante, fue la fermentación alcohólica la auténtica llave que abrió el estudio científico de las enzimas. *Lavoisier* y *Gay-Lussac* establecieron que este proceso era una transformación química con una estequiometría bien definida. Libro: "Fundamentos de Bioquímica" Universidad de Valencia, 2007.

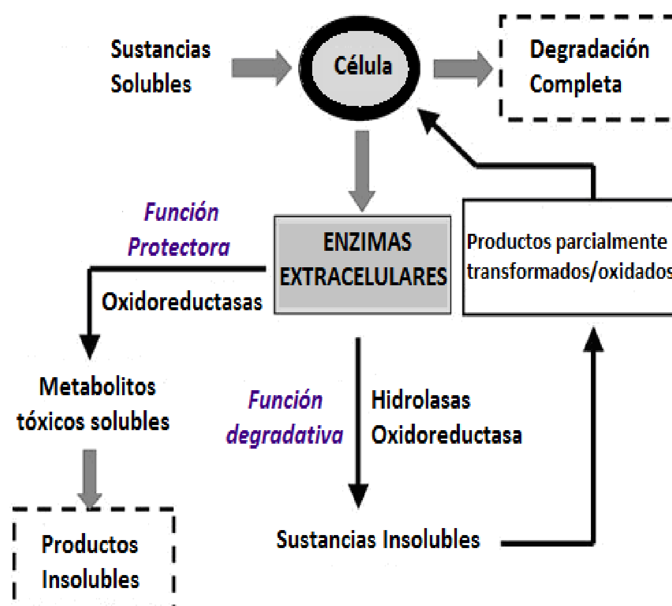


Figura I.10. Implicaciones de las enzimas extracelulares en la degradación biológica (Gianfreda *and* Rao, 2004).

Asimismo, el estudio de las actividades enzimáticas, ayuda a optimizar las condiciones operacionales para alcanzar una mayor eficiencia en el proceso de biodegradación (Li *and* Chróst, 2006; Anupama *et al*., 2008; Molina-Muñoz *et al*., 2010); ya que, la actividad de las enzimas hidrolíticas puede variar en respuesta a los cambios originados en la disponibilidad de nutrientes, las condiciones de aceptor de electrones, el pH o la temperatura (Burgess *and* Pletschke, 2008; Gómez-Silván *et al*., 2013).

En los últimos años, se ha analizado la relación existente entre algunos parámetros operacionales de los MBRs y las actividades hidrolíticas presentes en los

sistemas (Molina-Muñoz *et al*., 2010; Calderón *et al*., 2012a; Calderón *et al*., 2013; Gómez-Silván *et al*., 2013). Sin embargo, no se le ha puesto la suficiente atención a la actividad biológica que sucede en los tanques de los MBBRs (Pal *et al*., 2012), ni al estudio de las actividades enzimáticas de los microorganismos allí presentes, ni tampoco a la relación existente entre dichos cambios biológicos y los parámetros fisicoquimicos operacionales de los sistemas MBMBRs.

2.2.2. Eliminación de nutrientes: el nitrógeno

El nitrógeno, es un componente esencial de los seres vivos, ya que forma parte de moléculas orgánicas como los aminoácidos, proteínas, ácidos nucleicos, etc. La mayor parte del nitrógeno en la tierra, se encuentra en forma de dinitrógeno molecular (N_2) en la atmósfera y disuelto en los océanos. Sin embargo, su asimilación no puede llevarse a cabo por la mayoría de los seres vivos, quedando reducida a un grupo de microorganismos procariotas pertenecientes a los dominios *Bacteria* y *Archaeae* (Martínez-Espinosa *et al*., 2011; Vlaeminck *et al*., 2011). Debido a ello, la fijación biológica del nitrógeno, catalizada por la enzima *nitrogenasa* (NIF), es un paso crucial en el ciclo del nitrógeno, ya que es el único proceso biológico capaz de convertir el nitrógeno atmosférico en biodisponible (Vlaeminck *et al*., 2011). No obstante, la fijación de nitrógeno, también puede ocurrir por procesos naturales, mediante la acción de los rayos y la actividad volcánica, así como, por procesos antropogénicos, como la producción y empleo de fertilizantes nitrogenados y la combustión de combustibles fósiles (Howarth, 2004). Estas actividades antropogénicas, han contribuido enormemente a un desequilibrio del ciclo del nitrógeno, incrementando considerablemente la concentración del mismo en el medio ambiente (Jetten *et al*., 2009; Martínez-Espinosa *et al*., 2011).

De forma más concreta, en los ecosistemas acuáticos (lagos, ríos y océanos), el nitrógeno es considerado el principal responsable del proceso de eutrofización. Además, la toxicidad que presentan algunos compuestos nitrogenados, tales como el amonio y el nitrito, los convierte en una importante amenaza para los seres vivos que habitan estos ecosistemas (Daims *et al*., 2006). En este contexto, cabe destacar, que la descarga de los efluentes de aguas residuales tratadas, ha sido considerada la mayor fuente de compuestos nitrogenados en los ecosistemas acuáticos (Ahmed, 2012). Es por ello, que uno de los principales objetivos de los tratamientos de las aguas residuales consiste en incrementar lo máximo posible la tasa de eliminación de compuestos nitrogenados.

La eliminación del nitrógeno de las aguas residuales comprende tres principales procesos (**Figura I.11**): 1) Nitrificación *(nitritación y nitratación),* 2) Desnitrificación y 3) Oxidación anaerobia de amonio (anammox) (Daims *et al*., 2006).

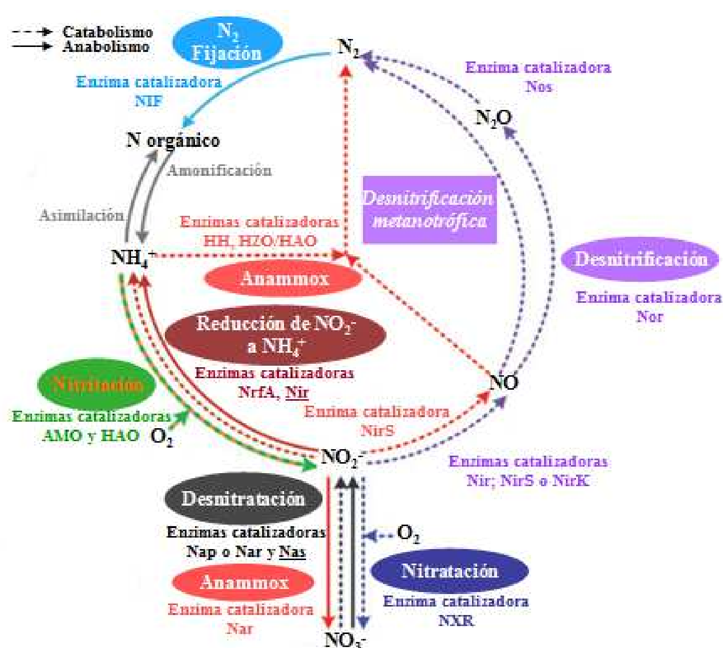


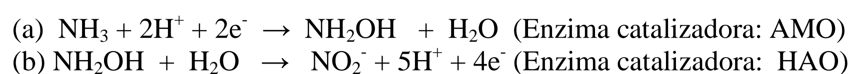
Figura I.11. Esquema global del ciclo del nitrógeno (Vlaeminck *et al*., 2011).

Nitrificación

El proceso de nitrificación, comprende la oxidación aeróbica del amonio (NH_4^+) a nitrito (NO_2^-), conocida como *nitritación* y la posterior oxidación del nitrito a nitrato (NO_3^-), conocida como *nitratación* (**Figura I.11**).

La primera etapa de nitrificación (***nitritación***), es llevada a cabo por bacterias aerobias quimiolitoautótrofas oxidadoras de amonio (AOB), pertenecientes a los géneros *Nitrosomonas* y *Nitrosospira* (clase *Betaproteobacteria*) y al género *Nitrosococcus* (clase *Gammaproteobacteria*) (Koops *and* Pommerening-Röser, 2001; Kowalchuk *and* Stephen, 2001). Además, el amonio también puede ser oxidado por arqueas oxidadoras de amonio (AOA), agrupadas filogenéticamente en un nuevo filo denominado *Thaumarchaeota* (11) (Pester *et al*., 2011; Vlaeminck *et al*., 2011).

La conversión de amonio a nitrito, comienza con la oxidación del amonio a hidroxilamina (a), estando esta reacción catalizada por la enzima monooxigenasa (AMO). Posteriormente, la hidroxilamina es convertida a nitrito por la enzima oxidoreductasa (HAO) (b).



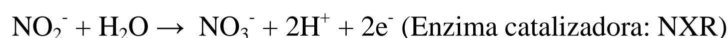
(a) $NH_3 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$ (Enzima catalizadora: AMO)
(b) $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$ (Enzima catalizadora: HAO)

(11): Las arqueas pertenecientes al Philum *Thaumarchaeota*, son numéricamente dominantes y cosmopolitas de los océanos Atlántico Norte y Pacífico Norte. Se han encontrado especies asociadas como simbiontes a la esponja marina *Cenarchaeum symbiosum*. Además, algunos miembros de este grupo de arqueas fijan el carbono inorgánico y pueden oxidar el amoníaco aeróbicamente, por lo que podrían ser responsables de una parte significativa de la producción quimioautotrófica en la oscuridad de los océanos (Swan *et al*., 2014).

La enzima AMO, está compuesta por tres subunidades (AMO-A, AMO-B y AMO-C), las cuales están codificadas respectivamente, por los genes (*amoA*, *amoB* y *amoC*) (Klotz *et al.,* 1997). El gen *amoA* es ampliamente utilizado en la mayoría de estudios moleculares basados en la detección de AOB (Kowalchuk *and* Stephen, 2001; Francis *et al*., 2007). Cabe resaltar, además, que algunas AOB, también poseen las enzimas nitrito reductasa (Nir K) y óxido nítrico reductasa (NOR), por lo que bajo condiciones anóxicas, pueden llevar a cabo algunos pasos de la desnitrificación (Vlaeminck *et al*., 2011).

La oxidación del nitrito a nitrato (***nitratación***), es llevada a cabo por bacterias aerobias quimiolitoautótrofas oxidadoras de nitrito (NOB); este grupo de bacterias son filogenéticamente heterogéneas y se encuentran en un amplio abanico de ecosistemas acuáticos y terrestres (Lücker *et al*., 2010). Se han descrito diversos géneros de NOB tales como: *Nitrobacter* (*Alfaproteobacteria*), *Nitrococcus* (*Gammaproteobacteria*), *Nitrogea* (*Betaproteobacteria*), *Nitrospira* (filo *Nitrospirae*), *Nitrolancetus* (*filo Chloroflexi*) y *Nitrospina*, ésta última asociada filogenéticamente a la clase *Deltraproteobacteria* y propuesta para un nuevo filo denominado *Nitrospinae* (Lücker *et al*., 2013).

La nitratación, es catalizada por la nitrito oxidoreductasa (NXR); esta enzima está compuesta a su vez por dos subunidades: la subunidad *alfa* NxrA y la subunidad *beta* NxrB, las cuales son codificadas respectivamente por los genes *nxrA* y *nxrB*.

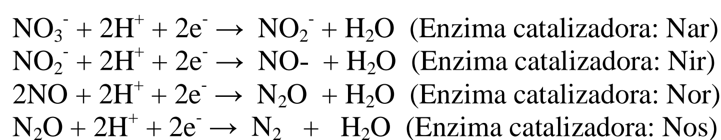


$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$ (Enzima catalizadora: NXR)

Desnitrificación

La desnitrificación, es un proceso generosamente extendido en la naturaleza que consiste en la reducción del nitrito (*desnitritación*) o nitrato (*desnitratación*) a óxidos de nitrógeno gaseosos y dinitrógeno (**Figura I.11**). A diferencia de la nitrificación, la desnitrificación tiene lugar bajo condiciones anóxicas y es llevada a cabo principalmente por organismos heterótrofos (Francis *et al*., 2007). Las especies bacterianas desnitrificantes, se encuentran repartidas entre aproximadamente más de 50 géneros dentro de la clase *Proteobacteria*. Entre ellos, los más estudiados son: *Alcalígenes, Paracoccus, Pseudomonas* y *Thiobacillus.* Es de destacar que este proceso también puede ser realizado por algunos miembros de arqueas, hongos y diversos géneros de foraminíferos bentónicos (Cervantes-Carrillo *et al*., 2000; Francis *et al*., 2007; Kraft *et al*., 2011).

Los cuatro tipos de enzimas *nitrógeno reductasas* que catalizan las diferentes etapas del proceso de desnitrificación son: nitrato reductasa (Nar), nitrito reductasa (Nir), óxido nítrico reductasa (Nor) y óxido nitroso reductasa (Nos). Estas enzimas están, generalmente, inducidos de forma secuencial (Philippot *et al*., 2002):



$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$ (Enzima catalizadora: Nar)
$NO_2^- + 2H^+ + 2e^- \rightarrow NO- + H_2O$ (Enzima catalizadora: Nir)
$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$ (Enzima catalizadora: Nor)
$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$ (Enzima catalizadora: Nos)

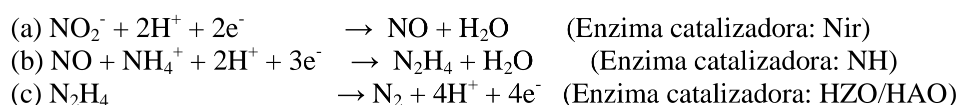
Dado el amplio espectro de genes que codifican las enzimas nitrógeno reductasas (Ej. *narG*, *nirS*, *nirK*, *norB* y *nosZ*), la mayoría de bacterias y arqueas desnitrificantes son capaces de realizar todas las etapas del proceso (Francis *et al*., 2007). Cabe destacar que recientemente se ha descrito una nueva ruta de *desnitrificación metanotrófica* en bacterias relacionadas filogenéticamente con la división NC10. En esta ruta, el nitrito es reducido a óxido nítrico y seguidamente a dinitrógeno mediante la acción de una enzima hasta ahora desconocida (Vlaeminck *et al*., 2011) (**Figura I.11**).

Asimismo, también existe otra ruta denominada *reducción desasimilatoria del nitrato a amonio* (dissimilatory nitrate reduction to ammonium, DNRA), mediante la cual el nitrato es reducido a nitrito y parte de este nitrito a amonio (**Figura I.11**). Este proceso, se ve favorecido frente al proceso de desnitrificación, cuando la concentración de nitrato es limitante en comparación con la concentración de carbono orgánico (elevados ratios de C/ NO_3^-) (Kraft *et al*., 2011; Rütting *et al*., 2011). No obstante, se han estudiado recientemente los procesos que regulan la reducción de nitrito a amonio y la información disponible parece indicar que este proceso está relativamente extendido entre las bacterias (Vlaeminck *et al*., 2011). Las enzimas nitrito-reductasa de las bacterias fermentadoras (Nir) y penta-homo-citocromo C nitrito-reductasa (NrfA) han sido descritas como catalizadores de la reducción de nitrito a amonio (Mohan *et al*., 2004).

Anammox

La oxidación anaerobia del amonio (anammox), consiste en la formación de dinitrógeno (N_2) a partir de nitrito y amonio, bajo condiciones anaerobias (**Figura I.11**). El primer paso del proceso anammox, consiste en reducir el nitrito a óxido nítrico

mediante la acción de la enzima nitrito óxido reductasa (Nir) (a). Posteriormente, el NO se combina con el amonio para producir hidracina, estando este paso catalizado por la enzima hidracina hidrolasa (HH) (b). Finalmente, las enzimas hidracina/hidroxilamina oxidoreductasa (HZO/HAO) transforman la hidracina en dinitrógeno (c) (Jetten *et al*., 2009). Asimismo, una enzima nitrato/nitrito oxido reductasa (Nar), oxida parte del nitrito a nitrato (Vlaeminck *et al*., 2011).



(a) $NO_2^- + 2H^+ + 2e^- \rightarrow NO + H_2O$ (Enzima catalizadora: Nir)
(b) $NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O$ (Enzima catalizadora: NH)
(c) $N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$ (Enzima catalizadora: HZO/HAO)

El proceso anammox, se lleva a cabo mediante un grupo de bacterias quimiolitoautótrofas pertenecientes al orden *Planctomycetales*, dentro del cual se agrupan cinco géneros: Candidatus *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus* y *Jettenia* (Kraft *et al*., 2011). Las bacterias anammox, son microorganismos anaerobios obligados de crecimiento lento, capaces de prosperar en todo tipo de ecosistemas que posean zonas anaerobias y bajas concentraciones de sustrato (Jetten *et al*., 2009). Estos microorganismos han sido descritos en ecosistemas acuáticos, incluyendo sedimentos marinos y de agua dulce, así como en plantas de tratamiento de aguas residuales (Kraft *et al*., 2011).

3. Estudio de la diversidad bacteriana de las aguas residuales

Durante muchas décadas, el diseño de los sistemas biológicos de depuración se optimizaba basándose en el estudio de parámetros físico-químicos, y aunque no se tuviera una información detallada de la comunidad microbiana responsable del proceso de depuración, obviamente obtenían beneficios de la misma (Gilbride *et al*., 2006).

Este hecho, podía llevar a suponer que no era esencial el estudio de la composición y estructura de las comunidades microbianas en estos sistemas, sin embargo, no podemos obviar que, gracias al estudio de las mismas, sabemos la identidad de determinados microorganismos relacionados con problemas de sedimentación del fango, las espumas y el bulking, así como la de otros microorganismos responsables de la eliminación de determinados compuestos orgánicos y nutrientes (Gilbride *et al*., 2006). De acuerdo con Sanz *and* Köchling (2007), un buen ejemplo de la importancia del estudio de estas comunidades, es el descubrimiento de *Brocadia anammoxidans*, capaz de oxidar el amonio a dinitrógeno (N_2) bajo condiciones anóxicas. Este descubrimiento, ha revolucionado las investigaciones en la eliminación de nitrógeno en las plantas de tratamiento de aguas residuales.

Los métodos tradicionales empleados en Microbiología para el estudio de la diversidad microbiana, se fundamentan en el uso del microscopio y las técnicas dependientes de cultivo. Estas últimas, se basan en el empleo de diversos medios artificiales o naturales preparados en el laboratorio para el cultivo, aislamiento y estudio de los microorganismos. La combinación de ambas metodologías, puso de manifiesto que no todas las bacterias de un determinado ecosistema podían ser cultivadas, ya que comparando el número de células observadas en el microscopio y el número de unidades formadoras de colonias desarrolladas en un medio de cultivo, se encontraban diferencias denominadas "*great plate count anomaly*" que podían alcanzar varios órdenes de magnitud (Handelsman, 2004; Stewart, 2012). Pese a los esfuerzos de simular en los medios de cultivo las condiciones ambientales en las que habitan los microorganismos de interés, es difícil recrear los requerimientos nutricionales, las condiciones físico-químicas y las complejas relaciones simbióticas entre los microorganismos que ocurren en condiciones naturales (Sanz *and* Köchling, 2007;

Stewart, 2012). Por ello, y gracias al desarrollo de los métodos independientes de cultivo en las últimas décadas del siglo XX, podemos afirmar que existe una gran diversidad bacteriana y que hasta el momento, sólo se ha podido cultivar en laboratorio una pequeña fracción de la misma, del orden del 1% (Giovannoni *and* Stingl, 2005). No obstante, pese a sus limitaciones, el empleo de la metodología clásica, en el estudio de la diversidad microbiana en las plantas de tratamiento de aguas residuales, ha permitido la identificación de numerosos microorganismos eucariotas y procariotas, así como, el conocimiento de la relación existente entre las bacterias filamentosas y los procesos de *bulking* (Gilbride *et al.*, 2006; Sanz *and* Köchling 2007).

3.1. Estudios de la diversidad bacteriana basados en métodos independientes de cultivo

3.1.1. Generalidades

El gen 16S ARNr

Woese *et al.* (1987) fueron pioneros en la descripción de los genes bacterianos ARNr, describiéndolos como “relojes moleculares”, debido a su universalidad, su conformación extremadamente conservada, su capacidad para regular actividades funcionales celulares y por presentar distintas regiones con diversos grados de variabilidad, lo que permite medir y establecer relaciones filogenéticas.

Los tres tipos de ARNr presentes en los ribosomas procariotas (5S, 16S y 23S), (**Figura I.12**) de acuerdo con sus tasas de sedimentación, tienen longitudes de secuencia de alrededor de 120, 1500 y 3300 nucleótidos respectivamente (Amann *et al.*, 1995).

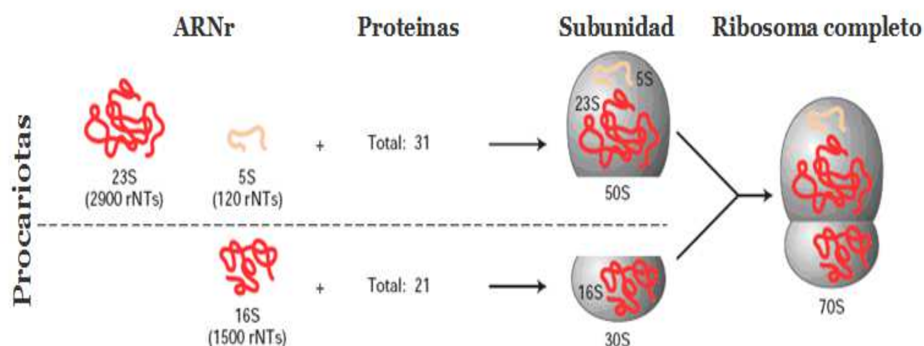


Figura I.12. Estructura y componentes del ribosoma procariota

Los 1500 pares de bases que constituyen el gen 16S ARNr son una parte estructural de la subunidad pequeña 30S ribosomal (SSU, small subunit) y consisten en ocho regiones altamente conservadas (U-U8) y nueve regiones variables en todo el dominio bacteriano (Jonasson *et al*., 2002). Hasta el momento, el gen 16S ARNr, está considerado el principal marcador filogenético para el estudio de la estructura y composición de las comunidades bacterianas (Větrovský *and* Baldrian, 2013). Esto se debe principalmente a las siguientes características; 1) La longitud de su secuencia, permite una fácil y rápida secuenciación; 2) Se encuentra conservado en todas las bacterias, manteniendo la misma función en todas ellas; 3) Presenta zonas variables, que permiten suficiente diversificación para el establecimiento de una clasificación taxonómica. Asimismo, la presencia de zonas conservadas, permite el diseño de cebadores (primers); 4) Sólo se ve ligeramente afectado por transferencia horizontal de genes (Woese *et al*., 1987; Tringe *and* Hugenholtz, 2008; Mizrahi-Man *et al*., 2013; Větrovský *and* Baldrian, 2013).

El gen 16S ARNr, presenta algunos aspectos que limitan la interpretación de resultados, uno de los más importantes es que el número de copias por genoma puede variar entre 1 y 15 (Větrovský *and* Baldrian, 2013). A su vez, muchas bacterias relacionadas filogenéticamente, pueden poseer diferentes potenciales metabólicos, mientras que otras distribuidas en diferentes linajes filogenéticos comparten características fisiológicas (Ej. las bacterias desnitrificantes). Por ello, el estudio de la diversidad bacteriana en las plantas de tratamiento de aguas residuales, debe ser abordado no sólo con el estudio del gen 16S ARNr, sino también con genes *funcionales* que permitan identificar los miembros responsables de las principales funciones implicadas en el proceso de depuración (Wagner *et al*., 2002).

Reacción en cadena de la polimerasa

El gran avance técnico que aceleró el estudio de la biodiversidad a nivel molecular, surgió de una gran idea desarrollada por *Kary Banks Mullis* (12) durante una noche del mes de abril de 1983, la "*reacción en cadena de la polimerasa*" (PCR, Polymerase Chain Reaction) (Mullis, 1990).

(12): Bioquímico estadounidense, le fue concedido el Premio Nobel en Química en 1993 junto a Michael Smith por sus aportaciones sobre la Reacción en cadena de la Polimerasa. En 1994 dijo: " *I was working for Cetus, making oligonucleotides. They were heady times. Biotechnology was in flower and one spring night while the California buckeyes were also in flower I came across the polymerase chain reaction. I was driving with Jennifer Barnett to a cabin I had been building in northern California. She and I had worked and lived together for two years. She was an inspiration to me during that time as only a woman with brains, in the bloom of her womanhood, can be. That morning she had no idea what had just happened. I had an inkling. It was the first day of the rest of my life. From there it's a single sentence. I worked as a consultant, got the Nobel Prize, and have now turned to writing*". (http://www.nobelprize.org).

La PCR, es una técnica de Biología Molecular, cuyo objetivo es la obtención de un gran número de copias de una secuencia de ADN, bajo un número de ciclos que comprenden las etapas de: 1) *Desnaturalización* del ADN por calor, 2) *Hibridación* de los cebadores con la región complementaria del ADN molde que se desea amplificar y 3) *Extensión* de la región comprendida entre los cebadores, catalizada por la enzima ADN polimerasa (DNA polymerase) (Saiki *et al*., 1987). Los cebadores (oligonucleótidos sintéticos) y la enzima ADN polimerasa, son de vital importancia para la realización de esta técnica. Por ello, el desarrollo de cebadores específicos para determinados taxones y cebadores "*universales*", unido al empleo de la enzima ADN polimerasa termoestable y al desarrollo tecnológico del termociclador, permitieron la rápida aplicación de la PCR en el campo de la medicina, biología, biotecnología, ciencias forenses, etc. (Valones et al., 2009).

Vale la pena resaltar, que junto con la PCR, el método de secuenciación de Sanger (Sanger et al., 1977) representó la piedra angular de la producción de secuencias de ADN de los genes ARNr. Este método generó los primeros análisis en profundidad de las comunidades microbianas, facilitando la obtención y el depósito de un sin fin de secuencias de ADN del gen 16S ARNr en las bases de datos (NCBI, The National Center for Biotechnology Information y EMBL-EBI European Molecular Biology Laboratory-European Bioinformatics Institute).

3.1.2. Técnicas moleculares: *DGGE/TGGE y qPCR*

La Biología Molecular, se nutre de diversas técnicas para abordar la identificación de microorganismos y el estudio de la biodiversidad bacteriana a partir de una muestra de ADN medioambiental. Entre ellas, la "*Electroforesis en gel con*

gradiente de temperatura (TGGE) o desnaturalizante (DGGE)" (TGGE/DGGE; temperature / denaturating gradient gel electrophoresis) descrita por Muyzer *et al*., (1993), representa una de las técnicas más ampliamente utilizadas en el campo de la ecología microbiana (Sanz *and* Köchling, 2007). El empleo de la misma, proporciona el perfil de la diversidad genética de una comunidad microbiana, permitiendo el estudio de la estructura y dinámica de la misma (Wittebolle *et al*., 2005).

Esta técnica permite separar los fragmentos de ADN previamente amplificados por PCR, de igual longitud pero con diferentes secuencias de nucleótidos, permitiendo diferenciar las diversas poblaciones que componen la comunidad microbiana. La separación se fundamenta en la disminución de la velocidad electroforética de una doble cadena de ADN parcialmente desnaturalizada. El punto de desnaturalización de cada fragmento de ADN, depende de la composición de nucleótidos de cada secuencia, por lo que en un gel de poliacrilamida con un gradiente lineal de desnaturalización (DGGE) o temperatura (TGGE), los fragmentos de ADN con diferentes secuencias se detendrán en su migración electroforética en diferentes posiciones del gel (Muyzer *and* Smalla, 1998; Muyzer, 1999). Posteriormente, al objeto de obtener información filogenética sobre las poblaciones, se podrán secuenciar los fragmentos de ADN (bandas) obtenidos de los perfiles del TGGE o DGGE (Muyzer *and* Smalla, 1998).

En un TGGE o DGGE, el número de bandas, su posición precisa, y la intensidad de las mismas nos dan una estimación del número y la abundancia relativa de las poblaciones dominantes en la muestra (Boon *et al*., 2002). A diferencia de otras técnicas (p.e. clonación), con esta técnica se pueden estudiar las variaciones poblacionales a lo largo del tiempo o en función de las condiciones ambientales y/o experimentales (Muyzer, 1999). Sin embargo, el estudio de las comunidades

microbianas mediante TGGE/DGGE, presenta algunas limitaciones. Entre ellas, destacar que sólo son capaces de detectar las poblaciones mayoritarias que componen la comunidad estudiada (un 1% o más del total de la población) (Muyzer *and* Smalla, 1998). Además, puede ocurrir una co-migración de diferentes fragmentos de ADN, que conlleva la agrupación de diferentes secuencias en una misma banda, impidiendo la identificación filogenética de las poblaciones microbianas. Asimismo, no podemos obviar que un mismo microorganismo puede generar más de una banda, dado que puede poseer varias copias de genes ARNr (Marzorati *et al*., 2008). Finalmente, enfatizar que estas técnicas no proporcionan datos cuantitativos absolutos de las poblaciones (Muyzer *and* Smalla, 1998).

Para cuantificar la abundancia y expresión de marcadores genéticos taxonómicos (ARNr) y funcionales presentes en una muestra de ADN medioambiental, la "*reacción en cadena de la polimerasa cuantitativa en tiempo real*" (RT-qPCR, *Real Time quantitative Polymerase Chain Reaction*) constituye una técnica molecular ampliamente utilizada en los estudios de ecología microbiana (Smith *and* Osborn, 2009). Esta técnica, emplea sondas fluorescentes (Ej. TaqMan) o colorantes (Ej. SYBR green I), los cuales emiten una señal de fluorescencia en tiempo real que es proporcional al número de copias obtenido en cada ciclo de amplificación de PCR (Zhang *and* Fang, 2006; Smith *and* Osborn, 2009). El SYBR green I, es el colorante más utilizado en los estudios de muestras ambientales; este fluorocromo presenta una gran afinidad al ADN de doble cadena, siendo indetectable la emisión de fluorescencia cuando el ADN es de cadena simple (Smith *and* Osborn, 2009; Valones *et al*., 2009). Para una cuantificación absoluta del gen de interés, es necesario realizar una curva de calibrado estándar, la cual consiste en hacer diluciones seriadas de un patrón de concentración conocida. Como patrón o estándar, se puede emplear ADN genómico de

un cultivo puro, si el número de copias del gen y el tamaño del genoma son conocidos, o un plásmido que contenga el gen diana (Zhang *and* Fang, 2006; Fraga *et al*., 2008). Esta técnica, presenta una gran especificidad, sensibilidad, reproducibilidad y permite un rápido análisis de cuantificación, así como un mayor control de la calidad del proceso (Valones *et al*., 2009).

3.1.3. Metagenómica: *Tecnologías de nueva generación*

Handlesman *et al*. (1998), acuñaron el término *metagenómica* para describir la metodología que permite analizar una colección de genes secuenciados de una muestra medioambiental como si se tratara de un único genoma. En otras palabras, la metagenómica consiste en el análisis genético directo de los genomas presentes en una muestra ambiental (Thomas *et al*., 2012). En los inicios de esta ciencia, los estudios metagenómicos permitían la obtención de secuencias genéticas de comunidades complejas realizando una genoteca de clones y secuenciando los clones individualmente, siendo común la secuenciación de 500 clones por muestra para tener un número significativo de secuencias que permitiera una cuantificación realista del ecosistema estudiado. Eso conlleva la realización de múltiples pasos antes de obtener el resultado final: amplificación, digestión enzimática, ligación, transformación, cultivo de clones, extracción de plásmido, amplificación, extracción del pADN y por último secuenciación por el método Sanger. La clonación era un paso clave en los estudios de metagenómica, dado que el método de secuenciación de Sanger sólo puede secuenciar especímenes individuales y una muestra ambiental puede contener cientos de individuos (Shokralla *et al*.,2012).

En los últimos años, la rápida y sustancial reducción de los costes de los procedimientos de nueva generación, denominados comúnmente "*Next Generation Sequencing*" (NGS), está acelerando la obtención de secuencias de metagenomas ambientales eludiendo los pasos anteriormente comentados (Morozova *and* Marra, 2008; Ansorge, 2009; Thomas *et al*., 2012). Estas nuevas tecnologías, permiten una lectura rápida y eficiente de miles de secuencias de ADN. En comparación con la secuenciación de Sanger el coste por base es inferior en varios órdenes de magnitud, sin embargo, los fragmentos de lectura que se obtienen son de menor tamaño. La longitud de los fragmentos de lectura representa una de las principales limitaciones de estas tecnologías (Ansorge, 2009).

Las tecnologías NGS pueden ser clasificadas en dos categorías. La primera comprende las tecnologías basadas en PCR, dentro del cuál se encuentran cuatro plataformas comerciales: Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, EEUU), HiSeq 2000 (Illumina Inc., San Diego, CA, EEUU), AB SOLiD System (Life Technologies Corp., Carlsbad, CA, EEUU) y Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, EEUU). El segundo grupo está compuesto por las tecnologías denominadas "*Single Molecule Sequencing*" (SMS), las cuales son independientes de PCR y no incluyen la amplificación previa a la secuenciación. En este grupo se encuentran dos sistemas descritos recientemente: HeliScope (Helicos Bio-Sciences Corp., Cambridge, MA, EEUU) y PacBio RS SMRT system (Pacific Biosciences, Menlo Park, CA, EEUU) (Shokralla *et al*., 2012). En la **Figura I.13**, se muestra el desarrollo histórico de las tecnologías NGS, indicando las diferentes plataformas.

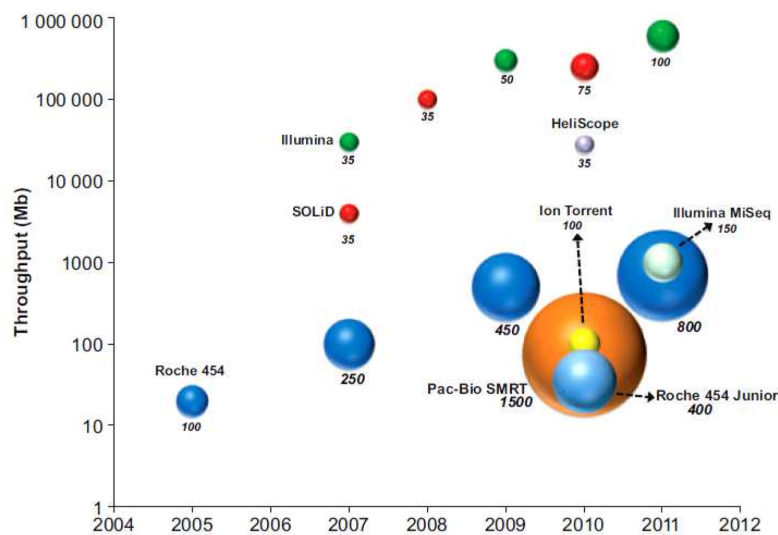


Figura I.13. Desarrollo y evolución histórica de las tecnologías NGS (Shokralla *et al.*, 2012). El diámetro de las burbujas indica la longitud de lectura de cada tecnología en pares de bases (pb). Los colores corresponden a las diferentes plataformas y permiten observar las mejoras en cada una de ellas en términos de longitud de lectura (pb).

Las tecnologías NGS dependientes de PCR, difieren entre ellas, sin embargo, todas se fundamentan en dos pasos principales: La preparación de la librería de fragmentos de ADN y la detección de los nucleótidos incorporados (Shokralla *et al.*, 2012).

3.1.3.1. Pirosecuenciación e Illumina

Pirosecuenciación o Secuenciación 454

Es una técnica de secuenciación masiva de ADN, desarrollada a finales de la década de 1990 (Ronaghi *et al.*, 1996, 1998; Ronaghi *and* Elahi, 2002; Clarke, 2005).

La tecnología 454 (454 Genome Sequencer, Roche Diagnostics Corp., Brandford, CT, EEUU), fue lanzada al mercado en 2005 (Margulies et al., 2005),

siendo la primera de las "*Next Generation Sequencing*" (Ansorge, 2009). Esta técnica, se basa en la secuenciación por síntesis, que mide por quimioluminiscencia el pirofosfato inorgánico (PPi) liberado, lo que permite una rápida determinación de secuencias en tiempo real.

La síntesis de la cadena complementaria de ADN se monitoriza utilizando como molde un ADN de cadena simple, esta amplificación es llevada a cabo mediante PCR en emulsión (emPCR). Para ello, en primer lugar los fragmentos de ADN se ligan en ambos extremos con adaptadores específicos, denominados genéricamente A y B (Paso 1.**Figura I.14 (A)**). El adaptador B, permite la inmovilización de los fragmentos de ADN en una "perla". Seguidamente, la perla con los fragmentos de ADN se emulsiona con los reactivos de amplificación en una micela de agua y aceite al objeto de amplificar clonalmente los fragmentos de ADN mediante emPCR (Paso 2.**Figura I.14 (A)**). Tras la emPCR, cada perla con su fragmento amplificado se incuba en una placa "picotiter" que contiene la enzima ADN polimerasa, ATP sulfurilasa, luciferasa, apirasa, más los sustratos adenosina-5´-fosfosulfato (APS) y luciferina (Paso 3.**Figura I.14 (A)**).

Los nucleótidos se añaden de forma consecutiva a la reacción y, en caso de incorporación, se libera PPi. En cada ciclo, un solo tipo de dNTP es añadido (dATP, dTTP, dCTP o dGTP) y más de uno puede ser incorporado. La ATP-sulfurilasa convierte cuantitativamente el PPi en ATP en presencia de APS. El ATP generado permite la conversión de la luciferina en oxiluciferina por acción de la luciferasa, generando luz visible en cantidades proporcionales a la cantidad de ATP presente (**Figura I.14 (B)**). La luz emitida es detectada por una cámara CCD y puede ser analizada mediante un programa informático. Cada señal luminosa detectada en forma

de pico es proporcional al número de nucleótidos incorporados. La degradación de dNTPs no incorporados es llevada a cabo por la enzima apirasa (Ronaghi *et al*., 1996, 1998; Mardis, 2008; Morozova *and* Marra, 2008; Ansorge, 2009).

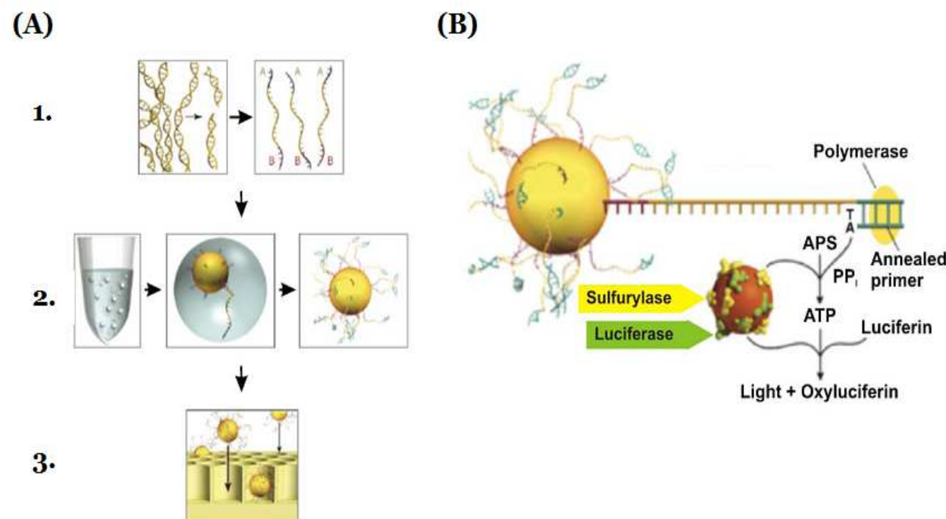


Figura I.14. **(A)** Esquema metodológico de la pirosecuenciación 454. **(B)** Reacción de pirosecuenciación tras la incorporación de nucleótidos (dNTPs).

Illumina

Esta técnica, formalmente conocida como Solexa, fue introducida en el mercado en 2007. Al igual que la pirosecuenciación 454, se basa en la secuenciación por síntesis. En illumina, la síntesis de la cadena complementaria de ADN también se monitoriza utilizando como molde un ADN de cadena simple, sin embargo, en esta técnica se utiliza la amplificación en puente. Para ello, los fragmentos con sus adaptadores (Paso 1. **Figura I.15**) son colocados en una "celda de flujo" que contiene en su superficie adaptadores con la secuencia de oligonucleótidos complementarios a los adaptadores de los fragmentos de ADN, permitiendo la unión de los mismos por un extremo a su

adaptador complementario (Paso 2. **Figura I.15**). Una vez hibridados los fragmentos con sus adaptadores complementarios, los cuales actuaran como cebadores, se lleva a cabo la amplificación mediante PCR de tipo puente para obtener la librería de fragmentos clonados (Paso 3. **Figura I.15**) (Mardis, 2008; Ansorge, 2009). En la secuenciación por síntesis con la plataforma illumina los cuatro tipos de dNTPs son adicionados simultáneamente a la reacción en cada uno de los ciclos de secuenciación. Cada dNTP está marcado con un fluorocromo y tienen inactivado químicamente su extremo 3´, por lo que tan solo una base puede ser incorporada por la polimerasa en cada uno de los ciclos. Mediante un proceso químico el fluorocromo es eliminado y permite la incorporación de la siguiente tanda de nucleótidos en el próximo ciclo (Ansorge, 2009; Shokralla *et al*., 2012).

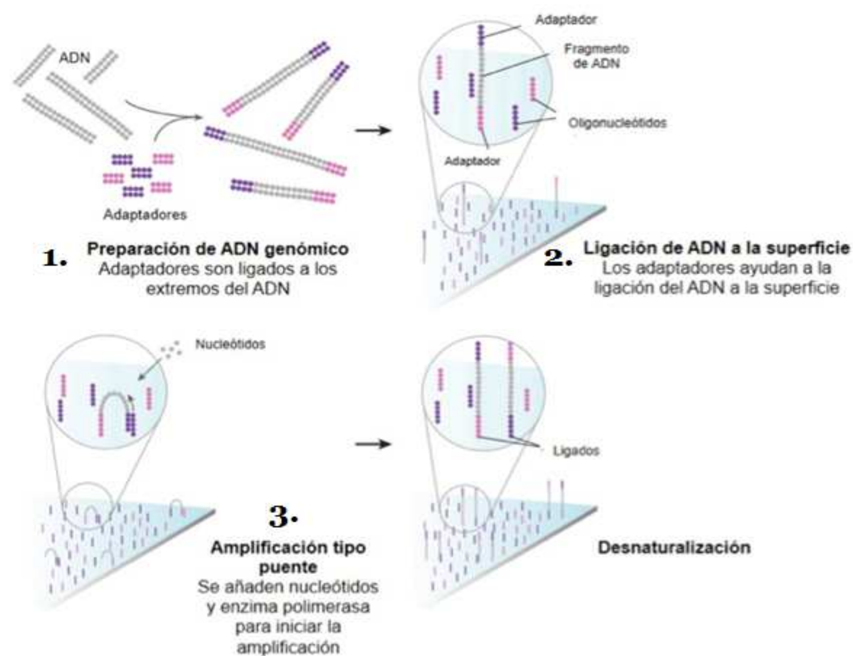


Figura I.15. Creación de la librería de fragmentos de ADN para la secuenciación por la plataforma illumina. PCR tipo puente (Mardis, 2008).

4. Diversidad bacteriana en las plantas de tratamiento de aguas residuales

En las plantas de tratamiento de aguas residuales, las comunidades microbianas desarrolladas se caracterizan por presentar una amplia y compleja diversidad genética y metabólica, permitiendo la degradación de la mayor parte de los contaminantes orgánicos (Erijman *et al.*, 2011). Asimismo, estas comunidades son dinámicas, permitiendo una continua adaptación de las poblaciones microbianas a las nuevas condiciones ambientales u operacionales. Esta característica, es fundamental en las plantas de tratamiento de aguas residuales, ya que éstas están sometidas a continuas fluctuaciones.

Sin embargo, la "*estabilidad biológica*" del proceso de depuración no sólo parece deberse a la gran diversidad microbiana y a su dinámica, sino también a la extensa redundancia funcional que caracteriza a las comunidades microbianas desarrolladas en las plantas de tratamiento de aguas residuales. La redundancia funcional, se garantiza gracias a la presencia de un reservorio de especies diferentes capaces de realizar las mismas funciones bioquímicas. Esta característica, permite a las comunidades microbianas amortiguar las fluctuaciones que tienen lugar en los sistemas de depuración, ya que gracias a la redundancia funcional, la eficiencia del proceso no depende de una sola especie o de las especies dominantes (Briones *and* Raskin, 2003; Curtis *and* Sloan., 2006; Erijman *et al.*, 2011).

La composición de estas comunidades microbianas en los sistemas CAS y MBRs, parece mostrar un alto grado de similitud, estando ambas caracterizadas por la presencia de los filos *Proteobacteria, Actinobacteria, Bacterioidetes, Firmicutes, Acidobacteria, Chloroflexi y Verrucomicrobia* (Xia *et al.*, 2010; Zhang *et al.*, 2012;

Kim *et al*., 2013; Ye *and* Zhang, 2013). En los sistemas MBBRs, la composición de la comunidad microbiana, parece diferir de la descrita para los sistemas CAS y MBRs, principalmente debido a la existencia de dos tipos de comunidades: la comunidad de la biopelícula, la cuál parece estar dominada por microorganismos anaerobios de la clase *Clostridia* y reductores de sulfato de la clase *Deltaproteobacteria* y la comunidad en suspensión, dominada por microorganismos aerobios del filo *Proteobacteria* (Biswas *and* Turner, 2012). Sin embargo, aunque esto constituye la generalidad, los filos anteriormente mencionados de los sistemas CAS y MBRs también se han descrito en los sistemas MBBRs, tanto en la biopelícula como en suspensión. (Biswas *and* Turner, 2012; Biswas *et al.*, 2013). No obstante, Pal *et al*. (2012) y Fu *et al*. (2010) describieron los filos *Proteobacteria*, *Nitrospira* y *Bacterioidetes* como dominantes en la comunidad bacteriana de la biopelícula formada en un sistema MBBR. Asimismo, en otro estudio de la diversidad bacteriana de la biopelícula en un MBBR, se observó una dominancia de los filos *Proteobacterias* y *Firmicutes* (Calderón *et al.*, 2012b). Sin embargo, de acuerdo con Pal *et al*. (2012), son pocos los estudios de biodiversidad realizados en los sistemas que combinan biomasa bacteriana suspendida y adherida (biopelícula), enfatizando que, la información disponible sobre la comunidad bacteriana desarrollada en ambos tipos de biomasa (suspendida y adherida) es aún más limitada (Kwon *et al*., 2010; Biswas *et al*., 2014).

En referencia a los MBMBRs, de acuerdo con la revisión publicada por Ivanovic *and* Leiknes en 2012, los estudios realizados en estos sistemas se han enfocado principalmente en el rendimiento de eliminación de materia orgánica y nutrientes (N y P), así como en el estudio del fouling de membrana. Los resultados recopilados por estos autores concluyen que los procesos de biopelícula en los biorreactores de membrana son potencialmente beneficiosos, sin embargo parece que el estudio de las

comunidades microbianas responsables del proceso de depuración en estos sistemas aún no se ha realizado en profundidad.

Referencias

Ahmed, Z., 2012. Microbial Communities in nutrient-removing membrane bioreactors, A review. Journal of Environmental Science and Technology, 5, 16-28.

Alleman, J.E., 1983. The History of Fixed-Film Wastewater Treatment Systems. http,//web.deu.edu.tr/atiksu/ana52/biofilm4.pdf

Allison, D.G., Gilbert, P., Lappin-Scott, H.M., Wilson, M. and Scourfield, M., 2000. Community Structure and Co-operation in Biofilms. Cambridge University Press, Cambridge.

Amann, R.I., Ludwing, W. And Schleifer, K.H. 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. Microbiological Reviews, 59, 143-169.

Andreottola, G., Foladori, P., Ragazzi, M., Tatano, F., 2000. Experimental comparison between MBBR and activated sludge system for the treatment of municipal wastewater. Water Science and Technology, 41, 375-382.

Ansorge, W.J., 2009. Next-generation DNA sequencing techniques. New Biotechnology, 25, 195-203.

Anupama, V.N., Amrutha, P.N., Chitra, G.S., Krishnakumar, B., 2008. Phosphataseactivity in anaerobic bioreactors for wastewater treatment. Water Research, 42, 2796-2802.

BCC, 2011. Membrane Bioreactors, Global Markets. BCC report MST047C.

Bin, Z., Baosheng, S., Min, J., Taishi, G., Zhenghong, G., 2008. Extraction and analysis of extracellular polymeric substances in membrane fouling in submerged MBR. Desalination, 227, 286-294.

Biswas, K., Turner, S.J., 2012. Microbial community composition and dynamics of moving bed biofilm reactor systems treating municipal sewage. Applied and Environmental Microbiology, 78, 855-864.

Biswas, K., Taylor, M.W., Turner, S.J., 2014. Successional development of biofilms in moving bed biofilm reactor (MBBR) systems treating municipal wastewater. Applied Microbiology and Biotechnology, 98, 1429-1440.

Boon, N., De Windt, W., Verstraete, W., Top, E.M., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiology Ecology, 39, 101-112.

Briones, A., and Raskin, L., 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology, 14, 270-276.

Burgess, J.E., Pletschke, B.I., 2008. Hydrolytic enzymes in sewage sludge treatment,a mini-review. Water SA, 34, 343-349.

Cadoret, A., Conrad, A., Block, J.C., 2002. Availability of low and high molecular weightsubstrate to extracellular enzymes in whole and dispersed activated sludges. Enzyme and Microbial Technology, 31, 179-186.

Calderón, K., González-Martínez, A., Montero-Puente, C., Reboleiro-Rivas, P., Poyatos, J.M., Juárez-Jiménez, B., Martínez-Toledo, M.V., Rodelas, B., 2012a. Bacterial community structure and enzyme activities in a membrane bireactor (MBR) using pure oxygen as an aeration source. Bioresource Technology, 103, 87-94.

Calderón, K., Martín-Pascual, J., Poyatos, J.M., Rodelas, B., 2012b. Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. Bioresource Technology, 121, 119-126.

Calderón, K., Reboleiro-Rivas, P., Rodríguez, F.A., Poyatos, J.M., González-López, J., Rodelas, B., 2013. Comparative analysis of the enzyme activities and the bacterial community structure based on the aeration source supplied to an MBR to treat urban wastewater. Journal of Environmental Management, 128, 471-479.

Cervantes-Carrillo, F., Pérez-Trevilla, J., Gomez, J., 2000. Avances sobre la eliminación biológica del nitrógeno de las aguas residuales. Revista Latinoamericana de Microbiología, 42, 73-82.

Chan, Y.J., Chong, M-F., Law, C.L., Hassell, D.G., 2009. A review on anaerobic–aerobic treatment of industrial and municipal wastewater. Chemical Engineering Journal, 155, 1-18.

Chang, CY., Tanong, K., Xu, J., Shon, H., 2011. Microbial community analysis of an aerobic nitrifying-denitrifying MBR treating ABS resin wastewater. Bioresource Technology, 102, 5337-5344.

Cheng, K-C., Demirci, A., Catchmark, J.M., 2010. Advances in biofilm reactors for production of value-added products. Applied Microbiology and Biotechnology, 87, 445-456.

Chu, L. and Wang, J., 2011. Comparison of polyurethane foam and biodegradable polymer as carriers in moving bed biofilm reactor for treating wastewater with a low C/N ratio. Chemosphere 83, 63-68.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H., 2005. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Research, 39, 4797-4807.

Clarke, S.C., 2005. Pyrosequencing, nucleotide sequencing technology with bacterial genotyping applications. Expert Review of Molecular Diagnostics, 5, 947-953.

Cosenza, A., Di Bella, G., Mannina, G., Torregrossa, M., 2013. The role of EPS in fouling and foaming phenomena for a membrane bioreactor. Bioresource Technology, 147, 184-192.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial Biofilms. Annual Review of Microbiology 49, 711-745.

Costerton, J.W., 1999. Introduction to biofilm. International Journal of Antimicrobial Agents, 11, 217-221.

Cunha, A., Almeida, A., Coelho, F.J.R.C., Gomes, N. C. M., Oliveira, V., Santos, A.L., 2010. Bacterial Extracellular Enzymatic Activity in Globally Changing Aquatic Ecosystems. In, Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. In, Mendez-Vilas, A. (Ed.), Formatex Microbiology Series, vol. 1. Badajoz, Spain, pp. 124-135.

Curtis, T.P. and Sloan, W.T., 2006. Towards the design of diversity, stochastic models for community assembly in wastewater treatment plants. Water Science & Technology, 54, 227-236.

Daims, H., Taylor, M.W., Wagner, M., 2006. Wastewater treatment, a model system for microbial ecology. TRENDS in Biotechnology, 24, 483-489.

Davey, M.E., O'toole, G.A., 2000. Microbial Biofilms, from Ecology to Molecular Genetics. Microbiology and Molecular Biology Reviews, 64, 847-867.

Delrue, F., Stricker, A.E., Mietton-Peuchot, M., Racault, Y., 2011. Relationships between mixed liquor properties, operating conditions and fouling on two full-scale MBR plants. Desalination, 272, 9-19.

Di Trapani, D., Mannina, G., Torregrossa, M., Viviani., G., 2010. Comparison between hybrid moving bed biofilm reactor and activated sludge system, a pilot plant experiment. Water Science and Technology, 61, 891-902.

Donlan, R.M., 2002. Biofilms, Microbial Life on Surfaces. Emerging Infectious Diseases, 8, 881-890.

Drewsa, A., Leeb, C-H., Kraumea, M., 2006. Membrane fouling – a review on the role of EPS. Desalination 200, 186-188.

Drews, A., 2010. Membrane fouling in membrane bioreactors- characterization, contradictions, causes and cures. Journal of membrane Science, 363, 1-28.

Erijman, L., Figuerola, E.L.M., Guerrero, L.D., Ayarza, J.M., 2011. Impacto de los recientes avances en el análisis de comunidades microbianas sobre el control del proceso. Revista Argentina de Microbiología, 43, 127-135.

Fischer, A., 1900. The structure and functions of bacteria. Translated into english by A. Coppen Jones. Oxford at the Clarendon Press.

Flemming, H-C., 2009. Why Microorganisms Live in Biofilms and the Problem of Biofouling. Marine and Industrial Biofouling, 4, 3-12.

Flemming, H-C., Wingender, J., 2010. The biofilm matrix. Nature Reviews Microbiology, 8, 623-633.

Fraga, D., Meulia, T. and Fenster, S., 2008. Real-Time PCR. Current Protocols, Essential Laboratory Techniques, 1, 10.3.1-10.3.34.

Francis, C.A., Beman, J.M., Kuypers, M.M.M., 2007. New processes and players in the nitrogen cycle, the microbial ecology of anaerobic and archaeal ammonia oxidation. The ISME Journal, 1, 19-27.

Fu, B., Liao, X., Ding, L., Ren, H., 2010. Characterization of microbial community in an aerobic moving bed biofilm reactor applied for simultaneous nitrification and denitrification. World Journal of Microbiology and Biotechnology, 26, 1981-1990.

Futselaar, H., Rosink, R., Smith, G. and Koens, L., 2013. The anaerobic MBR for sustainable industrial wastewater management. Desalination and Water Treatment, 51, 4-6.

Gao, D-W., Fu, Y., Tao, Y., Wu, W-M., An, R. and Li, X-X., 2009. Current research and development of controlling membrane fouling of MBR. African Journal of Biotechnology, 8, 2993-2998.

Gernaey, K.V., van Loosdrecht, M.C.M., Henze, M., Lind, M., Jørgensen, S.B., 2004. Activated sludge wastewater treatment plant modelling and simulation, state of the art. Environmental Modelling & Software, 19, 763-783.

Gianfreda, L. and Rao, M.A., 2004. Potential of extra cellular enzymes in remediation of polluted soils, a review. Enzyme and Microbial Technology 35, 339-354.

Gilbride, K.A., Lee, D.Y., Beaudette, L.A., 2006. Molecular techniques in wastewater, Understanding microbial communities, detecting pathogens, and real-time process control. Journal of Microbiological Methods, 66, 1-20.

Giovannoni, S.J., and Stingl, U., 2005. Molecular diversity and ecology of microbial plankton. Nature, 437, 343-348.

Gómez-Silván, C., Arévalo, J., Pérez, J., González-López, J., Rodelas, B., 2013. Linking hydrolytic activities to variables influencing a submerged membrane bioreactor (MBR) treating urban wastewater under real operating conditions. Water Research, 47, 66-78.

Han, Y., Liu, J., Guo, X., Li, L., 2012. Micro-environment characteristics and microbial communities in activated sludge flocs of different particle size. Bioresource Technology, 124, 252-258.

Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J., Goodman, R.M., 1998. Molecular biological access to the chemistry of unknown soil microbes, a new frontier for natural products. Chemistry Biology, 5, 245-249.

Handelsman, J., 2004. Metagenomics, Application of Genomics to Uncultured Microorganisms. Microbiology and Molecular Biology Reviews, 68, 669-685.

Handelsman, J., 2007. Metagenomics and Microbial Communities. Encyclopedia of the Life Sciences. John Wiley and Sons Ltd., Chichester, UK.

Henze, M., van Loosdrecht, M.C.M., Ekama, G.A., 2008. Biological Wastewater Treatment, Principles, Modeling, and Design. IWA Publishing, London, United Kingdom.

Howarth, R.W., 2004. Human acceleration of the nitrogen cycle, drivers, consequences, and steps toward solutions. Water Science and Technology, 49, 7-13.

Ivanovic, I., Leiknes, T., 2008. Impact of aeration rates on particle colloidal fraction in the biofilm membrane bioreactor (BF-MBR). Desalination, 231, 182-190.

Ivanovic, I. and Leiknes, T., 2011. Membrane reactor design as a tool for better membrane performance in a biofilm MBR (BF-MBR). Desalination and Water Treatment, 25, 259-267.

Ivanovic, I. and Leiknes, T.O., 2012. The biofilm membrane bioreactor (BF-MBR)—a review. Desalination and Water Treatment, 37, 288-295.

Jang, N., Ren, X., Kim, G., Ahn, C., Cho, J., Kim, I.S., 2007. Characteristics of soluble microbial products and extracellular polymeric substances in the membrane bioreactor for water reuse. Desalination, 202, 90-98.

Jarvis, P., Jefferson, B., Gregory, J., Parsons, S.A., 2005. A review of floc strength and breakage. Water Research, 39, 3121-3137.

Jefferson, K.K., 2004. What drives bacteria to produce a biofilm?. FEMS Microbiology Letters, 236, 163-173.

Jetten, M.S.M., van Niftrik, L., Strous, M., Kartal, B., Keltjens, J.T., Op den Camp, H.J.M., 2009. Biochemistry and molecular biology of anammox bacteria. Critical Reviews in Biochemistry and Molecular Biology, 44, 65-84.

Johnson, L.R., 2008. Microcolony and biofilm formation as a survival strategy for bacteria. Journal of Theoretical Biology, 251, 24-34.

Jonasson, J., Olofsson, M. and Monstein, H.J., 2002. Classification, identification and subtyping of bacteria based on pyrosequencing and signature matching of 16S rDNA feagments. Acta Pathologica, Microbiologica et Inmunologica Scandinavica, 110, 263-272.

Judd, S., 2008. The status of membrane bioreactor technology. Trends in Biotechnology, 26, 109-116.

Judd, S.J. and Judd, C., 2010. The MBR Book (2nd ed.), Principles and applications of membrane bioreactors in water and wastewater treatment. Elsevier.

Kermani, M., Bina, B., Movahedian, H., Amin, M.M., Nikaein, M., 2008. Application of Moving Bed Biofilm Process for Biological Organics and Nutrients Removal from Municipal Wastewater. American Journal of Environmental Sciences, 4, 675-682.

Khan, S.J., Ilyas, S., Javid, S., Visvanathan, C., Jegatheesan, V., 2011. Performance of suspended and attached growth MBR systems in treatinghigh strength synthetic wastewater. Bioresource Technology, 102, 5331-5336.

Kim, T.S., Jeong, J.Y., Wells, G.F., Park, H.D., 2013. General and rare bacterial taxa demonstrating different temporal dynamic patterns in an activated sludge bioreactor. Applied Microbiology and Biotechnology, 97, 1755-1765.

Klotz MG, Alzerreca J, Norton JM. 1997. A gene encoding a membrane protein exists upstream of the amoA/amoB genes in ammonia oxidizing bacteria, a third member of the amo operon? FEMS Microbiology Letters, 150, 65-73.

Koops, H.P., Pommerening-Röser, A., 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiology Ecology, 37, 1-9.

Kowalchuk, G.A. and Stephen, J.R., 2001. AMMONIA-OXIDIZING BACTERIA, A Model for Molecular Microbial Ecology. Annual Review of Microbiology, 55, 485-529.

Kraemer, J.T., Menniti, A.L., Erdal, Z.K., Constantine, T.A., Johnson, B.R., Daigger, G.T., Crawford, G.V., 2012. A practitioner's perspective on the application and research needs of membrane bioreactors for municipal wastewater treatment. Bioresource Technology, 122, 2-10.

Kraft, B., Strous, M., Tegetmeyer, H.E., 2011. Microbial nitrate respiration – Genes, enzymes and environmental distribution. Journal of Biotechnology, 155, 104-117.

Kwon, S., Taek-Seung, K., Gi Hyeon, Y., Joon-Hong, J., Hee-Deung, P., 2010. Bacterial Community Composition and Diversity of a Full-Scale Integrated Fixed-Film Activated Sludge System as Investigated by Pyrosequencing. Journal of Microbiology and Biotechnology, 20, 1717-1723.

Lappin-Scott, H. M., 1999. Claude e. zobell-his life and contributions to biofilm microbiology. In 8th International Symposium on Microbial Ecology (p. 6).

Lazarova, V. and Manem, J., 1995. Biofilm characterization and activity analysis in water and wastewater treatment. Water Research, 29, 2227-2245.

Le-Clech, P., Chen, V., Fane, T.A.G., 2006. Fouling in membrane bioreactors used in wastewater treatment. Journal of Membrane Science, 284, 17-53.

Le-Clech, P., 2010. Membrane bioreactors and their uses in wastewater treatments. Applied Microbiology and Biotechnology, 88, 1253-60.

Lee, W-N., Kang, I-J., Lee, C-H., 2006. Factors affecting filtration characteristics in membranecoupled moving bed biofilm reactor. Water Research, 40, 1827-1835.

Leiknes, T. and Knudsen, E.C.S., 2000. Microfiltration of effluentproduced by a moving bed biofilm reactor by an immersed hollow fiber membrane module. Proceedings EuroMembrane, Jerusalem, Israel, September.

Leiknes, T. and Ødegaard, H., 2001. Moving bed biofilm membrane reactor (MBB-M-R), characteristics and potentials of a hybrid process design for compact wastewater treatment plants. Proceedings of Engineering with membranes, Vol.I, Granada, Spain, June 3-6.

Leiknes,T.O., Ødegaard, H., 2007. The development of a biofilm membrane bioreactor. Desalination, 202, 135-143.

Le-Minh, N., Coleman, H.M., Khan, S.J., van Luer, Y., Trang, T.T.T., Watkins, G., Stuetz, R.M., 2010a. The application of membrane bioreactors as decentralised systems for removal of endocrine disrupting chemicals and pharmaceuticals. Water Science and Technology, 61, 1081-1088.

Le-Minh, N., Khan, S.J., Drewes, J.E., Stuetz, R.M., 2010b. Fate of antibiotics during municipal water recycling treatment processes. Water Research, 44, 4295-4323.

Levstek, M., Platz, I., 2009. Influence of carrier type on nitrification in the movingbed biofilm process. Water Science and Technology, 59, 875-882.

Li, Y., Chróst, R.J., 2006. Microbial enzymatic activities in aerobic activated sludgemodel reactors. Enzyme and Microbial Technology, 39, 568-572.

Li, J., Li, Y., Ohandja, D-G.,Yang, F., Wong, F-S., Chuad, H-C., 2008. Impact of filamentous bacteria on properties of activated sludge and membrane-fouling rate in a submerged MBR. Separation and Purification Technology, 59, 238-243.

Liao, G-Q., Kraemer, J.T. and Bagley, D.M., 2006. Anaerobic Membrane Bioreactors, Applications and Research Directions. Critical Reviews in Environmental Science and Technology, 36, 489-530.

Liu, Y., Tay, J-H., 2002. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. Water Research, 36, 1653-1665.

Liu, Y. and Fang, H.H.P., 2003. Influences of Extracellular Polymeric Substances (EPS) on Flocculation, Settling, and Dewatering of Activated Sludge. Critical Reviews in Environmental Science and Technology, 33, 237-273.

Lücker, S., Wagner, M., Maixnera, F., Pelletierb, E., Kocha, H., Vacherie, B., Rattei, T., Damsté, J.S.S., Spieck, E., Paslierb, D.L., Daims, H., 2010. A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. Proceedings of the National Academy of Sciences, 107, 13479-13484.

Makowska, M., Spychała, M., and Mazur, R., 2013. Removal of Carbon and Nitrogen Compounds in Hybrid Bioreactors, Biomass Now - Cultivation and Utilization. Dr. Miodrag Darko Matovic (Ed.), ISBN, 978-953-51-1106-1, InTech, DOI, 10.5772/53582. Disponible en, http,//www.intechopen.com/books/biomass-now-cultivation-and-utilization/removal-of-carbon-and-nitrogen-compounds-in-hybrid-bioreactors.

Malaeb, L., Le-Clech, P., Vrouwenvelder, J.S., Ayoub, G.M., Saikaly, P.E., 2013. Do biological-based strategies hold promise to biofouling control in MBRs? Water Research 47, 5447- 5463.

Mannina, G. and Viviani, G., 2009. Hybrid moving bed biofilm reactors, an effective solution for upgrading a large wastewater treatment plant. Water Science & Technology, 60, 1103-1116.

Manser, R., Gujer, W., Siegrist, H., 2005. Consequences of mass transfer effects on the kinetics of nitrifiers. Water Research 39, 4633-4642.

Mardis, E.R., 2008. Next-Generation DNA Sequencing Methods. Annual Review of Genomics and Human Genetics, 9, 387-402.

Margulies, M., Egholm1, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F. and Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature, 437, 376-380.

Martín-Pascual, J., López-López, C., Cerdá, A., González-López, J., Hontoria, E., Poyatos, J.M., 2011. Comparative kinetic study of carrier type in a moving bed system applied to organic matter removal in urban wastewater Treatment. Water, Air and Soil Pollution, 223, 1699-1712.

Martínez-Espinosa, R.M., Cole, J.A., Richardson, D.J., Watmough, N.J., 2011. Enzymology and ecology of the nitrogen cycle. Biochemical Society Transactions, 39, 175-178.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints, practical tools for microbial ecology. Environmental Microbiology, 10, 1571-1581.

McSwain, B.S., Irvine, R.L., Hausner, M., Wilderer, P.A., 2005. Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge. Applied and Environmental Microbiology, 71, 1051-1057.

Meng, F., Zhang, H.,Yang, F., Li, Y., Xiao, J., Zhang, X., 2006. Effect of filamentous bacteria on membrane fouling in submerged membrane bioreactor. Journal of Membrane Science, 272, 161-168.

Meng, F., Chae, S.R., Drews, A., Karume, M., Shin, H.S., Yang, F., 2009. Recent advances in membrane bioreactors (MBRs), Membrane fouling and membrane material. Water Research, 43, 1489-1512.

Mizrahi-Man, O., Davenport, E.R., Gilad, Y., 2013. Taxonomic Classification of Bacterial 16S rRNA Genes Using Short Sequencing Reads, Evaluation of Effective Study Designs. PLOS ONE, 8(1), e53608.

Mohan, S. B., Schmid, M., Jetten, M. S. M., Cole, J., 2004. Detection and widespread distribution of the nrfA gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiology Ecology, 49, 433-443.

Molina-Muñoz, M., Poyatos, J.M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E., González-López, J., 2010. Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions. Bioresource Technology, 101, 696-704.

Morgenroth, E., Kommedal, R., Harremoës, P., 2002. Processes and modelling of hydrolysis of particulate organic matter in aerobic wastewater treatment—a review. Water Science and Technology, 45, 25-40.

Morozova, O. and Marra, M.A., 2008. Applications of next-generation sequencing technologies in functional genomics. Genomics, 92, 255-264.

Mullis, K.B., 1990. The unusual origin of polymerase chain reaction. Scientific American, 262, 56-65.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59, 695-700.

Muyzer, G. and Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek, 73, 127-141.

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology, 2, 317-322.

Nadell, C.D., Xavier, J.B., Foster, K.R., 2009. The sociobiology of biofilms. FEMS Microbiology Reviews, 33, 206-24.

Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. Journal of Biotechnology, 80, 1-33.

Ødegaard, H., Rusten, B. and Westrum, T., 1994. A new moving bed bofilm reactor-Applications and results. Water Science and Technology, 29, 157-165.

Ødegaard, H., 1999. The moving bed biofilm reactor. In Water Environmental Engineering and Reuse of Water, Edited by, Igarashi, T., Watanabe, Y., Asano, T. and Tambo, N., 250–305. Sapporo, Hokkaido University Press.

Ødegaard, H., 2006. Innovations in wastewater treatment, the moving bed biofilm process. Water Science and Technology, 53, 17-33.

Onnis-Hayden, A., Majed, N., Schramm, A., Gu, A.Z., 2011. Process optimization by decoupled control of key microbial populations, Distribution of activity and abundance of polyphosphate-accumulating organisms and nitrifying populations in a full-scale IFAS-EBPR plant. Water Research, 45, 3845-3854.

Pal, L., Kraigher, B., Brajer-Humar, B., Levstek, M., Mandic-Mulec, I., 2012. Total bacterial and ammonia-oxidizer community structure in moving bed biofilm reactors treating municipal wastewater and inorganic synthetic wastewater. Bioresource Technology, 110, 135-143.

Panikov, N. S., 1995. Microbial growth kinetics. Chapman & Hall, London, United Kingdom.

Pauwels, B., Ngwa, F.F., Deconinck, S., Verstraete, W., 2006. Effluent Quality of a Conventional Activated Sludge and a Membrane Bioreactor System Treating Hospital Wastewater. Environmental Technology, 27, 395-402.

Percival, S.L., Malic, S., Cruz, H., Williams, D.W., 2011. Introduction to Biofilms. Biofilms and Veterinary Medicine, 6, 41-68.

Pester, M., Schleper, C., Wagner, M., 2011. The Thaumarchaeota, an emerging view of their phylogeny and ecophysiology. Current Opinion in Microbiology, 14, 300-306.

Phattaranawik, J. and Leiknes, T., 2009. Double-Deck Aerated Biofilm Membrane Bioreactor with Sludge Control for Municipal Wastewater Treatment. AIChE Journal, 55, 1291-1297.

Phillips, P.L., Wolcott, R.D., Fletcher, J., Schultz, G.S., 2011. Biofilms Made Easy. Wounds International, 1, 1-6.

Philippot, L., 2002. Denitrifying genes in bacterial and archaeal genomes. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1577, 355-376.

Plattes, M., Henry, E., Schosseler, P.M., Weidenhaupt, A., 2006. Modelling and dynamic simulation of a moving bed bioreactor for the treatment of municipal wastewater. Biochemical Engineering Journal, 32, 61-68.

Qiqi, Y., Qiang, H. and Ibrahim, H.T., 2012. Review on Moving Bed Biofilm Processes. Pakistan Journal of Nutrition, 11, 804-811.

Radjenović, J., Matošić, M., Mijatović, I., Petrović, M., Barceló, D., 2008. Membrane Bioreactor (MBR) as an AdvancedWastewater Treatment Technology. The Handbook of Environmental Chemistry. Berlin-Heidelberg, Vol. 5, Part S/2, pp. 37-101.

Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Research, 43, 831-841.

Rahimi, Y., Torabiana, A., Mehrdadia, N., Habibi-Rezaieb, M., Pezeshkc, H., Nabi-Bidhendi, G-R., 2011. Optimizing aeration rates for minimizing membrane fouling and its effect on sludge characteristics in a moving bed membrane bioreactor. Journal of Hazardous Materials, 186, 1097-1102.

Raszka, A., Chorvatova, M., Wanner, J., 2006. The role and significance of extracellular polymers in activated sludge. Part I, Literature review. Acta hydrochimica et hydrobiologica, 34, 411-424.

Rodgers, M. and Zhan, X.M, 2003. Moving-medium biofilm reactors. Reviews in Environmental Science and Bio/Technology, 2, 213-224.

Rodgers, M., Lambe, A., Xiao, L., 2006. Carbon and nitrogen removal using a novel horizontal flow biofilm system. Process Biochemistry, 41, 2270-2275.

Ronaghi, M., Karamohamed, S., Pettersson, B., Uhlen, M. and Nyren, P., 1996. Real-time DNA sequencing using detection of pyrophosphate release. Analytical Biochemistry, 242, 84-89.

Ronaghi, M., Uhlen, M. and Nyren, P., 1998. A sequencing method based on real-time pyrophosphate. Science, 281, 363-365.

Ronaghi, M. and Elahi, E., 2002. Pyrosequencing for microbial typing. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences, 782, 67-72.

Rosenberger, S., Kruger, R., Manz, W., Szewzyk, U. and Kraume, M., 2002. Performance of a bioreactor with submerged membranes for aerobic treatment of municipal waste water. Water Research, 36, 413-420.

Rosso, D., Lothman, S.E., Jeung, M.K.,Pitt, P., Gellner, W.J., Stone, A.L., Howard, D., 2011. Oxygen transfer and uptake, nutrient removal, and energy footprint of parallel full-scale IFAS and activated sludge processes. Water Research, 45, 5987-5996.

Rütting, T., Boeckx, P., Müller, C., Klemedtsson, L., 2011. Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. Biogeosciences, 8, 1779-1791.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., 1988. Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science, 239, 487-491.

Salehizadeh, H., Shojaosadati, S.A., 2001. Extracellular biopolymeric flocculants recent trends and biotechnological importance. Biotechnology Advances, 19, 371-385.

Sanger, F., Nicklen, S. and Coulson, A.R., 1977. DNA sequencing with chain terminating inhibitors. Proceeding of the National Academy of Science of the United States of America, 74, 5463-5467.

Sanz, J.L., Köchling, T., 2007. Molecular biology techniques used in wastewater treatment, An overview. Process Biochemistry, 42, 119-133.

Schäfer, A.I., Fane, A.G., Waite, T.D., 2000. Fouling effects on rejection in the membrane filtration of natural waters. Desalination, 131, 215-224.

Schramm, A., Santegoeds, C.M., Nielsen, H.K., Ploug, H., Wagner, M., Pribyl, M., Wanner, J., Amann, R., de Beer, D., 1999. On the Occurrence of Anoxic Microniches, Denitrification, and Sulfate Reduction in Aerated Activated Sludge. Applied and Environmental Microbiology, 65, 4189-4196.

Sheng, G-P., Yu, H-Q., Li, X-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems, A review. Biotechnology Advances, 28, 882-894.

Shokralla, S., Spall, J.L., Gibson, J.F. and Hajibabaei, M., 2012. Next-generation sequencing Technologies for environmental DNA research. Molecular Ecology, 21, 1794-1805.

Singh, R., Paul, D., Jain, R.K., 2006. Biofilms, implications in bioremediation. TRENDS in Microbiology, 14, 389-397.

Smith, C.J. and Osborn, A.M., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiology Ecology, 67, 6-20.

Sponza, D.T., 2002. Extracellular polymer substances and physicochemical properties of flocs in steady- and unsteady-state activated sludge systems. Process Biochemistry, 37, 983-998.

Stewart, E.J., 2012. Growing Unculturable Bacteria. Journal of Bacteriology, 194, 4151-4160.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W., 2002. Biofilms as complex differentiated communities. Annual Review of Microbiology, 56, 187-209.

Strange, P., 2010. Earth closet and Great Stinks, The science of Victorian sewage. Disponible en, http,//www.lablit.com/article/613.

Sutherland, I.W., 2001. The biofilm matrix – an immobilized but dynamic microbial environment. TRENDS in Microbiology, 9, 222-227.

Swan, B.K., Chaffin, M.D., Martinez-Garcia, M., Morrison, G.M., Field, E.K., Poulton, N.J., Masland, E.D.P., Harris, C.C., Sczyrba, A., Chain, P.S.G., Koren, S., Woyke, T., Stepanauskas, R. 2014. Genomic and Metabolic Diversity of Marine Group I Thaumarchaeota in the Mesopelagic of Two Subtropical Gyres. PLoS ONE, 9 (4), e95380.

Thomas, T., Gilbert, J. and Meyer, F., 2012. Metagenomics - a guide from sampling to data analysis. Microbial Informatics and Experimentation, 2, 1-12.

Tiyasha, Shaktibala and Suraj Kr. Bhagat, 2013. Photon-Filtration, A New Approach of Waste Water Treatment. International Journal of Engineering and Innovative Technology, 3, 447-453.

Tringe, S.G. and Hugenholtz, P., 2008. A renaissance for the pioneering 16S rRNA gene. Current Opinion in Microbiology, 11, 442-446.

Valentín-Vargas, A., Toro-Labrador, G., Massol-Deyá, A.A., 2012. Bacterial Community Dynamics in Full-Scale Activated Sludge Bioreactors, Operational and Ecological Factors Driving Community Assembly and Performance. PLoS ONE 7(8), 12.

Valones, M.A.A., Guimarães, R.L., Cavalcanti Brandão1, L.A., de Souza, P.R.E., Carvalho, A.D.A.T., Crovela, S., 2009. Principles and applications of polymerase chain reaction in medical diagnostic fields, a review. Brazilian Journal of Microbiology, 40, 1-11.

Větrovský, T., Baldrian, P., 2013. The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses. PLoS ONE, 8(2), e57923.

Visvanathan, C. and Abeynayaka, A., 2012. Developments and future potentials of anaerobic membrane bioreactors (AnMBRs). Membrane Water Treatment, 3, 1-23.

Vlaeminck, S.E., Hay, A.G., Maignien, L., Verstraete, W., 2011. In quest of the nitrogen oxidizing prokaryotes of the early Earth. Environmental Microbiology, 13, 283-295.

Wagner, M., Loy, A., Nogueira, R., Purkhold, U., Lee, N. and Daims, H., 2002. Microbial community composition and function in wastewater treatment plants. Antonie van Leeuwenhoek, 81**,** 665-680.

Wang, R.C., Wen, X.H., Qian, Y., 2005. Influence of carrier concentration on the performance and microbial characteristics of a suspended carrier biofilm reactor. Process Biochemistry, 40, 2992-3001.

Wang, Z., Wu, Z., Tang, S., 2009. Extracellular polymeric substances (EPS) properties and their effects on membrane fouling in a submerged membrane bioreactor. Water Research 43, 2504-2512.

Wang, Z., Wang, P., Wang, Q., Wu, Z., Zhou, Q.,Yang, D., 2010. Effective control of membrane fouling by filamentous bacteria in a submerged membrane bioreactor. Chemical Engineering Journal, 158, 608-615.

Wang, Z., Han, X., Ma, J., Wang, P., Mei, X. and Wu, Z., 2013. Recent advances in membrane fouling caused by extracellular polymeric substances, a mini-review. Desalination and Water Treatment, 51, 5121-5131.

Weber, S.D., Ludwig, W., Schleifer, K.H., Fried, J., 2007. Microbial Composition and Structure of Aerobic Granular Sewage Biofilms. Applied and Environmental Microbiology, 73, 6233-6240.

Wittebolle, L., Boon, N., Vanparys, B., Heylen, K., De Vos, P. and Verstraete, W., 2005. Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. Journal of Applied Microbiology, 99, 997-1006.

Woese, C.R., 1987. Bacterial evolution. Microbiology and Molecular Biology Reviews, 51, 221-271.

Xia, S., Duan, L., Song, Y., Li, J., Piceno, Y.M., Andersen, G.L., Alvarez-Cohen, L., Moreno-Andrade, I., Huang, C.L., Hermanowicz, S.W., 2010. Bacterial Community Structure in Geographically Distributed Biological Wastewater Treatment Reactors. Environmental Science and Technology, 44, 7391-7396.

Yang, S.,Yang, F., Fu, Z., Lei, R., 2009. Comparison between a moving bed membrane bioreactor and a conventional membrane bioreactor on organic carbon and nitrogen removal. Bioresource Technology, 100, 2369-2374.

Ye, L., and Zhang, T., 2013. Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. Applied Microbiology and Biotechnology, 97, 2681-2690.

Ying, W., Yang, F., Bick, A., Oron, G., Herzberg, M., 2010. Extracellular Polymeric Substances (EPS) in a Hybrid Growth Membrane Bioreactor (HG-MBR), Viscoelastic and Adherence Characteristics. Environmental Science and Technology, 44, 8636-8643.

Yu, T., Lei, Z., De-Zhi, S., 2006. Functions and behaviors of activated sludge extracellular polymeric substances (EPS), a promising environmental interest. Journal of Environmental Sciences, 8, 420-427.

Yu, G-H., He, P., Shao, L., Lee, D., 2008a. Extracellular enzymes in sludge flocs collected at 14 full-scale wastewater treatment plants. Journal of Chemical Technology and Biotechnology, 83, 1717-1725.

Yu, G-H., He, P-J., Shao, L-M., He, P-P., 2008b. Stratification Structure of Sludge Flocs with Implications to Dewaterability. Environmental Science and Technology, 42, 7944-7949.

Zhang, J. and Cao, Z., 2010. Effect of Filamentous bacteria on the Operation of membrane Bioreactor (MBR). 2nd Conference on Environmental Science and information Application Technology, 380-383.

Zhang, T. and Fang, H.H.P., 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Applied Microbiology and Biotechnology, 70, 281-289.

Zhang, T., Shao, M. F., Ye, L., 2012. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME journal, 6, 1137-1147.

II. OBJETIVOS

El agua dulce es un recurso natural de vital importancia para los seres vivos, y necesario para el desarrollo económico. Es por ello, que la escasez de los recursos hídricos y la contaminación de los mismos, constituyen dos de los principales problemas medioambientales del siglo XXI. Ante esta problemática, la legislación ambiental es cada vez más estricta en referencia a los contaminantes orgánicos y la concentración de nutrientes (N y P) en los efluentes tratados de las aguas residuales, ya que estos, constituyen una importante amenaza para la calidad de este recurso natural. En consecuencia, el desarrollo y estudio de nuevos sistemas de depuración biológica para mejorar la calidad de los efluentes, es una necesidad real y creciente, no sólo para permitir de nuevo su uso, sino también para proteger la salud pública y el medioambiente.

En este contexto, y como **objetivo global de esta investigación**, se planteó estudiar las actividades enzimáticas y la dinámica de las comunidades microbianas implicadas en el proceso de depuración de un biorreactor de membrana con lecho móvil (MBMBR, *Moving Bed Membrane Bioreactor*), construido a nivel de planta piloto para el tratamiento de aguas residuales urbanas.

Para alcanzar este objetivo global, se marcaron los siguientes **objetivos específicos**:

1. Estudio de la influencia de las condiciones de operación sobre las actividades enzimáticas (fosfatasa ácida, fosfatasa alcalina y α-glucosidasa) de la biomasa suspendida y la biopelícula adherida del MBMBR.

2. Estudio de la comunidad bacteriana desarrollada en la biomasa suspendida y la biopelícula adherida del MBMBR, mediante técnicas de TGGE y pirosecuenciación 454. Influencia de los parámetros operacionales.

3. Estudio cuantitativo de las bacterias nitrificantes (AOB y NOB) y desnitrificantes desarrolladas en la biomasa suspendida y la biopelícula adherida del MBMBR, mediante qPCR. Influencia de los parámetros operacionales.

Estos objetivos han sido desarrollados en el ámbito del proyecto de investigación denominado: *Procesos Microbianos en Biorreactores de Membrana con Lecho Fluidificado en Tratamiento de Aguas Residuales,* con referencia CTM2009-11929-C02-02.

Ecological Engineering 61 (2013) 23–33

Contents lists available at ScienceDirect

Ecological Engineering

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

Enzymatic activities in a moving bed membrane bioreactor for real urban wastewater treatment: Effect of operational conditions

P. Reboleiro-Rivas[a,c,*], J. Martín-Pascual[b,c], B. Juárez-Jiménez[a,c], J.M. Poyatos[b,c], E. Hontoria[b,c], B. Rodelas[a,c], J. González-López[a,c]

[a] Department of Microbiology, Faculty of Pharmacy, University of Granada, Granada, Spain
[b] Department of Civil Engineering, University of Granada, Granada, Spain
[c] Institute of Water Research, University of Granada, Granada, Spain

III. CHAPTER 1

Enzymatic activities in a Moving Bed Membrane Bioreactor for real urban wastewater treatment: effect of operational conditions

1. Introduction

The population in developed countries is constantly growing and consequently, the production of wastewater has become a huge environmental problem. Similarly, environmental legislation is becoming increasingly stringent concerning organic pollutant and nutrient (C, N and P) concentrations in wastewater effluent (Onnis-Hayden *et al.*, 2011; Pal *et al.*, 2012). In recent decades, to obtain a better quality of effluent, wastewater treatments based on conventional activated sludge have been improved by the development of new technologies.

Among these technologies, Membrane Bioreactor (MBR) systems combine a conventional biological treatment of activated sludge and membrane filtration technology (Chang *et al.*, 2011; Yang *et al.*, 2009). Membrane filtration replaces the clarification process and confers several advantages to the system compared to Conventional Activated Sludge Processes (CASPs), (Calderón *et al.*, 2012a; Molina-Muñoz *et al.*, 2010; Yang *et al.*, 2009). Alternatively, wastewater treatments based on biofilm processes, such as Moving Bed Biofilm Reactors (MBBRs), are emerging due to their efficiency in the removal of organic carbon, ammonium, nitrates, and nitrites (Di Trapani *et al.*, 2010; Ødegaard, 2006). MBBRs are based on the addition of carriers to the activated sludge system. The carriers move freely in the bioreactor and are gradually colonised by the biomass. Thus, suspended and attached growth processes occur together (Chu *et al.*, 2011; Martín-Pascual *et al.*, 2011; Pal *et al.*, 2012; Wang *et al.*, 2005). To improve the efficiency of MBRs and MBBRs systems further, a combination of biofilm processes and membrane filtration technology (MBMBR system) is a viable alternative, as reported by various authors in recent years (Ivanovik *and* Leiknes, 2008; Leiknes *and* Ødegaard, 2007; Ødegaard, 2006).

Microbial communities in a MBMBR system develop in different types of aggregates, such as suspended flocs or attached biofilms. The immobilisation of microorganisms promotes the development of slow-growing microorganisms, such as nitrifying bacteria, and can support an appropriate environment for both aerobic and anoxic microorganisms (Watanabe *et al*., 1992). Therefore, differences in the biological activity of the mixed liquor and the biofilm can occur. Consequently, in a heterogeneous biological system such as a MBMBR, the process of biodegradation might be enhanced, or at least altered.

In nature, the biodegradation process begins with the hydrolysis of macromolecules performed by extracellular microbial enzymes. This initial process is considered as the main rate-limiting step in organic matter degradation, since an important fraction of organic matter present in the influent must be hydrolysed by enzymes before it can be utilised by bacterial metabolism (Anupama *et al*., 2008; Chróst *and* Siuda, 2006; Guellil *et al*., 2001; Morgenroth *et al*., 2002). Furthermore, as reported Burgees *and* Pletschke (2008), the enzymes immobilised on extracellular polymeric substances (EPS), flocs or carriers are more stable. In this context, the first step to achieve a better characterisation of the biodegradation process involved in a MBMBR system is to measure biomass concentration and enzymatic activities in both suspended biomass (SB) and attached biofilm (AB).

The enzymatic activities of α-glucosidase, acid phosphatase and alkaline phosphatase have an essential role in biological wastewater treatment, since carbohydrates comprise a large fraction of the organic matter and an important proportion of the total phosphorous is present in organic form. The study of these enzyme activities not only provides knowledge concerning the hydrolysis process, but

can also help to optimise operational conditions to achieve a better biodegradation of sludge (Anupama *et al*., 2008; Li *and* Chróst, 2006; Molina-Muñoz *et al*., 2010).

In recent years, the effect of different operational parameters, including carrier filling ratio (CFR), on the efficiency of organic pollutant and nutrient removal in MBBRs and MBMBRs has been studied (Di Trapani *et al*., 2010; Hem *et al*., 1994; Martín-Pascual *et al*., 2011; Ødegaard, 2006; Wang *et al*., 2005; Yang *et al*., 2009). However, little attention has focussed on the analysis of biological activity and species composition variation in MBBRs (Pal *et al*., 2012) and, to our knowledge, attempts to link enzymatic activity variations to changes of the different operational parameters have not been performed in a MBMBR system.

For these reasons, we investigated the evolution of α-glucosidase, acid phosphatase and alkaline phosphatase activities in both SB and AB in a MBMBR system with a 20% and 35% (v/v) CFR under four different operational conditions. Furthermore, the AB contribution to the total enzymatic activities in the MBMBR was calculated. Finally, in order to understand the influence of physico-chemical parameters and CFR on the enzymatic activities, a multivariate statistical analysis was performed.

2. Materials and Methods

2.1 Experimental set-up

2.1.1 Pilot-scale experimental plant

To carry out this research, a pilot-scale experimental plant was configured as shown in **Fig. III.1.** This consisted of one aerobic MBMBR system composed of two bioreactors: a moving bed bioreactor (MBBR) and a membrane bioreactor (MBR),

where the biodegradation and clarification process, take place, respectively. The MBBR system consisted of a cylindrical tank with an operating volume of 358 L, where the carriers moved freely by aeration, whereas the MBR was composed of three ultrafiltration membrane modules of hollow fibre (Zenon®). The membranes were submerged in a sludge volume of 87 L under continuous aeration. Recirculation was performed, to maintain the same concentration of total suspended solids in each reactor. Finally, the effluent was collected in a back-washing tank.

To approach real conditions as closely as possible, the MBMBR system was located in the municipal WWTP "Puente de los Vados" (Emasagra S.A.,Granada, Spain). Specifically, the influent was pumped from the primary settler to the MBBR. The mean composition of wastewater was determined by standard methods (APHA, 2005). A control device was available to monitor the temperature in the MBBR.

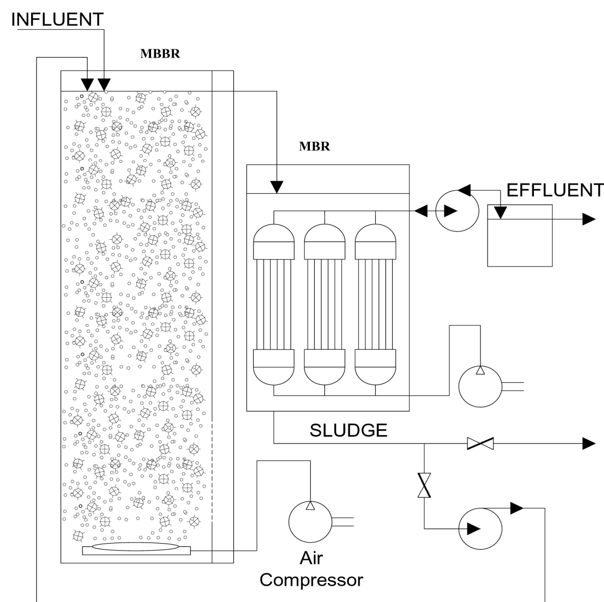


Figure III.1. Diagram of the Moving Bed Membrane Biofilm Reactor (MBMBR) used in this study.

The carrier K1, developed by AnoxKaldness (Norway), was used as a support material. Its use has been widely reported for different types of wastewater and appears to be one of the most commonly used in MBBRs (Ivanovic *and* Leiknes, 2008; Levstek *and* Plazl 2009; Martín-Pascual *et al*., 2011; Ødegaard *et al*., 2006; Pal *et al*., 2012). The K1 carrier is a small cylinder of high-density polyethylene, with a cross inside and "fins" in the external surface and an effective surface area of 800 m^2/m^3 (Ødegaard *et al*., 2006).

To achieve a good diffusion of substrates through the biofilm as reported by Levsket *and* Plazl (2009), two CFRs of K1 (20% or 35%, v/v) were used, and 3.8% or 6.65% of mixed liquor volume was displaced from the moving bed bioreactor, respectively. In agreement with Martín-Pascual *et al*. (2011), the units of carriers (K1) per m^3 were 1,030,000.

2.1.3 Operating conditions

To perform a comparative study between a 20% and 35% (v/v) CFR, eight experimental phases (designated as experiments 1 to 8), were conducted in the MBMBR system. For this, four operational conditions with 20% (v/v) CFR (experiments 1, 2, 3 and 4) or 35% (v/v) CFR (experiments 5, 6, 7 and 8) were performed. Two different concentrations of Mixed Liquor Total Suspended Solids (MLTSS) and two Hydraulic Retention Times (HRTs) were combined: 10 h (experiments 1, 3, 6 and 7) or 24 h (experiments 2, 4, 5 and 8). Experiments with 20% (v/v) CFR were conducted in 2011, whereas those with 35% (v/v) CFR were conducted in 2012 (**Table III.1**).

Table III.1. Experimental design and operational conditions of the eight experiments conducted in the MBMBR system. CFR: Carrier Filling Ratio; HRT: Hydraulic Retention Time; MLTSS: Mixed Liquor Total Suspended Solids.

	Experiment	Sampling period (steady operation)			HRT (h)	MLTSS (mg/L)
		Starting date	Ending date	Days		
CFR 20%	1	23 March 2011	13 April 2011	22	10	ca. 2,500
	2	6 June 2011	26 June 2011	22	24	ca. 2,500
	3	21 October 2011	9 November 2011	20	10	ca. 4,500
	4	19 November 2011	6 December 2011	18	24	ca. 4,500
CFR 35%	5	1 April 2012	20 April 2012	20	24	ca. 2,500
	6	7 May 2012	26 May 2012	20	10	ca. 2,500
	7	16 July 2012	4 August 2012	20	10	ca. 4,500
	8	10 September 2012	1 October 2012	22	24	ca. 4,500

To evaluate the influence of the attached biofilm on the enzymatic activities, the MBMBR system was continuously operated throughout the study with either 20% or 35% (v/v) CFR. Furthermore, the experimental phases were conducted from lower to higher MLTSS concentrations (ca. 2,500 mg/L to ca. 4,500 mg/L). Inflow rates of 45.5 and 18.96 L/h were used to maintain the same biomass concentration at a HRT of 10 h and 24 h, respectively. In addition, different purges of 35, 18, 20, 8, 24, 52, 25 and 8 L/day were required after stabilisation of the system in experiments 1 to 8, respectively. After completing each experimental phase, the membranes were cleaned with sodium hypochlorite (1 g/L) as previously reported by Poyatos *et al*. (2010).

To compare the nutrient biotransformation rates of the MBMBR system under different operational conditions, samples were collected during the steady period of each experiment. According to Calderón *et al*. (2012a), the stability of the MBMBR system was considered when the MLTSS concentration was constant.

2.2 Biofilm recovery

The evolution of microbial enzymatic activities in attached biofilm as well as Biofilm Total Solids (BTS) and Biofilm Volatile Solids (BVS) were studied by removing the biofilm from the supporting material, according to the methods described by Martín-Pascual *et al*. (2011). Fifty units of carriers with adhered biofilm were collected in sterile conditions, using a sieve-sampling device as recommended by Calderón *et al*. (2012b), from different areas of the MBMBR and were placed in flasks with 50 mL sterile saline water (0.9% NaCl). These carrier samples were vortexed for 1 min, sonicated for 3 min, and the resultant biofilm suspensions were collected by centrifuging for 5 min at 3000 *g*. This process was repeated twice. The biofilm pellet obtained after the last centrifugation was resuspended in 50 ml of sterile saline water (0.9% NaCl).

2.3 Physico-Chemical analysis

Influent and effluent water samples were obtained every 24 h for analytical studies. Biological Oxygen Demand at 5 days (BOD_5), Chemical Oxygen Demand (COD), MLTSS, Mixed Liquor Volatile Suspended Solids (MLVSS), BTS and BVS, were determined according to Standard Methods for the Examination of Waste and Wastewater (APHA, 2005). The pH was determined using a Crison pH 25 pH meter (Crison instruments S.A., Barcelona, Spain). The BTS and BVS concentrations in the MBBR were calculated according to Plattes *et al*. (2006).

2.4 Evolution of microbial enzymatic activities

The activities of α-glucosidase, acid phosphatase and alkaline phosphatase of both suspended biomass (SB) and attached biofilm (AB) were analysed twice a week, so six samplings were performed for each experiment. For the enzymatic activity assays of SB, a volume of mixed liquor (c.a. 300 mL) was collected across different areas of the MBMBR in a sterile flask and stored at 4°C until transferred to the laboratory. The enzymatic activity assays of AB were performed on the biofilm suspensions retrieved from carrier samples as described in section 2.2.

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 phosphatase (acetate-acetic, pH 4.8, or carbonate-bicarbonate, pH 9.6, respectively). α-glucosidase was measured by a colorimetric method (Goel *et al.*, 1998), with Tris-HCl buffer (pH 7.6) and 1% *p*-nitrophenyl α-D-glucopyranoside as a substrate for the reaction. All the enzymatic activity assays were performed in 15-ml screw-cap tubes and conducted in triplicate (3 samples and 1 blank). A volume of 2 ml of buffer and 1 ml of substrate (*p*-nitrophenyl phosphate or *p*-nitrophenyl α-D-glucopyranoside) were added to 1 ml of SB or AB. The tubes were incubated at 37°C. Different incubation times were used for phosphatases and α-glucosidase determination (30 min and 60 min, respectively). After incubation, acid and alkaline phosphatase activities were stopped by adding 2 ml of 0.2 M NaOH. α-Glucosidase activity was stopped by heating the tubes in boiling water bath for 5 min. Finally, the samples were centrifuged for 15 min at 3000 *g* and the supernatant absorbance was measured at 410 nm using a Hitachi U-2900 spectrophotometer (Hitachi High Technologies Europe, Krefeld, Germany). For both

phosphatases and α-glucosidase, standard curves were performed using known concentrations of *p*-nitrophenol. All chemicals required for the enzymatic activity analyses were provided by Sigma-Aldrich (St. Louis, MO, USA).

All the enzymatic activities were measured separately in SB and AB fractions, and were expressed per L of mixed liquor (Li *and* Chróst, 2006) or per L of carriers (Plattes *et al*., 2006), respectively. In order to evaluate the % of contribution of each specific fraction of the biomass (SB or AB) to the total enzymatic activities per L of the MBMBR system, the effective mixed liquor volumes in the biorreactor were calculated by subtracting the amount displaced by the carrier media (3.8% for a CFR of 20%, 6.65% for a CFR of 35%). The hydrolytic activity of AB in 1L of the MBMBR was calculated taking into account the number of carrier units per L of bioreactor (1,030 carriers) and the CFR (20 or 35%, v/v) according to the formula described by Plattes *et al*. (2006) :

Hydrolytic activity of AB/ L of bioreactor = (Hydrolytic activity/carrier) × (1,030 carriers/L of carriers) × (0.2 or 0.35 L of carriers/L of bioreactor)

2.5 Statistical analysis

Analysis of variance (ANOVA) was performed using the software package STATGRAPHICS 5.0 (STSC, Rockville, MD, USA). A 95% significance level ($p < 0.05$) was used for data analysis.

Multivariate statistical analysis was performed using CANOCO for windows v.4.5 software (ScientiaPro, Budapest, Hungary). To select the correct ordination

method (unimodal or linear), a preliminary detrended correspondence analysis (DCA) was performed. DCA revealed a linear, rather than a unimodal response of the enzymatic activities, thus a redundancy analysis (RDA) as a linear constrained ordination method was chosen for data analysis (Leps *and* Smilauer, 1999).

RDA was performed to search for patterns in the set of species data (enzymatic activities) and to assess their relationship with the environmental data (system variables). The Monte Carlo permutation test option was used to obtain the statistical significance of each operational parameter in the canonical axes. With the aim of knowing whether the enzymatic activities from suspended biomass and attached biofilm were affected by the same or different system variables, RDA was performed separately for each sample type. The following environmental variables were used for the RDA: HRTs (10h or 24h), CFR (20% or 35%, v/v), pH, Temperature, COD and BOD_5. MLTSS and MLVSS were used in the RDA analysis for the enzymatic activities of suspended biomass. Similarly, BTS and BVS were used in RDA analysis for the enzymatic activities of attached biofilm. Among these variables, MLTSS and BVS were removed from the final analyses due to their strong linear correlation with MLVSS and BTS, respectively. In addition, HRT and CFR were expressed as nominal variables. All the non-nominal environmental variables were transformed to log (x+1), except pH.

3. Results and Discussion

3.1 Operational parameters

Table III.2 (**A**) shows the mean values of MLTSS, MLVSS, BTS, BVS, temperature and pH measured in the bioreactor at different HRTs (10 or 24h) and CFRs (20% or 35%, v/v).

ANOVA showed that the mean temperature and concentrations of solids in the biofilm (BTS and BVS) varied significantly among the four experiments performed with a 20% (v/v) CFR. However, among the experiments performed with a 35% (v/v) CFR (experiment 5, 6, 7 and 8), no significant differences in BTS were observed between experiments 6 and 7. In our study, the highest concentrations of BTS and BVS were detected in experiment 4, where the MLTSS and HRT were 4,431 mg/L and 24 h, respectively (**Table III.2**). In addition, it is noticeable that the biofilm solid concentration (BTS and BVS) in the experiments with 20% (v/v) CFR increased over time. In contrast, during the last experiment performed with a 35% (v/v) CFR (experiment 8), a decrease in biofilm solid (BTS and BVS) was observed.

In MBBR systems, it has been reported that the growth of attached biofilms is an active process, in which attachment and detachment occur at the same time, and at a steady state, biofilm growth occurs when both processes reach a balance (Plattes *et al*., 2006; Wang *et al*., 2005). Consequently, changes in operational conditions, for instance, CFR, might disturb this balance. In this context, Di Trapani *et al*. (2010) operating at a 30% (v/v) CFR, reported a decrease in the attached biofilm concentration in connection with an increased rate of detachment. Furthermore, Wang *et al*. (2005) reported that at higher CFR (> 30%), collision and abrasion among the carriers was more severe. Therefore, it might be that the detachment processes observed in experiment 8 relate to the CFR in the bioreactor.

Table III.2. (**A**) Operational parameters: Hydraulic Retention Time (HRT), Mixed Liquor Total Suspended Solid and Mixed Liquor Volatile Suspended Solid (MLTSS and MLVSS, respectively), Biofilm Total Solid and Biofilm Volatile Solid (BTS and BVS, respectively), Temperature (T) and pH of the eight experiments conducted in a MBMBR system with a 20% (v/v) or 35% (v/v) CFR. (**B**) Organic matter: Biological Oxygen Demand at 5 days (BOD_5) and Chemical Oxygen Demand (COD), in the influent and effluent, and BOD_5 and COD removal rates (%) in the eight experiments conducted in the MBMBR system with a 20% (v/v) or 35% (v/v) FRC. Experiments 1, 2, 3 and 4 were conducted with a 20% (v/v) CFR (CFR 20%) and experiments 5, 6, 7 and 8 with a 35% (v/v) CFR (CFR 35%). Values shown are means ± standard deviation. LSD: least significant difference (Student´s test, $p < 0.05$). Data followed by the same lower case-letter do not significantly differ among experiments with the same CFR.

A

	Exp	HRT (h)	MLTSS (mg/L)	MLVSS (mg/L)	BTS (mg/L)	BVS (mg/L)	T °C	pH
CFR 20%	1	10	2275±107.09[a]	1836±163.78[a]	2679±257.92[a]	2242±363.68[a]	17.8±1.6[c]	7.33±0.12[b]
	2	24	2403±158.07[a]	1960±173.32[a]	4229±323.2[b]	3642±372.59[b]	26.4±1.05[d]	7±0.08[a]
	3	10	4308±297.68[b]	3556±336.43[b]	5211±370.58[c]	4600±482.36[c]	13.77±1.91[b]	7.3±0.17[b]
	4	24	4431±359.46[b]	3635±346.33[b]	7273±432[d]	6360±430.58[d]	10.62±2.45[a]	6.9±0.35[a]
	LSD		303.66	324.28	423.64	499.9	2.2	0.26
CFR 35%	5	24	2769±81.26[a]	2332±98.73[a]	4359±203.93[a]	3633±183.48[a]	13.3±2.42[a]	7.31±0.1[b]
	6	10	2649±106,33[a]	2138±126.6[a]	5262±278.39[c]	4448±559.65[b]	20.3±3.04[b]	7.35±0.07[b]
	7	10	4346±198.29[b]	3735±148.94[b]	5316±216.67[c]	4866±208[c]	28.2±1.5[c]	7.03±0.19[a]
	8	24	4505±110.96[b]	3921±253.01[b]	4660±155.03[b]	3988±302.16[a]	23.4±2.18[d]	7.03±0.1[a]
	LSD		158.8	201.51	262.53	417.83	2.84	0.15

B

	Exp	BOD_5 influent (mg/L)	BOD_5 effluent (mg/L)	COD influent (mg/L)	COD effluent (mg/L)	BOD_5 removal (%)	COD removal (%)
CFR 20%	1	396.66±53.91[b]	21.33±6.88[c]	529.18±71.14[b]	68.05±10.61[d]	94.6±2.02[a]	87.01±2.3[a]
	2	350±86.48[ab]	8.7±3.01[ab]	463.12±60.91[ab]	35.22±9.89[c]	97.44±0.99[bc]	92.36±2.24[b]
	3	316.66±65.62[ab]	9.5±8.87[b]	426.57±39.39[a]	27.51±14.67[b]	97±3.15[ab]	93.33±4.01[bc]
	4	296.7±99.93[a]	2.16±0.75[a]	428.27±129.6[a]	14.38±1.45[a]	99.21±0.32[c]	96.4±1.1[c]
	LSD	94.59	7.02	99.17	12.46	2.34	3.16
CFR 35%	5	383.33±60.88[bc]	5.66±1.21[a]	493.39±65.13[c]	20.67±4.93[a]	98.51±0.33[b]	95.8±1.12[b]
	6	406.7±50.06[c]	14±5.65[b]	552.05±59.4[c]	66.49±12.61[b]	96.44±1.8[a]	87.7±3.21[a]
	7	271.68±32.5[a]	5.16±0.98[a]	358.15±29.46[a]	23.47±10.81[a]	98.1±0.4[b]	93.38±3.2[b]
	8	341.59±36[b]	4.5±1.04[a]	432.02±36.6[b]	18.44±6.62[a]	98.7±0.32[b]	95.61±1.93[b]
	LSD	55.74	3.59	60.154	11.17	1.13	3.04

Exp.: Experiment

Table III.2B shows the mean values of organic matter concentrations (COD and BOD_5) measured in the influent and effluent of the MBMBR system as well as the COD and BOD_5 removal rates. It is notable that the biotransformation of organic matter in the MBMBR system expressed as COD and BOD_5 was efficiently produced and that the effluent values under all experimental conditions assayed were within the limits allowed by the European Legislation (91/271/CEE, 1991). These results suggest that independently of the HRT (10 or 24 h) and CFR (20% or 35%, v/v) in the MBMBR system, a high quality of permeates can be produced.

3.2 Enzymatic activities

Previous reports have described that when attached biofilm and activated sludge systems are combined, the microorganisms involved in the biodegradation processes can grow both as suspended cell aggregates (flocs), as single cells in the mixed liquor and also as attached biofilm in the carriers (Di Trapani *et al*., 2010; Nicolella *et al*., 2000). Therefore, to understand the hydrolytic processes in the MBMBR system, the evolution of enzymatic activities within the eight experiments was assessed in both suspended biomass (SB) and attached biofilm (AB) samples. **Figs. III.2** and **III.3** show the overall mean enzymatic values of SB and AB obtained in each experiment. The four experiments conducted with 20% (v/v) CFR are shown in **Fig. III.2** and those conducted with 35% (v/v) CFR are presented in **Fig. III.3**.

Different hydrolysis levels of alkaline phosphatase were obtained, depending on the origin of the sample (SB and AB) and the statistical analysis of variance revealed significant differences between SB and AB samples ($p < 0.05$) in all experiments (**Figs. III.2** and **III.3**). In contrast, the enzymatic activities of acid phosphatase and α-

glucosidase were not significantly different in any experiment between SB and AB samples (**Figs. III.2** and **III.3**). However, with some exceptions, the mean alkaline phosphatase, acid phosphatase and α-glucosidase activities were higher in SB samples than AB samples (**Figs. III.2** and **III.3**).

The hydrolase enzymes such as glucosidases and phosphatases in activated sludge have been associated mainly with suspended single cells and flocs (Goel *et al*., 1998; Li *and* Chróst, 2006) and it is known that large parts of extracellular enzymes are retained in extracellular polymeric substances (EPS) in both attached biofilms and mixed liquor flocs (Burgess *et al*., 2008; Flemming *and* Wingender, 2010). As reported by various authors (Cadoret *et al*., 2002; Dimock *and* Morgenroth, 2006), high molecular weight compounds have a limited diffusion through EPS. In addition, Morgenroth *et al*. (2002) suggested that the mixed liquor flocs have a more open structure and a larger relative surface area compared to attached biofilms. In view of this, higher rates of hydrolytic activities in the suspended biomass might relate to a better diffusion of substrate in the flocs, as well as to the contribution of suspended single cells to the hydrolytic activity. However, this preliminary suggestion must be treated with caution, because changes in enzymatic activities might also reflect changes in cellular physiology as well as changes in the microbial community (i.e., changes in the microbial populations responsible for the production of enzymes). In this context, analysis of the microbial population in both SB and AB is in progress.

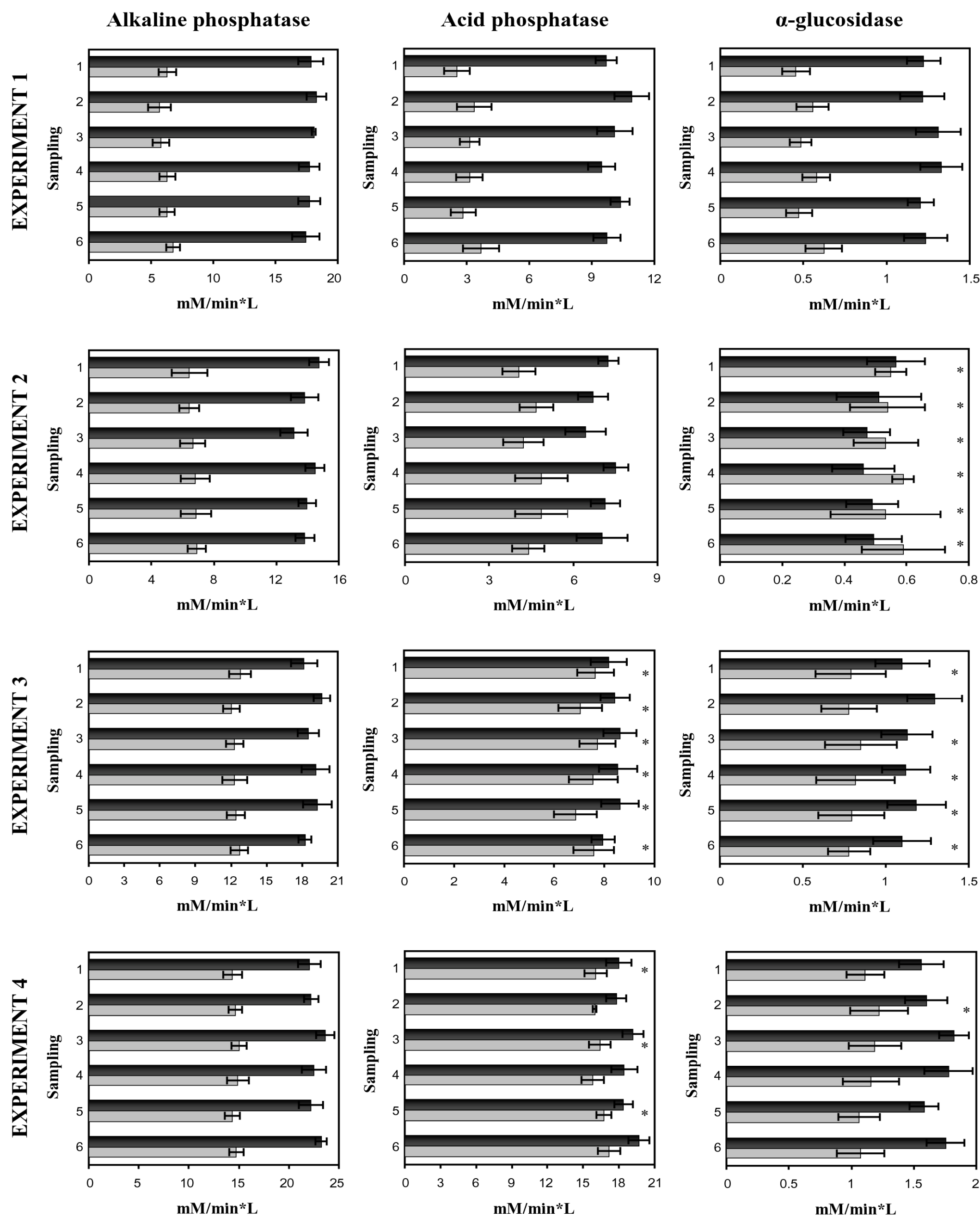


Figure III.2. Alkaline phosphatase, acid phosphatase and α-glucosidase measured in suspended biomass (SB: black colour , mM/ min*L of mixed liquor) and attached biofilm (AB: gray colour, mM/min*L at 100% volumetric CFR) in experiments 1, 2, 3 and 4 conducted in the MBMBR system with a 20% (v/v) CFR. Two samplings were performed per week.
*No significant differences between SB and AB in the same sampling, according to Student's test ($p > 0.05$).

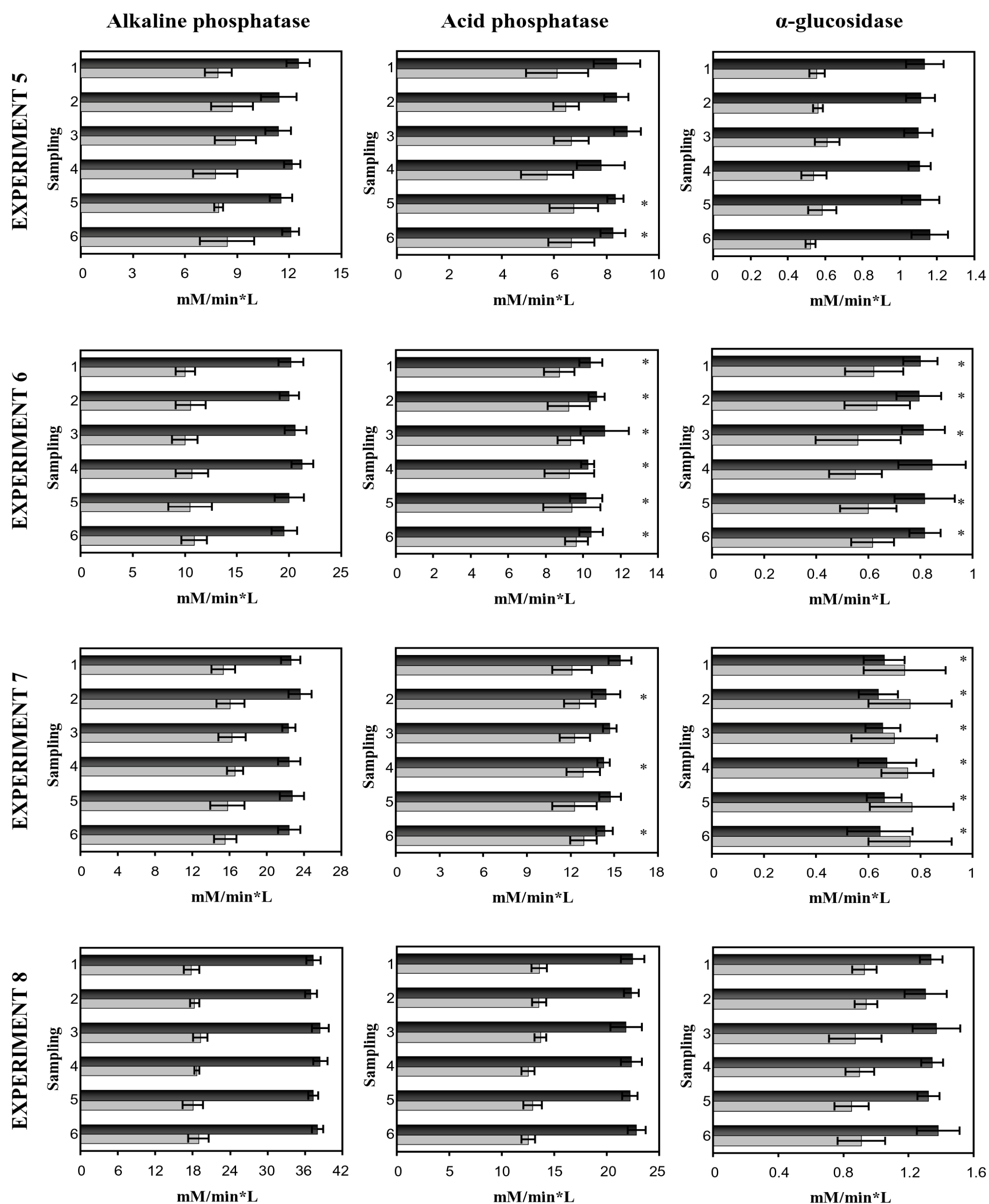


Figure III.3. Alkaline phosphatase, acid phosphatase and α-glucosidase measured in suspended biomass (SB: black colour, mM/ min*L of mixed liquor) and attached biofilm (AB: gray colour, mM/min*L at 100% volumetric CFR) in experiments 5, 6, 7 and 8 conducted in the MBMBR with a 35% (v/v) CFR. Two samplings were performed per week.

*No significant differences between SB and AB in the same sampling, according to Student's test (p> 0.05).

The temporal evolution of the activities of acid phosphatase, alkaline phosphatase and α-glucosidase of the SB and AB in each experiment are also presented in **Figs. III.2** and **III.3**. When the hydrolysis levels were compared in the attached biofilm for samples within the same experiment, no significant differences ($p > 0.05$) were observed. The same trend was observed for suspended biomass samples. The mean values of hydrolytic activity in each experiment conducted with either a 20% or 35% (v/v) CFR are shown in **Table III.3**. As can be observed, the hydrolytic activities in suspended biomass varied significantly among the four experiments conducted with either a 20% (v/v) or 35% (v/v) CFR, suggesting that the hydrolytic enzymes might be affected by changes in the HRT and biomass concentration. It can also be seen that in the experiments conducted with a MLTSS concentration of c.a. 2,500 mg/L (experiment 1, 2, 5 and 6), a higher hydrolytic activity was obtained at a low HRT (10 h) (experiment 1 and 6), with the exception of α-glucosidase, which was higher in experiment 5 (HRT 24 h) compared to experiment 6 (HRT 10 h). However, at a MLTSS concentration of c.a. 4,500 mg/L (experiments 3, 4, 7 and 8), higher hydrolytic values were observed with a HRT of 24 h (experiments 4 and 8).

For attached biofilm samples, alkaline phosphatase, acid phosphatase and α-glucosidase activities tended to increase either with increasing BTS concentration or over time. However, alkaline phosphatase and α-glucosidase activities showed no significant differences between experiments 1 and 2 conducted with a 20% (v/v) CFR, although the BTS concentration was lower in experiment 1 compared to experiment 2 (c.a. 2,679 and 4,229 mg/L, respectively). In addition, no significant differences were observed in α-glucosidase activity levels between experiments 5 and 6. This greater stability of the extracellular enzymes of the attached biofilm is to be expected among experiments, since the biofilm matrix (EPS) has been described as a “protective barrier”

against adverse environmental conditions (Flemming *and* Wingender, 2010). Accordingly, Guelli de Souza (2012) reported that biofilms are less affected by environmental changes such as nutrient concentration, temperature, pH, metabolic products and toxic substances, than suspended cultures.

Table III.3. Mean ± standard deviation of the enzymatic activities (mM/min*L) alkaline phosphatase, acid phosphatase and α-glucosidase measured in suspended biomass (SB) and attached biofilm (AB) in the MBMBR. Experiments 1, 2, 3 and 4 were conducted with a 20% (v/v) CFR (CFR 20%) and experiments 5, 6, 7 and 8 with a 35% (v/v) CFR (CFR 35%). LSD: least significant difference (Student´s test, $p < 0.05$). Data followed by the same lower case-letter do not significantly differ among experiments with the same CFR.

	Exp.	Alkaline phosphatase		Acid phosphatase		α-glucosidase	
		SB	AB	SB	AB	SB	AB
CFR 20%	1	17.85±0.76[b]	6.18±0.7[a]	10.05±0.75[c]	3.12±0.69[a]	1.25±0.11[c]	0.53±0.09[a]
	2	13.99±0.79[a]	6.68±0.76[b]	7.03±0.63[a]	4.51±0.7[b]	0.49±0.09[a]	0.55±0.1[a]
	3	18.84±1[c]	12.42±0.75[c]	8.39±0.62[b]	7.38±0.77[c]	1.15±0.15[b]	0.8±0.17[b]
	4	22.63±1.1[d]	14.66±0.74[d]	18.63±1.02[d]	16.35±0.84[d]	1.68±0.17[d]	1.14±0.18[c]
	LSD	0.615	0.489	0.513	0.501	0.09	0.093
CFR 35%	5	11.84±0.74[a]	8.27±1.04[a]	8.32±0.61[a]	6.4±0.83[a]	1.12±0.08[c]	0.56±0.05[a]
	6	20.26±1.11[b]	10.45±1.26[b]	10.52±0.72[b]	9.26±0.94[b]	0.81±0.08[b]	0.59±0.11[a]
	7	22.67±1.05[c]	15.93±1.25[c]	14.64±0.69[c]	12.49±1.05[c]	0.65±0.07[a]	0.75±0.13[b]
	8	37.75±1.1[d]	18.48±1.16[d]	22.37±0.89[d]	13.46±0.65[d]	1.34±0.09[d]	0.9±0.1[c]
	LSD	0.67	0.786	0.492	0.585	0.055	0.067

Exp-: Experiment

The total enzymatic activity in the bioreactor (SB+AB) and the specific contribution of the AB were calculated, taking into account the real volume of mixed liquor and biofilm-colonized carriers in the MBMBR (**Table III.4**). Thus, the alkaline phosphatase, acid phosphatase and α-glucosidase activities in the MBMBR were slightly improved by the presence of attached biofilm in all the experimental conditions assayed in our study. Moreover, our results suggest that this beneficial effect was more

evident in experiments conducted with a 35% (v/v) CFR (experiments, 5, 6, 7 and 8), since a higher contribution percentage was observed. This might be due to differences in the biofilm structure that developed, or to differences in the microbial community diversity as a consequence of different CFRs (20% or 35%, v/v).

Table III.4. Mean ± standard deviation (mM/min*L) of the enzyme activities of alkaline phosphatase, acid phosphatase and α-glucosidase measured in the MBMBR system (suspended biomass + attached biofilm) and contribution of the biofilm (%) to the total hydrolytic activities, in the eight experiments conducted in a MBMBR system. LSD: least significant difference (Student´s test, $p < 0.05$). Data followed by the same lower case-letter do not significantly differ among experiments.

	Alkaline phosphatase		Acid phosphatase		α-glucosidase	
Exp.	**MBMBR**	**Biofilm Contribution (%)**	**MBMBR**	**Biofilm Contribution (%)**	**MBMBR**	**Biofilm Contribution (%)**
1	18.41±0.21^{c}	6.72±0.52^{a}	10.29±0.53^{c}	6.06±0.78^{a}	1.31±0.05^{e}	8.05±0.94^{a}
2	14.8±0.53^{b}	9.04±0.44^{b}	7.65±0.39^{a}	11.82±0.79^{b}	0.59±0.04^{a}	18.85±1.46^{d}
3	20.6±0.54^{d}	12.07±0.56^{c}	9.55±0.25^{b}	15.5±0.9^{c}	1.27±0.07^{de}	12.64±0.86^{b}
4	24.71±0.67^{f}	11.88±0.19^{c}	21.14±0.77^{g}	15.47±0.34^{c}	1.85±0.11^{g}	12.32±0.86^{b}
5	13.94±0.36^{a}	20.77±1.48^{e}	10.01±0.41^{bc}	22.36±0.78^{e}	1.24±0.02^{d}	15.84±0.96^{c}
6	22.57±0.54^{e}	16.21±0.69^{d}	13.06±0.35^{d}	24.84±0.87^{f}	0.97±0.01^{c}	21.58±1.27^{f}
7	26.74±0.46^{g}	20.85±0.58^{e}	18.04±0.29^{e}	24.26±0.96^{f}	0.87±0.01^{b}	29.91±0.87^{g}
8	41.71±0.76^{h}	15.51±0.25^{d}	25.46±0.26^{f}	18.05±0.76^{d}	1.57±0.03^{f}	20.07±0.75^{e}
LSD	0.622	0.812	0.512	0.924	0.06	1.19

Exp.: Experiment

3.3 Statistical multivariate analysis: influence of physico-chemical parameters on enzymatic activities.

To obtain a better understanding of the influence of the operational parameters on the enzymatic activities, as well as the relationship among the operational variables

in our MBMBR system, multivariate statistical analysis was performed. The influence of the simultaneous variation of physico-chemical parameters (BTS, MLVSS, COD, BOD_5, pH, temperature, and HRT) and CFR (20% or 35%, v/v) on the enzymatic activities measured in the SB and AB in the eight experiments was analysed by RDA and the biplot diagrams generated are shown in **Fig. III.4** (**A** and **B**).

According to the results of the Monte Carlo permutation test, MLVSS (p = 0.002), temperature (p = 0.018), COD (p = 0.024) and HRT (p = 0.034) were the significant factors explaining the variation in hydrolytic enzyme activity levels in SB. For the AB, the enzymatic activities were significantly affected by BTS (p = 0.002), CFR (p = 0.004) and COD (p = 0.004).

Concerning the RDA of enzymatic activities in the SB, the first canonical ordination axis (horizontal) was mainly correlated with MLVSS and COD, and the second canonical ordination axis (vertical) was correlated with temperature and HRT. The first and second canonical ordination axes (COA) described 59.4% and 2.4% of the variation of the enzymatic activities data, respectively (96% of their relation to the environment explained by the first axis and 3.9% by the second one). For the AB analysis, the first COA was correlated with BTS, CFR and COD, and described 66.7% of the variation of the enzymatic activities data (96% of their relation to the environment). The second axis was mainly correlated to temperature and described 2.8% of the variation of the enzymatic activities data (4% of their relation to the environment).

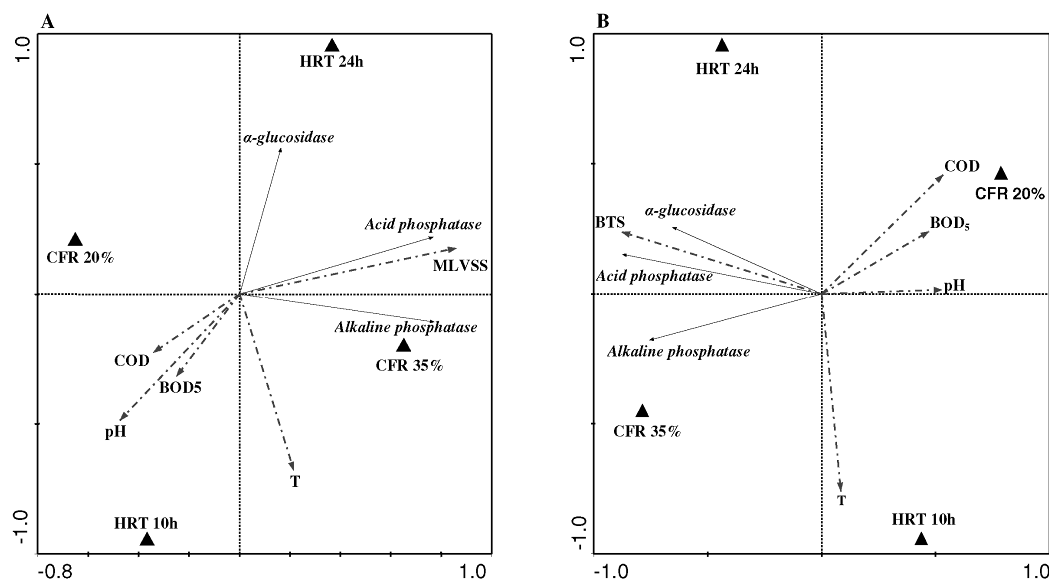


Figure III.4. Redundancy analyses (RDA) of suspended biomass (**A**) and attached biofilm (**B**). The ordination diagrams (biplots) show operational conditions (dotted arrows and triangle) and enzymatic activities (straight arrows), measured in the eight experiments performed in the MBMBR. Operational parameters: pH, Temperature (T), Mixed Liquor Volatile Suspended Solids (MLVSS), Biofilm Total Solids (BTS), Biological Oxygen Demand at 5 days in influent water (BOD_5), Chemical Oxygen Demand in influent water (COD), Hydraulic Retention time (HRT: 10 and 24h) and CFR (v/v) (20% or 35%). HRT and CFR are shown as nominal variables (triangle).

Both RDA analyses (SB and AB) revealed a positive correlation of the enzymatic activities with the concentrations of suspended solids in the mixed liquor or the biofilm, and a negative correlation with the COD in the influent. Moreover, a significant positive correlation was found between the hydrolytic activities in the AB samples and the increase of the CFR from 20 to 35%. MLVSS and BST were the variables displaying the highest correlations to the first COA in the respective RDA analyses (**Fig. III.4**).

It is known that an increase in the biomass concentration of mixed liquor promotes an increase in the concentration of hydrolytic enzymes (Li *and* Chróst, 2006),

thus, a positive correlation between the suspended solid concentration and enzymatic activities was expected. In agreement with this, Anupama *et al*. (2008) reported a positive influence of MLTSS concentration on phosphatase activity. As for the positive correlation between enzymatic activities in the attached biofilm and their total solid concentration (BTS), the immobilisation of the enzymes in the EPS possibly favoured the hydrolytic process. Consequently, an increase in the solid concentration in the biofilm would determine an increase in α-glucosidase, acid and alkaline phosphatase activities.

Comparing both RDA analyses, a strong negative correlation between solid concentration and the organic load of the influent (COD and DBO_5) was clear, thus the arrows point in the opposite direction (**Fig. III.4 A** and **B**). In fact, the higher COD values and the lowest concentrations of solids in the mixed liquor and biofilms were recorded simultaneously during experiments 1 and 2 (20% (v/v) CFR) and experiments 5 and 6 (35% (v/v) CFR). Therefore, decreased values of the assayed hydrolytic activities were observed in both SB and AB, with the exception of α-glucosidase in SB. Mosquera-Corral *et al*. (2003) reported that polymeric compounds are an inherent part of the COD in urban wastewater, where specialised groups of microorganisms might develop in response to changes in organic matter concentration (Biswas *and* Turner, 2012). In our study, this combination of higher organic load rates in the influent with a lower solid concentration in the bioreactor might indicate a higher food/microbial (F/M) rate in the suspended biomass and consequently, a higher substrate availability for microorganisms in the bioreactor as described by Gómez-Silván *et al*. (2013). However, these authors also reported that in studies conducted in MBR systems, bacteria might become more competitive under nutrient-limiting conditions and thus might increase the efficiency of the enzymes. Similarly, Anupama *et al*. (2008) observed an increase in

phosphatase activity in continuous and fed-batch anaerobic bioreactors, in response to the stress created by starvation conditions. However, these authors did not work under different COD levels in the same bioreactor. In contrast, Morgenroth *et al*. (2002) reported a positive correlation between extracellular hydrolytic enzymes and COD in activated sludge plants. Therefore, it could be suggested that the negative influence of COD on hydrolytic activity at lower solid concentration might be correlated with a lower efficiency of the enzymes, as competitive conditions did not occur in the microbiota of the system.

Regarding the influence of the CFR on hydrolytic activities, the RDA results confirmed the positive correlation between the higher CFR (35%, v/v) and the α-glucosidase, acid and alkaline phosphatase levels in attached biofilm. In contrast, no significant influence of this parameter on hydrolytic activities was observed in the suspended biomass, possibly due to a constant balance between attachment and detachment processes, with the exception of experiment 8, in which the detachment process was observed.

Calderón *et al*. (2012b) reported that the CFR is one of the most important parameters influencing the bacterial community structure of attached biofilm in MBBR systems. In agreement with these authors, Wang *et al*. (2005) (using scanning electronic microscopy), observed differences in morphology and composition of biofilm as a function of the CFR (20% or 50%, v/v). In addition, Wang *et al*. (2005) also reported changes in biofilm activities (heterotrophs, ammonium oxidisers and nitrite oxidisers) at different CFR (from 10% to 70%, v/v), possibly due to changes in biofilm structure and microbial composition. According to these previous results (Calderon *et al*., 2012b; Wang *et al*., 2005), our study suggests that changes in the structure and bacterial

community of the biofilm might explain the different hydrolytic activities detected in attached biofilm at 20% and 35% (v/v) CFR.

According to the results of the RDA analyses, temperature and HRT only contributed to the explanation of the distribution of the level of enzymatic activities in the SB samples (**Fig. III.4**). Moreover, the influence of these parameters on the hydrolytic activities was limited, since they were mostly correlated to the second COA. In this context, taking into account that the solid concentration is the most influential variable in our MBMBR system, a negative correlation of the solid concentration with the temperature and a positive one with a HRT of 24 h was observed in both RDA analyses. This suggests that changes in temperature and HRT might have a major effect on α-glucosidase, acid phosphatase and alkaline phosphatase of the suspended biomass when a low biomass concentration was maintained in our MBMBR system.

Temperature has been described as one of the major factors affecting the biodegradation process in wastewater treatment and the influence of temperature on bacteria aggregated as suspended biomass has been widely reported (Calderón *et al.*, 2012a; Cirja *et al.*, 2008; Cydzik-Kwiatkowska *et al.*, 2012). In contrast, in the case of attached biofilm, a minor influence of this parameter has been described (Guelli de Souza, 2012). In agreement with this, our results from multivariate analyses showed no significant effect of temperature on enzyme activities measured in the attached biofilm. In studies conducted in MBR systems in which the seasonal enzymatic variation was recorded, an increase in the enzymatic activities was observed at higher temperatures, with the exception of α-glucosidases (Gómez-Silván *et al.*, 2013; Molina-Muñoz *et al.*, 2010). In this study, higher α-glucosidase activity in the suspended biomass was also observed at lower temperatures. In fact, α-glucosidase activity was strongly correlated

with the second COA (vertical), which showed a strong negative correlation with temperature (**Fig. III.4A**). This effect on the α-glucosidase was clearly noticed in suspended biomass along experiments 2 and 7 run at higher temperatures (26.4 and 28.2°C, respectively), as low activity was recorded regardless of the concentration of solids and HRT.

Morgenroth *et al*. (2002) described that HRT appears to have an important influence in the levels of hydrolytic enzyme activities in biofilm reactors, since the retention time of the substrates can be significantly smaller than that of bacteria in the biofilm. However, in our case, no influence of HRT was observed for any of the hydrolases assayed in the attached biofilm, possibly due to diffusion of the substrates in the biofilm being slower than in the mixed liquor flocs.

Overall, the results presented here demonstrate that the levels of extracellular enzymatic activities were not influenced by the same variables in the SB and AB samples, under the operational conditions tested. Hydrolytic activities in the SB fraction were more sensitive to changes in MLVSS, COD, T and HRT, while they were mainly affected by BTS, CFR and COD in the AB fraction.

References

Anupama, V.N., Amrutha, P.N., Chitra, G.S., Krishnakumar, B., 2008. Phosphatase activity in anaerobic bioreactors for wastewater treatment. Water Res. 42, 2796–2002.

APHA, AWWA, WEF, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, D.C.

Biswas, K., Turner, S.J., 2012. Microbial community composition and dynamics of moving bed biofilm reactor systems treating municipal sewage. Appl. Environ. Microbiol. 78, 855–864.

Burgess, J.E., Pletschke, B.I., 2008. Hydrolytic enzymes in sewage sludge treatment: A mini-review. Water SA. 34, 343–349.

Cadoret, A., Conrad, A., Block, J.C., 2002. Availability of low and high molecular weight substrate to extracellular enzymes in whole and dispersed activated sludges. Enzyme Microb. Technol. 31, 179–186.

Calderón, K., González-Martínez, A., Montero-Puente, C., Reboleiro-Rivas, P., Poyatos, J.M., Juárez-Jiménez, B., Martínez-Toledo, M.V., Rodelas, B., 2012a. Bacterial community structure and enzyme activities in a membrane bireactor (MBR) using pure oxygen as an aeration source. Bioresource Technol. 103, 87–94.

Calderón, K., Martín-Pascual, J., Poyatos, J.M., Rodelas, B., 2012b. Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. Bioresource Technol. 121, 119–126.

Chang, CY., Tanong, K., Xu, J., Shon, H., 2011. Microbial community analysis of an aerobic nitrifying-denitrifying MBR treating ABS resin wastewater. Bioresource Technol. 102, 5337–5344.

Chu, L., Wang, J., 2011. Comparison of polyurethane foam and biodegradable polymer as carriers in moving bed biofilm reactor for treating wastewater with a low C/N ratio. Chemosphere 83, 63–68.

Chróst, R.J., Siuda, W., 2006. Microbial production, utilization, and enzymatic degradation of organic matter in the upper trophogenic water layer in the pelagial zone of lakes along the eutrophication gradient. Limnol. Oceanogr. 51,749–762.

Cirja, M., Ivashechkin, P., Schäffer, A., F.X. Corvini, P., 2008. Factors affecting the removal of organic micropollutants from wastewater in conventional treatment plants (CTP) and membrane bioreactors (MBR). Rev. Environ. Sci. Biotechnol. 7, 61–78.

Cydzik-Kwiatkowska, A., Zielinska, Wojnowska-Baryla, I., 2012. Impact of operational parameters on bacterial community in a full-scale municipal wastewater treatment plant. Pol. J. Microbiol. 61, 41–49.

Dimock, R., Morgenroth, E., 2006. The influence of particle size on microbial hydrolysis of protein particles in activated sludge. Water Res. 40, 2064–2074.

Di Trapani, D., Mannina, G., Torregrossa, M., Viviani., G., 2010. Comparison between hybrid moving bed biofilm reactor and activated sludge system: a pilot plant experiment. Water Sci. Technol. 61, 891–902.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.

Goel, R., Mino, T., Satoh, H., Matsuo, T., 1998. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. Water Res. 32, 2081–2088.

Gómez-Silván, C., Arévalo, J., Pérez, J., González-López, J., Rodelas, B., 2013. Linking hydrolytic activities to variables influencing a submerged membrane bioreactor (MBR) treating urban wastewater under real operating conditions. Water Res. 47, 66–78.

Guellil, A., Boualam, M., Quiquampoix, H., Ginestet, P., Audic, J.M., Block, J.C., 2001. Hydrolysis of wastewater colloidal organic matter by extracellular enzymes extracted from activated sludge flocs. Water Sci. Technol. 43, 33–40.

Guelli de Souza, S., 2012. Application of biofilm in the degradation of contaminants in industrial effluents-A review. Open J. Biochem. Biotechnol. 1, 1–10.

Hem, L.J., Rusten, B., Ødegaard, H., 1994. Nitrification in a moving bed biofilm reactor. Water Res. 28, 1425–1433.

Ivanovic, I., Leiknes, T.O., 2008. Impact of aeration rates on particle colloidal fraction in the biofilm membrane bioreactor (BF-MBR). Desalination 231, 182–190.

Leiknes,T.O., Ødegaard, H., 2007. The development of a biofilm membrane bioreactor. Desalination 202, 135–143.

Lepš, J., Šmilauer, P., 1999. Multivariate analysis of ecological data. Faculty of Biological Sciences, University of South Bohemia, Ceske Budejovice.

Levstek, M. Plazl, I., 2009. Influence of carrier type on nitrification in the moving-bed biofilm process.Water Sci. Technol. 59, 875–882.

Li, Y., Chróst, R.J., 2006. Microbial enzymatic activities in aerobic activated sludge model reactors. Enzyme Microb. Technol. 39, 568–572.

Martín-Pascual, J., López-López, C., Cerdá, A., González-López, J., Hontoria, E., Poyatos, J.M., 2011. Comparative kinetic study of carrier type in a moving bed system applied to organic matter removal in urban wastewater Treatment. Water, Air, Soil Pollut. 223, 1699–1712.

Molina-Muñoz, M., Poyatos, J.M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E., González-López, J., 2010. Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions. Bioresource Technol. 101, 696–704.

Morgenroth, E., Kommedal, R., Harremoës, P., 2002. Processes and modelling of hydrolysis of particulate organic matter in aerobic wastewater treatment — a review.

Water Sci. Technol. 45, 25–40.

Mosquera-Corrala, A., Montràs, A., Heijnena, J.J., Van Loosdrecht, M.C.M., 2003. Degradation of polymers in a biofilm airlift suspension reactor. Water Res. 37, 485–492.

Nicolella, C., Van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. J. Biotech. 80, 1–33.

Ødegaard, H., 2006. Innovations in wastewater treatment: the moving bed biofilm process. Water Sci. Technol. 53, 17–33.

Onnis-Hayden, A., Majed, N., Schramm, A., Gu, A.Z., 2011. Process optimization by decoupled control of key microbial populations: Distribution of activity and abundance of polyphosphate-accumulating organisms and nitrifying populations in a full-scale IFAS-EBPR plant. Water Res. 45, 3845–3854.

Pal, L., Kraigher, B., Brajer-Humar, B., Levstek, M., Mandic-Mulec, I., 2012. Total bacterial and ammonia-oxidizer community structure in moving bed biofilm reactors treating municipal wastewater and inorganic synthetic wastewater. Bioresource Technol. 110, 135–143.

Plattes, M., Henry, E., Schosseler, P.M., Weidenhaupt, A., 2006. Modelling and dynamic simulation of a moving bed bioreactor for the treatment of municipal wastewater. Biochem. Eng. J. 32, 61–68.

Poyatos, J.M., Molina-Muñoz, M., González-López, J., Hontoria, E., 2010. Particle size distribution in permeate from a pilot-scale membrane bioreactor system under actual operating conditions. Part. Sci. Technol. 28, 309–318.

Wang, R.C., Wen, X.H., Qian, Y., 2005. Influence of carrier concentration on the performance and microbial characteristics of a suspended carrier biofilm reactor. Process Biochem. 40, 2992–3001.

Watanabe, Y., Masuda, S., Ishiguro, M., 1992. Simultaneous nitrification and denitrification in micro-aerobic biofilms. Water Sci. Technol. 26, 511–522.

Yang, S.,Yang, F., Fu, Z., Lei, R., 2009. Comparison between a moving bed membrane bioreactor and a conventional membrane bioreactor on organic carbon and nitrogen removal. Bioresource Technol. 100, 2369–2374.

IV. CHAPTER 2

Study of the structure and dynamic of total Bacteria and ammonia-oxidizing bacteria communities, involved in the depuration process of urban wastewater in moving bed membrane bioreactor: TGGE fingerprinting and 454-pyrosequencing methodologies.

1. Introduction

Nowadays, the biological wastewater treatments are one of the most common biotechnological processes, since they represent an effective, economical and environmentally friendly solution (Kermani *et al*., 2008; Chan *et al*., 2009; Khan *et al*., 2011; Valentín-Vargas *et al*., 2012). For this reason, in recent years, the idea of combining suspended and attached growth to improve biological process on well established wastewater treatment technologies represents an interesting solution. In this sense, different combinations of biofilm-based and membrane filtration technologies (hybrid MBRs) have been proposed (Kraume *and* Drews, 2010).

From a biological point of view, the incorporation of biofilm process confers several advantages to the MBR system such as higher biomass activity, higher biomass resistance to toxic or organics loads socks and improving of nitrification and denitrification processes, since the biofilm favours the development of slow growing bacteria, such as nitrifying bacteria (Wang *et al*., 2010; Rahimi *et al*., 2011; Ivanovic *and* Leiknes, 2012). Indeed, a recent review written by Ivanovic *and* Leiknes (2012) pointed out that the biofilm processes in MBRs are potentially useful to remove organic pollutants and nutrients (C, N and P) from urban and industrial wastewater effluents. One of the most innovative hybrid MBRs systems is the moving bed membrane bioreactor (MBMBR) described by Leiknes *and* Ødegaard in 2007 as a combination of the moving bed biofilm rector (MBBR) and the membrane technology. In essence, MBMBRs systems are based on the addition of freely moving support material (carriers) to the biological reactor of MBRs. Biofilm development takes place in the carrier.

The biological wastewater treatments feed on the metabolic activity of microorganisms for the transformation of toxic substances, the degradation of organic pollutants and the removal of nutrients from urban and industrial effluents (Wells *et al*., 2011; Valentin - Vargas *et al*., 2012). Consequently, it is generally accepted that the study of structure and dynamics of microbial communities is essential to understand the environmental or operational factors affecting efficiency and stability of the biological process, as well as to develop strategies to improve the performance of removing organic contaminants and nutrients (Wagner *and* Loy, 2002; Cydzik- Kwiatkowska *et al*., 2012; Biswas *et al*., 2014; Ibarbalz *et al*., 2013; Short *et al*., 2013; Vanwonterghem *et al*., 2014). However, the identification of microorganisms responsible for biotransformation processes in complex environments, such as wastewater treatment plants (WWTPs), remains today one of the major challenges in the field of microbiology and environmental engineering (de los Reyes, 2010).

Bearing in mind the above commented and taking into account that the microbial communities in a MBMBR system develop in different types of aggregates (suspended flocs and attached biofilms), it can be assumed that for efficient and cost-effective operation of MBMBR, the study of microbial community structure of the suspended biomass (SB) and the attached biofilm (AB) is essential.

To study the microbial communities in complex environments, the fingerprinting methodologies, such as TGGE and DGGE are two of the most used in the field of microbial ecology (Loisel *et al*., 2006; Marzorati *et al*., 2008). Specifically, these techniques have been widely used for the analysis of structure and dynamics of microbial communities present in WWTPs (Cortés-Lorenzo *et al*., 2006; Sanz *and* Köchling, 2007; Fernández *et al*., 2008; Polchan *et al*., 2010; Sánchez *et al*., 2011;

Calderón *et al*., 2012b; Calderón *et al*., 2013; De Vrieze *et al*., 2013; Gómez-Silván *et al*., 2014). Both these molecular techniques are very useful for the study of population dynamics over time and the analysis of population responses to environmental stress or operational changes, enabling to process and compare high number of samples (Wittebolle *et al*., 2005; Sanz *and* Köchling., 2007). However, it is also necessary to consider that only the dominant populations of the community analyzed are detected, and therefore, less abundant but potentially important species, are not revealed by these techniques (Boon *et al*., 2002). Although, according to van der Gast *et al*. (2008) the dominant populations could be the most ecologically important. These discrepancies, in fact, represent one of the main reasons why fingerprinting methods are generally combined with other molecular methodologies to obtain most comprehensive results (Köchling *and* Sanz., 2007; Miura *et al*., 2007a; Wojnowska-Baryla *et al*., 2010). In this regard, a combination of the Next Generation Sequencing (NGS) methods with TGGE technique could be a good option for further in-depth analysis of microbial populations' structure and dynamic. Because NGS methods provide thousands of sequences reads of one environmental sample, increasing the possibilities to detect the low abundance population present in the microbial community (McLellan *et al*., 2010; Shokralla *et al*., 2012).

The rapid and substantial NGS cost reduction as well as its continuous improvement in terms of reads length and accuracy is accelerating their use (Shokralla *et al*., 2012; Thomas *et al*., 2012). In fact, among the various NGS techniques, 454 pyrosequencing is being widely applied to study microbial diversity in conventional activated sludge systems (Hu *et al*., 2012; Zhang *et al*., 2012; Sánchez *et al*., 2013; Ye *and* Zhang, 2013). In addition, 454-pyrosequencing has been recently applied to the study of bacterial communities developed in suspended biomass and attached biofilm in

MBBR and fixed-film activated sludge (IFAS) systems (Kwon *et al*., 2010; Biswas *et al*., 2014).

Nevertheless, despite the large number of studies that have addressed the diversity and dynamics of microbial communities present in WWTPs, few studies have established a clear link between community structure and operational parameters (Polchan *et al*., 2010; Valentin-Vargas *et al*., 2012) and, according to Liang *et al*. (2010), more information about microbial community structure in hybrid MBRs is necessary for optimal design and operation. In addition, so far and to the best of our knowledge, attempts to link community structure variations to changes of the different operational parameters have not been performed in MBMBR systems.

For these reasons and considering that the bacteria are the dominant microorganisms and primarily responsible for the removal of organic contaminants and nutrients in WWTPs, as well as the key role of the ammonia-oxidizing bacteria (AOB) to removal of nitrogen, we studied the structure and the dynamic of total Bacteria and AOB communities in both SB and AB in a MBMBR system with a 20% and 35% (v/v) CFR under four different operational conditions. For that TGGE fingerprinting and 454-pyrosequencing methodologies were used. Finally, multivariate analysis was an important tool to understand the influence of physico-chemical parameters and CFR on the bacterial community structure.

2. Material and Methods

2.1. Experimental design

2.1.1. Pilot-scale experimental plant

The pilot-scale experimental plant used to perform this study has been described in detail in chapter 1 (sub-paragraph 2.1.1). In essence, the aerobic MBMBR system consisted of two bioreactors: a moving bed bioreactor (MBBR), in which the carriers moved freely by aeration in an operating volume of 358L and, a membrane bioreactor (MBR) composed of three ultrafiltration membrane modules of hollow fibre (Zenon®) submerged in 87 L of operating volume under continuous aeration. Sludge recirculation was performed to maintain the same concentration of total suspended solids in both reactors. Finally, the effluent was collected in a back-washing tank.

The MBMBR system worked with real urban wastewater and environmental conditions. To do this, MBMBR was installed in the municipal WWTP "Puente de los Vados" (Emasagra S.A., Granada, Spain). Specifically, the influent was pumped from the primary settler to the MBBR. The carrier K1, made from high density polyethylene, developed by AnoxKaldnes (Norway), was used as a support material. Characteristics of the carrier K1 were described in detail in chapter 1 (sub-paragraph 2.1.2).

2.1.2. Bioreactor operating conditions

In order to establish a rough link between operational conditions and microbial community structure in both SB and AB, different Carrier Filling Ratios (CFRs, 20% or 35%, v/v), Hydraulic Retention Times (HRT, 10h or 24h) and Mixed Liquor Total Suspended Solids (MLTSS, c.a. 2,500 mg/L or 4,500 mg/L) were tested as already

described in chapter 1 (sub-paragraph 2.1.3). Briefly, eight experimental phases (designated as experiment 1-8) were conducted in the MBMBR system. Four experimental phases were performed with 20% (v/v) CFR (experiments 1-4) conducted in 2011 or 35% (v/v) CFR (experiments 5–8) conducted in 2012. The same combinations of HRT and MLTSS were established for both CFRs (20% and 35%, v/v) and are detailed in **Table IV.1**. The sampling periods used in the present study were longer than those applied in the chapter 1. In this study sampling started when the MLTSS concentration required for each experiment was reached, while in the chapter 1 the sampling periods started when the system reached steady state conditions.

Table IV.1. Experimental design and operational conditions of the eight experiments conducted in the MBMBR system. CFR: Carrier Filling Ratio; HRT: Hydraulic Retention Time; MLTSS: Mixed Liquor Total Suspended Solids.

Experiment		Sampling period			HRT (h)	MLTSS (mg/L)
		Starting date	Ending date	Days		
CFR 20%	1	8 March 2011	13 April 2011	37	10	ca. 2,500
	2	12 May 2011	26 June 2011	46	24	ca. 2,500
	3	3 October 2011	9 November 2011	38	10	ca. 4,500
	4	11 November 2011	15 December 2011	35	24	ca. 4,500
FR 35%	5	8 March 2012	20 April 2012	44	24	ca. 2,500
	6	26 April 2012	26 May 2012	31	10	ca. 2,500
	7	3 July 2012	4 August 2012	33	10	ca. 4,500
	8	3 September 2012	1 October 2012	29	24	ca. 4,500

To monitor possible changes in the community structure of the attached biofilm over time, the MBMBR system was continuously operated throughout the study with either 20% or 35% (v/v) CFR. Inflow rates of 45.5 and 18.96 L/h were used to maintain the same biomass concentration at a HRT of 10 h and 24 h, respectively. To maintain the MLTSS concentration of the system in experiments 1–8, different purges of 35, 18, 20, 8, 24, 52, 25 and 8 L/day were required, respectively. After completing each experimental phase, the membranes were cleaned with sodium hypochlorite (1 g/L) as previously reported by Poyatos *et al*. (2010).

2.2. Biofilm recovery

Prior to study the bacterial diversity in AB, the biofilm was removed from the supporting material as reported in chapter 1: approximately 50 units of biofilm-colonized carriers were collected from different part of MBBR using a sieve sampling device as recommended by Calderón *et al*. (2012b) and were placed in sterile flaks with 50 mL of saline solution water (0.9% NaCl). Five of these carriers were placed in sterile flaks with 5 mL of saline solution water (0.9% NaCl), vortexed for 1 min, sonicated for 3 min, and the resultant biofilm suspensions were collected by centrifuging for 5 min at 3,000 × g. This process was repeated twice. The biofilm pellet obtained after last centrifugation was used for DNA extraction. The biofilm recovery to determinate its total solid (BTS) and volatile solids (BVS) was used same procedures with slight differences. All collected carriers were used and the final biofilm pellet obtained was resuspended in 50 mL of saline solution water (0.9% NaCl).

2.3. Physical-chemical analysis

Biological Oxygen Demand at 5 days (BOD_5), Chemical Oxygen Demand (COD), MLTSS, Mixed Liquor Volatile Suspended Solids (MLVSS), Biofilm Total Solids (BTS) and Biofilm Volatile Solids (BVS) were analyzed daily in influent, MBBR and effluent. All parameters were determinate according to Standard Methods for the Examination of Waste and Wastewater (APHA, 2005). BTS and BVS concentrations in the MBBR were calculated according to Plattes *et al*. (2006). Ammonium ion (NH_4^+) also was analyzed daily in influent and was determined by ionic chromatography using a conductivity detector (Methrom). Separation and dilution of the cation was carried out on a Metrosep C 4 column using as eluent a solution of dipicolinic acid, and distilled water as a regenerate. pH was determined using a Crison pH 25 pH metre (Crison instruments S.A., Barcelona, Spain). Bioreactor temperature was monitored by a control device available in the MBBR. **Table IV.2**. shows the mean values of MLTSS, MLVSS, BTS, BVS, temperature, pH and NH_4^+ measured in the bioreactor. Mean values of COD and BOD_5 measured in the influent and effluent of the MBMBR and COD and BOD_5 removal rates for each experiment are also sowed. In terms of biotransformation of organic matter (COD and BOD_5) high quality of effluent was obtained in all the experiments. Effluent values were within the limits allowed by the European Legislation (91/271/CEE, 1991) for effluent discharge to the environment.

Table IV.2. (**A**) Operational parameters: Hydraulic Retention Time (HRT), Mixed Liquor Total Suspended Solid and Mixed Liquor Volatile Suspended Solid (MLTSS and MLVSS, respectively), Biofilm Total Solid and Biofilm Volatile Solid (BTS and BVS, respectively), Temperature (T) and pH of the eight experiments conducted in a MBMBR system with a 20% (v/v) or 35% (v/v) CFR. (**B**) Organic matter: Biological Oxygen Demand at 5 days (BOD_5) and Chemical Oxygen Demand (COD), in the influent and effluent, and BOD_5 and COD removal rates (%) in the eight experiments. (**C**) Ammonia (NH_4^+) concentration in the influent. Values shown are means ± standard deviation. LSD: least significant difference (Student's test, $p < 0.05$). Data in columns with same superscript letter are not statistically different. *Exp.: Experiment.

A

Exp.	HRT (h)	MLTSS (mg/L)	MLVSS (mg/L)	BTS (mg/L)	BVS (mg/L)	T °C	pH
1	10	2172±218.89^{a}	1807±235.58^{a}	2585±314.6^{a}	2213±422.13^{a}	15.5±3.1^{b}	7.23±0.17^{bc}
2	24	2340±311.7^{a}	1925±276.03^{ab}	3901±324.25^{b}	3513±296.96^{b}	21±4.44^{d}	6.93±0.23^{a}
3	10	4175±280.8^{c}	3458±331.87^{d}	4935±804de	4363±829.46^{cd}	16.58±3.2^{bc}	7.09±0.15^{abc}
4	24	4288±252.7^{c}	3475±288.91^{d}	7163±520.94^{f}	6218±462.05^{e}	9.82±1.4^{a}	6.97±0.3^{ab}
5	24	2640±302.9^{b}	2226±286.77^{c}	4001±612.82^{bc}	3416±572.64^{b}	11.75±3^{a}	7.28±0.19^{c}
6	10	2686±133.52^{b}	2208±143.82^{c}	5013±562.75^{de}	4434±543.63^{cd}	19.9±2.5^{cd}	7.13±0.21^{abc}
7	10	4240±138.74^{c}	3603±170.2^{d}	5435±220.73^{e}	4872±232.64^{d}	26.3±1.3^{e}	6.94±0.18^{a}
8	24	4452±142.92^{c}	3996±171.9^{e}	4558±186.6^{cd}	3922±330.1^{bc}	23.5±2.6^{de}	6.97±0.25^{ab}
LSD		280.55	295.1	579.8	586.91	3.34	0.25

B

Exp.:	BOD_5 influent (mg/L)	BOD_5 effluent (mg/L)	COD influent (mg/L)	COD effluent (mg/L)	BOD_5 removal (%)	COD removal (%)
1	383.3±90.26^{b}	34.6±20.76^{b}	505.7±81.9^{c}	64.77±15.9^{c}	90.61±5.4^{a}	86.98±1.9^{a}
2	368.3±89.98^{ab}	5.5±0.84^{a}	522.7±81.8^{c}	34.1±9.85^{b}	98.42±0.47^{b}	93.33±2.46^{bc}
3	286.6±88.24^{a}	4.3±1.75^{a}	379.9±74.2^{a}	19.63±4.51^{ab}	98.43±0.73^{b}	94.7±1.5^{bc}
4	310±85.5^{ab}	2.2±0.75^{a}	443.5±108.2^{abc}	14.93±1.66^{a}	99.27±0.28^{b}	96.5±0.95^{c}
5	350±46.9^{ab}	5.5±1.8^{a}	498.8±53.6^{bc}	34.8±29.9^{b}	98.43±0.5^{b}	93±5.6^{b}
6	394±47.2^{b}	11±6.67^{a}	511.5±27.8^{c}	62.54±19.3^{c}	97.21±1.9^{b}	89.2±4.9^{a}
7	330±60.8^{ab}	6.6±1.7^{a}	400.6±26.5^{ab}	18.03±10.9^{ab}	97.18±1.9^{b}	94.3±3.9^{bc}
8	342±46.04^{ab}	6.4±4.4^{a}	445.6±26.6^{abc}	16.8±4.74^{ab}	98.01±1.6^{b}	96.2±1.1^{bc}
LSD	85.99	9.54	94.54	17.6	2.61	3.4

C

Exp.	NH_4^+ influent (mg/L)
1	66±16.73^{a}
2	84±32.68^{ab}
3	71±15.77^{ab}
4	87±28.60^{bc}
5	103±25.00^{c}
6	73.84±26.96^{ab}
7	80.61±28.29^{ab}
8	78±25.45^{ab}
LSD	22.55

2.4. Structure and dynamics of total bacteria and ammonia oxidizing bacteria by TGGE

To study the total Bacteria and the ammonia-oxidizing β-proteobacteria (AOB) communities present in the SB and AB from MBMBR system by TGGE fingerprinting, the gene encoding 16S rRNA was chosen. While it is true that the study of AOB can also be approached by amplification of the *amoA* gene, according to Purkhold *et al.* (2003), the specific partial amplification of partial 16S rRNA gene provides higher phylogeny inference resolution.

To obtain detailed information about the temporal evolution of both communities (total bacteria and AOB) for each experiment, one sample of SB and AB per week was used. The samples were denominated considering; the experiment (1-8), the sample type (SB or AB) and the sampling week in each experiment, e. g. for experiment 1, sample of SB taken in the second week of this experiment (1 SB2).

2.4.1. DNA extraction and PCR amplifications of partial bacterial and ammonia-oxidizing β-proteobacteria 16S rRNA genes

Genomic DNA from c.a. 250 mg of SB and AB samples was extracted using FastDNA® SPIN Kit for Soil and FastPrep® 24-Instrument (MPBiomedicals, Germany), according to the manufacturer‘s indications.

Taking into account the recommendations of others authors, to increase sensitivity and resolution of TGGE gels, a two-steps PCR (nested PCR) approach was performed for both (total bacteria and AOB) (Boon *et al.*, 2002; Molina-Muñoz *et al.*,

2009; Calderón *et al*., 2012a). All amplifications were done on Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany) and were performed using: TrueStart Hot Start Taq polymerase (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA), deoxyribonucleotides (dNTPs) (MBL, Córdoba, Spain), bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA, USA), dimethyl sulfoxide (DMSO) and HPLC-purified primers (Sigma Aldrich, St Louis, MO, USA).

For total Bacteria community, the target fragment for first PCR was the 16S rRNA gene. Extracted DNA (2-5 ng) was used as template and amplification was carried out using the universal primers fD1 and RD1 described by Weisburg *et al*. (1991). Subsequently, 1 µl of the first PCR product was used as template for second amplification step, for which P1-GC and P2 primers, described by Muyzer *et al*. (1993), were used to amplify the V3 hypervariable region of the 16S rRNA gene. Conditions for each PCR reaction were as previously described by Molina-Muñoz *et al*. (2009).

For AOB community, extracted DNA (2-5 ng) was used as a template for the first PCR, using the specific primers CTO189fAB, CTO189fC and CTO654r described by Kowalchuk *et al*. (1997). These primers allowed partial and specific amplification of the 16S rRNA gene belonging to the ammonia-oxidizing β-proteobacteria (AOB). PCR program was as previously described by Kowalchuk *et al*. (1997), and final concentrations of the different components in the reaction mix were: 24 mM of Taq Buffer, 1.5 mM of $MgCl_2$, 5% DMSO, 8 ng/µl of BSA, 0.2 nM of dNTPs mixture, 0.6 µM of each primer and 1 U of Taq DNA polymerase. Subsequently, 1 µl of a 1/30 dilution of amplified product from the first PCR was used as a template for the second PCR step, using universal primers targeting the V3 region as previously described by Molina-Muñoz *et al*. (2009).

The final amplified products were purified and/or concentrated (when required) using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). A final DNA concentration of (c.a. 100 ng) was loaded into each well for TGGE.

2.4.2. TGGE gels

TGGE runs were performed on a TGGE Maxi system (Whatman-Biometra, Goettingen, Germany). Denaturating gels (6% polyacrylamide gel electrophoresis with 20% deionised formamide, 2% glycerol and 8 M urea) were made and run with 2x Tris–acetate–EDTA buffer. All chemicals used were purchased from Sigma–Aldrich (St. Louis, MO, USA). The gels were run at 125 V for 18 h. For total Bacteria community, the temperature gradient applied for efficient separation of bands was optimized at 42-60 °C, while for AOB fingerprinting it was optimized at 38-50 ºC. Gel bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

2.4.3. Fingerprinting analysis

The band patterns generated by TGGE for total Bacteria and AOB were normalized, compared and clustered using the Gel Compar II image analysis software, version 5.102 (Applied Maths, Belgium). For proper normalization of band patterns of either total Bacteria or AOB, a common sample of the MBMBR system was used as an internal market in all gels. TGGE profiles were compared and subsequently clustered using a band assignment dependent method (Dice coefficient). This coefficient is based on band presence/absence, regardless of their intensity (Liu *et al.*, 2007). Bands were detected and assigned automatically, but bands assignments were performed manually.

In accordance with Calderón *et al.*, 2012a, 1% band position tolerance (relative to the total length of the gel) was applied for band assignment. 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. The relative intensity of the bands was also calculated using Gel Compar II.

For better understanding about population richness and evenness in the samples, several theoretical indices based on TGGE analysis were calculated for total Bactria and AOB communities. The range weighted richness index (*Rr*) was used to estimate the level of microbial diversity in each sample. This index was calculated based on the total number of bands in each TGGE pattern (N) and the temperature gradient (°C) between first and last band of each pattern (Tg), as described by Marzorati *et al*. (2008). The resulting values were divided by 100 (Gomez-Silvan *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).

Functional organization (*Fo*) index measures the relationship between functionality and community structure, allowing estimation of functional redundancy of the microbial communities analyzed by fingerprinting methods (Marzorati *et al*., 2008). This index (*Fo*) was calculated based on Pareto–Lorenz evenness curves, which were drawn based on the TGGE fingerprints, as previously described (Marzorati *et al*., 2008). 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).

Dynamics index (*Dy*) described also by Marzorati *et al*. (2008), indicates the stability of the microbial community over time. This index is based on the rate of change between consecutives TGGE profiles over a fixed time intervals and was determined using the matrix of similarities for the densiometric curves of the TGGE patterns as described Marzorati *et al*. (2008).

The Shannon–Wiener index of diversity (*H'*) (Shannon *and* Weaver, 1963), was calculated for each TGGE lane using the function described by Calderón *et al*. (2012a). This index is compiled from the relative intensity of a given band in the whole densitometric curve of the corresponding lane. Finally, the relative abundances of species present in a sample (*H'*) is calculated by adding the relative intensity of each band present on the corresponding TGGE line.

2.4.4. DNA sequencing of TGGE-isolated bands and Phylogenetic analysis

Individual bands visualised in silver-strained TGGE gels were picked up with sterile pipette tips, placed in 10 µl of DNase- and RNase-free water (Sigma-Aldrich, Germany) and directly used for re-amplification with the appropriate primers. Before sequencing, PCR products were electrophoresed in agarose gels and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The automated sequencing was carried out in an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, CA, USA). DNA sequences were analyzed using the biocomputing tools provided on-line by the by the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov). The BLASTn program (Altschul *et al*., 1997) was used for preliminary sequence

similarity analysis. The ClustalX version 2.0.3 software (Jeanmougin *et al*., 1998) was used for the aligning of sequences. Phylogenetic analyses were conducted using MEGA version 4 (Tamura *et al*., 2007). A p-distance based evolutionary tree was inferred using the Neighbor-Joining algorithm (Saitou *and* Nei, 1987). The bootstrap test was conducted to infer the reliability of branch order (Felsenstein, 1985). Bootstrap values below 50% are not shown in the tree.

2.5. Analysis of total bacterial and ammonia oxidizing bacteria by 454-pyrosequencing

A total of 9 SB and 9 AB samples corresponding to the last sample week of each experiment in the MBMBR system were selected in order to conduct an in-depth analysis of the microbial communities by 454-pyrosequencing of bacterial 16S rRNA genes. 454-pyrosequencing was conducted by Research and Testing Laboratory (Lubbock, USA) on a GS FLX+ platform (Roche, Branford, USA) using a modification of the primers 28F/519R targeting the V1-V3 hypervariable regions of bacterial 16S rRNA genes (28F: 5´-GAGTTTGATCNTGGCTCAG-3´, 519R: 5´-GTNTTACNGCGGCKGCTG-3´).

Denoising of pyrosequencing reads and chimera removal were performed by the sequencing facility applying the methods USEARCH (Edgar *at al*., 2010) and UCHIME (Edgar *et al*., 2011) respectively. The obtained filtered reads were subsequently processed using the QIIME pipeline (http://qiime.sourceforge.net/) version 1.7.0 (Caporaso *et al*., 2010). Firstly, retained pyrosequencing reads had to fulfil the following default quality requirements: include a perfect match to the sequence tag and the forward primer, be at least 200 bp in length, have no ambiguous

bases and have no homopolymer stretches longer than 6 nucleotides. Reverse primer sequences were removed using the option "-z truncate_only" of the script split_libraries.py. Once trimmed and assigned to samples, sequences were processed using the algorithm UCLUST (Edgar *at al*., 2010) in order to cluster the sequences in operational taxonomic units (OTUs) at the 97% identity level. A set of representative sequences of each OTU were subsequently aligned using PyNAST (DeSantis *et al*., 2006a) against the Greengenes core set (DeSantis *et al*., 2006b) version May2013. The taxonomic affiliation was assigned to each OTU using the Ribosomal Data Project (RDP) Classifier at a confidence threshold of 80% (Wang *et al*., 2007). In order to compute the diversity analysis, the number of reads of all samples was first normalized to the sample with the lowest number of reads (sample 6 AB5, 7050 reads) by using the QIIME script single_rarefaction_py. Rarefaction curves and alpha-diversity indices (OTUs number, Chao 1 and Shannon-Wiener) were calculated by using the QIIME script alpha_rarefaction.py.

2.6. Statistical analyses

Analysis of variance (ANOVA) was performed using the software package STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) with 95% of significance level ($p < 0.05$).

The Primer software (PRIMER-E, vs. 6.0, Plymouth, UK) was used to perform the analysis of similarity (ANOSIM). To execute ANOSIM analyses, the relative abundance data set were square-root transformed and sample resemblance matrices were generated using the Bray Curtis coefficient of similarity.

Multivariate statistical analysis was performed using CANOCO for windows v.4.5 software (ScientiaPro, Budapest, Hungary). To select the correct ordination

method (unimodal or linear), a preliminary detrended correspondence analysis (DCA) was performed. DCA revealed a linear, rather than a unimodal response of the different sets of biological data, thus a redundancy analysis (RDA) as a linear constrained ordination method was chosen for data analysis (Lepš *and* Šmilauer, 1999). RDA was performed to search for patterns in the set of species data (biological data) and to assess their relationship with the environmental data (system variables). The Monte Carlo permutation test option was used to obtain the statistical significance of each operational parameter in the canonical axes. The biological data analyzed throughout the whole set of samples were: 1. theoretical indices based on TGGE analysis (*Rr*, *Fo*, *Dy* and *H'*) and 2. relative abundance data set of OTUs obtained from 454-pyrosequencin analysis. The following environmental variables were used: HRTs (10 h or 24 h), CFR (20% or 35%, v/v), pH, temperature, COD and BOD_5. MLTSS, MLVSS, BTS and BVS. Due to the strong linear correlation between MLTSS and MLVSS, between BTS and BVS as well as between COD and BOD_5, one of the two parameters of each pair was removed from the final analyses. In this sense, to select the correct parameter, p-value was considered. In addition, HRT and CFR were expressed as nominal variables. All non-nominal environmental variables were transformed to log(x + 1), except pH.

3. Results and Discussion

3.1 Study of total Bacteria and AOB communities' structure by PCR-TGGE fingerprinting

To gain an overview of the dynamic of total Bacteria and AOB communities in both samples types (SB and AB) over the time and to address possible influence of

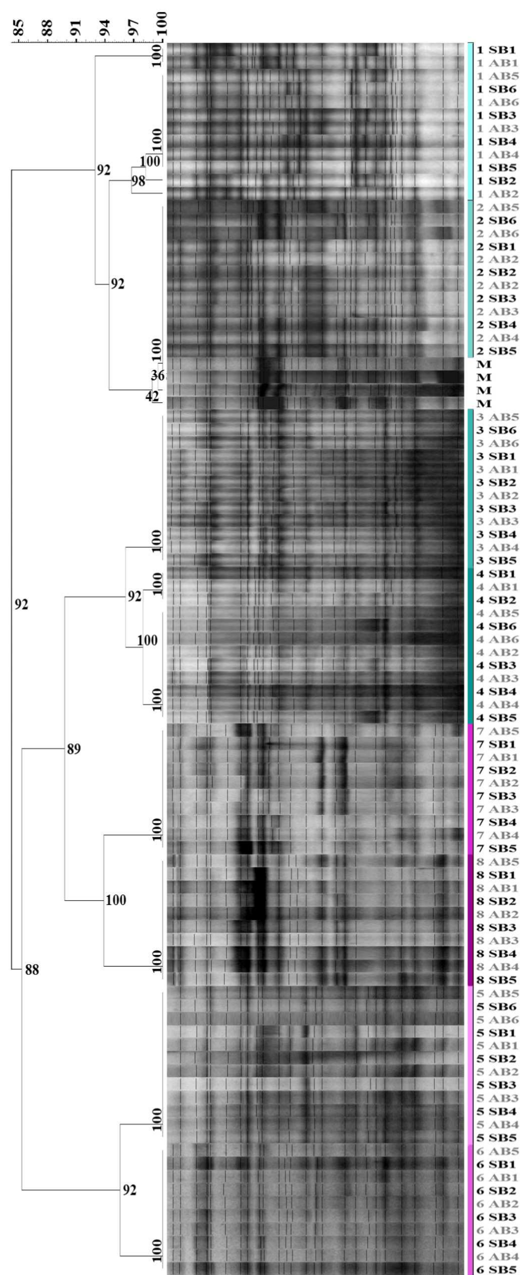
operational parameters on the evenness of these communities, TGGE fingerprinting was performed.

3.1.1 Cluster analysis of TGGE profiles

Figure IV.1. shows the TGGE profiles of total Bacteria (A) and AOB (B) communities with their corresponding dendrograms generated by the UPGMA cluster analysis of the TGGE banding patterns obtained for both samples types (SB and AB) in the eight experiments.

The internal markers (M) clustered with a 98.95% of similarity in total Bacteria fingerprinting and with a 99.9% of similarity in AOB (**Fig. IV.1 A** and **B**). Thus, significant differences in TGGE profiles for total Bacteria and AOB were consider below that levels. In both cluster analysis, it is remarkable that independently of the origin of the sample (SB or AB), significant similar band patterns (100% similarity) were observed for the same sampling day, with only minor exceptions. For total Bacteria community, the only observed exception was in experiment 4, in which the AB and SB in sampling 2 (4 AB2 and 4 SB2, respectively) showed a 98% of similarity. For AOB community, the SB samples taken in 1 and 2 sampling weeks of experiment 5 (5 SB1 and 5 SB 2) were grouped in an independent cluster showing a 78.5% of similarity with samples 5 AB1 and 5AB2, respectively. Moreover, AB sample taken in the sixth sampling week of experiment 2 (2 AB6) also was located in a different cluster respect to the SB sample (2 SB6), showing a similarity of 97.18%.

A. *Total Bacteria*



B. *AOB*

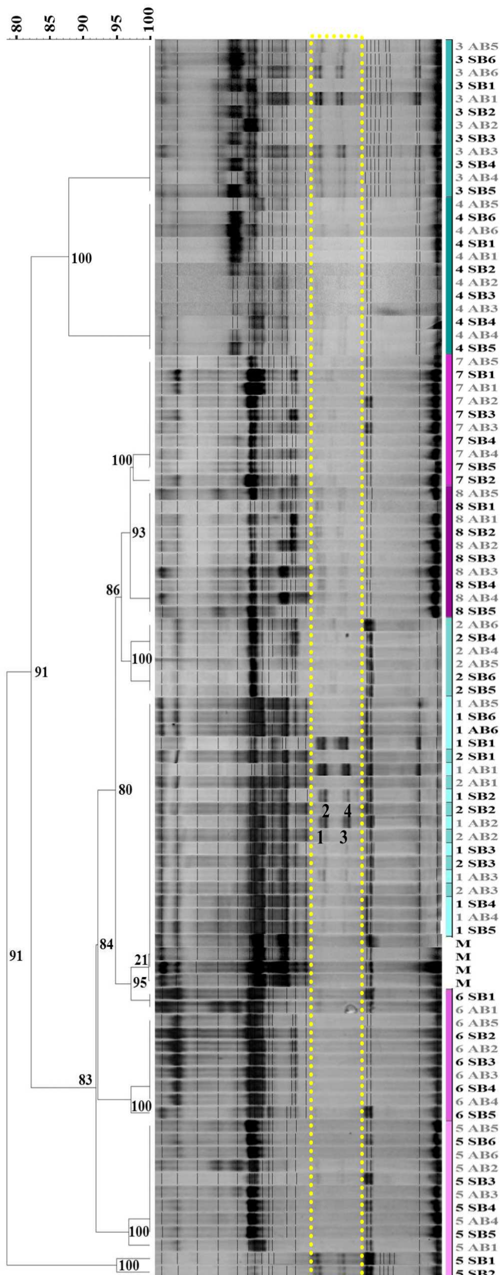


Figure IV.1. Dendrograms generated by UPGMA clustering (Dice correlation coefficient) of 16S rRNA based TGGE patterns from suspended biomass (SB) and attached biofilm (AB) samples taken during the eight experiments performed in the MBMBR system, using Gel Compar II v. 4.601 (Applied Maths, Belgium). **A**. Profiles of ***total Bacteria*** community based on the amplification and separation of the V3 hypervariable region of the 16S rRNA gene. **B.** Profiles of ***AOB*** community based on the specific amplification of the 16S rRNA gene belonging to the ammonium oxidizing β-Proteobacteria (AOB) and subsequent amplification of the V3 hypervariable region of the 16S rRNA gene. The scale bar indicates the percentage of similarity. Numbers in nodes represent the cophenetic correlation coefficient values**.** Experiments performed with 20% or 35% (v/v) CFR are marked in blue or pink colour scale, respectively. The samples nomenclature (from left to right) indicates the experiment, the samples type (SB or AB) and the sampling week. In black or grey colours are written the SB or AB samples, respectively. The band classes (1, 2, 3 and 4) highlighted within a yellow rectangle did not were phylogenetic related to AOB, thus they did were no include in cluster analysis.

Biomass immobilization on the support material allows microorganisms' retention in the system without being washed out, favouring the coexistence of mixed-species in the biofilm for a long time (Bassin *et al*., 2012). Moreover, biofilm morphology and structure (i.e., pore size and density) provides diverse microenvironments promoting the development of slow growing microorganisms (i.e. AOB) and the growth of both aerobic and anoxic microorganisms (Nicolella *et al.*, 2000; Fu *et al*., 2010; Wojnowska-Baryła *et al*., 2010; Biswas *and* Turner, 2012). Consequently, the biodiversity also could be promoted and therefore, differences between the microbial communities' structure from SB and AB could be expected. In fact, Biswas *and* Turner (2012) (using 16S rRNA gene clone libraries) observed differences between bacterial community of SB and AB in a MBBR. These authors reported that probably due to the low HRT used in MBBR systems, bacterial community in SB was dominated by aerobic bacteria, whereas in AB anaerobic bacteria were dominants. Later, Biswas *et al*. (2014) also observed these differences by pyrosequencing. However, in both studies, the differences were observed between SB and "*mature biofilm*". This last concept was defined by Biswas *et al*. (2014) as "*biomass on carriers residing in a functional MBBR system for several years*".

Nevertheless, similar community structure in SB and in "*early biofilm*", which were dominated by fast-growing aerobic bacteria during 48 days of study, was also reported by Biswas *et al*. (2014). As for similarity in the AOB community structure in SB and AB, this result was in agreement with a previous work done in a Fixed-Film Activated Sludge System (IFAS) reported by Short *et al*. (2013). These authors (using Functional Gene Microarray) compared the AOB diversity in SB and AB during four months and over this time period they are not observed differences between SB and AB communities. In concordance with the results obtained by Biswas *et al*. (2014), the time seems to be an important factor to find differences between communities' structure of SB and AB. In our case, the BTS and BVS concentrations obtained over time for 20% CFR (from experiment 1 to experiment 4, ten months) and for 35% CFR (from experiment 5 to experiment 8, eight months) showed the evolution of biofilm (**Table IV.2**), so differences could be expected in the last experiments with 20% or 35% CFR (v/v). Considering the few studies that approach the biodiversity of SB and AB together, we believe that this preliminary result obtained by TGGE must be considered with caution, since among the well know TGGE limitations, the co-migration of fragments with identical electrophoretic behaviour but different DNA sequences is very common (Marzorati *et al*., 2008; Loisel *et al*., 2006; Molina-Muñoz *et al*., 2009; De Vrieze *et al*., 2013).

Regarding the differences in community' structure among experiments, Dice coefficient-based analysis for total Bacteria (**Fig. IV.1A**) identified two main clusters, one with samples presenting a 93% of similarity and the other with 85.38% of similarity, respectively. In these clusters, samples taken in experiments 1 and 2 and samples of the experiments 3, 4, 5, 6, 7 and 8, respectively, grouped separately. It can be observed that the samples taken in the experiments performed with similar MLTSS

concentration (c.a. 2,500 mg/L or c.a. 4,500 mg/L) and CFR (20% or 35%, v/v) were correlated in the same cluster in which the samples were finally sub-clustered by experiment, suggesting that bacterial community might be affected by changes in the MLTSS and HRT. As for possible influence of CFR, it should be noted, that all the samples taken in the experiments 5, 6, 7 and 8, performed with a 35% (v/v) CFR, showed a similarity of 100% within the same experiment. However, when MBMBR worked with a 20% (v/v) CFR, the SB and AB samples of the first sampling week in the experiment 1 (1 SB1 and 1 AB1, respectively) were clustered in a different sub-cluster. Similar trend was observed in experiment 4 with the SB samples taken in the first weeks (4 SB1 and 4 SB2) and the AB sample of the first week (4 AB1). These observations seem to indicate that with a 35% (v/v) CFR higher stability of the bacterial community in each experiment can be obtained. In addition, experiments 3, 4, 7 and 8 performed with higher MLTSS concentration (c.a. 4,500mg/L) clustered with higher similarity index (89.79%) comparing with experiments 1, 2, 5 and 6 conducted with c.a. 2,500 mg/L of MLTSS, which showed a similarity of 84.1%. Emphasizing that experiments 1, 2, 3 and 4 were conducted with 20% of CFR (v/v) and experiments 5, 6, 7 and 8 with 35% CFR (v/v), these results suggest that the CFR could have a minor influence in bacterial community structure when the MBMBR worked with higher MLTSS concentration.

Dice coefficient-based analysis for AOB (**Fig. IV.1B**) revealed also a high similarity index (78.7%) among samples profiles through the 8 experiments. However, even if samples tended to cluster by experiment, higher variability of AOB community structure within the same experiment was observed compared to total Bacteria community. It is remarkable that the samples taken in experiments 1 and 2 were located in the same sub-cluster independently of HRT (10h and 24h, respectively) with the

exception of the samples taken in the last weeks of experiment 2 (2 AB6, 2 SB4, 2 AB4, 2 AB5, 2 SB6, 2 SB5) which grouped in a different sub-cluster. It is also significant that the first SB samples taken in experiment 5 (5 SB1 and 5 SB2) clustered separately. Considering the slow growth rate of AOB, time could be an important parameter to reach the functional stability of the AOB community within the same experiment. In fact, Wan *et al*. (2011) observed that the clustering of AOB community reflected a gradual evolution over the time. Similarly, Wittebolle *et al*. (2008) also observed this clustering trend. Concretely, these authors reported a higher functional stability of AOB community after the start-up period, observing increase of stability over the time during the steady period.

In general, it is not surprising to observe differences in the microbial communities' profiles among experiments, since in previous studies (using also DGGE or TGGE) it has been reported the influence of suspended biomass concentration, HRT or CFR in bacterial community of SB or AB developed in MBRs, MBBRs and Biofilm reactors (Molina Muñoz *et al*., 2009; Calderón *et al*., 2012b; Matos *et al*., 2012; Calderón *et al*., 2013; Cerrone *et al*., 2013). However, the strong similarity (84.1% and 78.7% for total Bacteria and AOB, respectively) observed in cluster analysis among all samples profiles was very remarkable.

According to Briones *and* Raskin (2003), by increasing the microorganisms' populations involved in the depuration process it is possible to ensure a greater adaptation of populations to disturbances. These authors also reported that the biofilm microbial community in bioreactors represents a good example of functional stability. Furthermore, it is also known that when suspended and attached growths are combined, biomass concentration increases in the system as a direct result of the biofilm presence

(Gavrilescu *and* Macoveanu, 2000; Mannina *et al*., 2009; Wang *et al*., 2010; Khan *et al*., 2011). In view of this, higher similarity or "*stability*" of the dominant populations observed among the experiments could be due to the coexistence of both SB and AB in the MBMBR.

3.1.2. Analysis of diversity, structure and functional organization of total Bacteria and AOB communities

It has been suggested that to perform an optimal design of wastewater treatment systems (WWTSs), a solid understanding of the ecological principles that characterize the microbial community is fundamental (Wang *et al*., 2012a). Indeed, it is known that high degree of diversity and functional redundancy tend to favour the stability of microbial communities, ensuring functionally more stable system performance (Briones *and* Raskin, 2003; Wittebolle *et al*., 2008). In short, accordingly to Ayarza *et al*. (2010) these characteristics, guarantee the presence of different microorganisms with the same ecological function, which can thrive under different environmental/operational conditions (Ayarza *et al*., 2010). For these reasons, to achieve a better knowledge about the evenness of the studied communities under different operational conditions, several indices (*Rr*, *Fo*, *H'* and *Dy*) based on TGGE cluster analysis were calculated. **Figure IV.2** shows the mean values of indices (*Rr*, *Fo*, *H'* and *Dy*) of SB and AB samples obtained in each experiment for total Bacteria (A) and AOB (B) communities. A multifactor ANOVA was performed to detect the possible significant differences in indices levels between sample type (SB and AB) as well as among experiments.

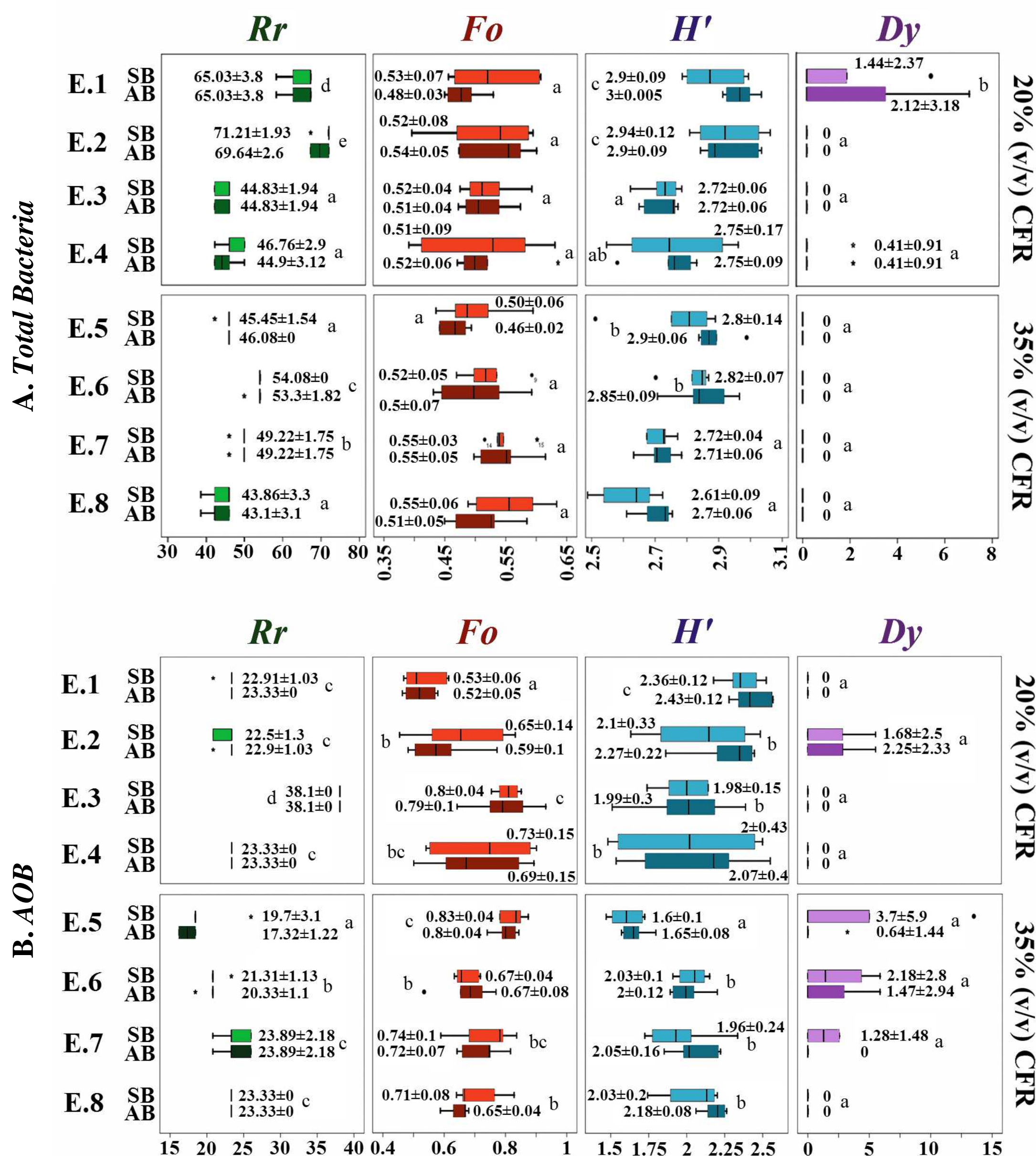


Figure IV.2. Box-and-Whisker plots of the ecological indices: Range weighted richness index (*Rr*), Functional organization (*Fo*), Shannon–Wiener (*H'*) and Dynamics index (*Dy*) calculated for suspended biomass (SB) and attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR with a 20% or 35% (v/v) CFR. **A**. Box-and-Whisker plots of the ecological indices for *total Bacteria* community and **B**. for *ammonium oxidizing β-Proteobacteria* (*AOB*) community. In plots, upper and lower bounds of the box denote the 75th and 25th percentiles, upper and lower bounds of bars represent the 90th and 10th percentiles, and outliers values are represented by circles. Average values ± standard deviations are shown near the boxes. Multifactor ANOVA did not show significant differences in indices levels between sample type (SB and AB). Thus, significant differences among experiments are indicated considering both samples type (SB and AB). Data followed by the same lower-case letter do not significantly differ according to the Student's test ($p < 0.05$).

In concordance with results of cluster analysis, for both communities (total Bacteria and AOB) the multifactor analysis of variance revealed no significant differences between indices of SB and AB samples ($p > 0.05$) in all experiments, indicating that species richness, functional organization, diversity degree and dynamic levels of total Bacteria and AOB communities were similar in both samples (SB or AB). The Range weighted richness index (*Rr*) for total Bacteria community was > 30 for all experiments (**Fig. IV.2A**). According to Marzorati *et al*. (2008) values of Rr > 30 define communities with high diversity, typical of very habitable environments such as those found in activated sludge. However, in concordance with Marzorati *et al*. (2008) AOB community was characterized by a medium range of diversity ($10 < Rr > 30$) in most of the experiments, with the exception of experiment 3 in which Rr was >30. Regarding functional organization (*Fo*), for total Bacteria community the index values scored around 0.5 in all the experiments, indicating a intermediate level of functional organization. The range between 0.45 and 0.8 is typical of communities with sufficient flexibility to react to changing environmental/operational conditions (Marzorati *et al*., 2008). Comparing *Fo* indices between total Bacteria and AOB communities, higher *Fo* values were obtained for AOB in almost every experiments (except E.1) (**Fig. IV.2B**), indicating a slightly higher functional organization of the AOB community. Therefore, AOB community was characterized as more specialized and probably more fragile to environmental/operational conditions changes (Marzorati *et al*., 2008). As for Shannon–Wiener index (*H'*), the average values obtained for total Bacteria community were in the range 2.5-3.1 (**Fig. IV.2A**), indicating a high diversity in all the experiments. The average values of *H'* for AOB communities were lower than those observed for total Bacteria community in all the experiments. Accordingly to Boon *et al*. (2002) low *H'* values indicate low diversity. Finally, the dynamics index (*Dy*) was calculated to know the stability of both communities (total Bacteria and AOB) within each experiment. For

both communities, low average rates of diversity change (*Dy*) were obtained in all experiments. Low values of *Dy* are characteristics of stable periods of operation in WWTSs (Marzorati *et al*., 2008). Indeed, it has been suggested that the functional stability of WWTSs and microbial community stability are interdependent (Falk *et al*., 2009; Levin Pal *et al*., 2012).

The differences observed in the various indices (*Rr*, *Fo* and *H'*) levels between the two communities (total Bacteria and AOB) could be expected, since it has been described that low diversity is common in nitrifying functional groups (Curtis *and* Sloan, 2006). Moreover, our results are in concordance with previous studies (Boon *et al*., 2002; Wan *et al*., 2011) in which also obtained lower levels of diversity in AOB community compared to total Bacteria community. In agreement with Boon *et al*. (2002), probably these results were a direct consequence of the lower number of bands in the TGGE fingerprinting of AOB compared to total Bacteria (**Fig. IV.1**). In addition, the average values of *H'* index obtained for AOB community were similar to those reported in previous studies dealing with AOB community developed in the SB in different WWTSs using DGGE (Ziembińska *et al*., 2009; Wan *et al*., 2011). It should also stated that the average values of *Rr*, *Fo* and *H'* obtained for total Bacteria community were similar to or higher than those described in works based on DGGE/TGGE in others WWTSs (Molina-Muñóz *et al*., 2009; Calderón *et al*., 2012a; Kim *et al*., 2012; Calderón *et al*., 2013).

To sum up, for all the experiments performed, the indices calculated for both sample types (SB and AB) revealed a total Bacteria community characterized by high species richness, medium functional organization, high diversity and strong stability. This suggests that the community developed in SB and AB of the MBMBR is able to

buffer the environment/operational changes. Consequently, these characteristics favour the stability of the WWTSs, promoting optimal depuration efficiency (Briones *and* Raskin, 2003; Curtis *and* Sloan, 2006; Miura *et al*., 2007a; Ayarza *et al*., 2010).

Nevertheless, despite of the indices in all the experiments were in the same range of functional organization, richness, diversity and dynamics, multifactor ANOVA (Student's test) revealed significant differences among some experiments in both communities (total Bacteria and AOB) (**Fig. IV.2 A** and **B**), suggesting the possible influence of environmental/operational parameters. Thus, to further support these observations, multivariate statistical analysis (RDA) was performed for total Bacteria and AOB communities. RDA correlated the simultaneous variation of physico-chemical parameters (BTS, MLTSS, BVS, COD, BOD_5, pH, temperature, HRT and ammonium influent concentration) and CFR (20% or 35%, v/v) to the indices (*Rr*, *Fo*, *H'* and *Dy*) measured in SB and AB in the eight experiments. The biplot diagrams generated for total Bacteria and AOB communities are shown in **Fig. IV.3** (**A** and **B**, respectively).

For total Bacteria, as revealed by the Monte Carlo permutation test, the significant parameters explaining the indices variations were: MLTSS (p = 0.002), COD (p = 0.002), temperature (p = 0.008), CFR (p = 0.012) and BTSS (p= 0.036). The first canonical ordination axis (horizontal) was mainly correlated with MLTSS, BTSS and CFR and the second canonical ordination axis (vertical) with COD and temperature. The first and second canonical ordination axes (COA) described 31.3% and 13.9% of the total variation of the indices values, respectively (69.1% of the indices-operational parameters relations explained by the first axis and 30% by the second one).

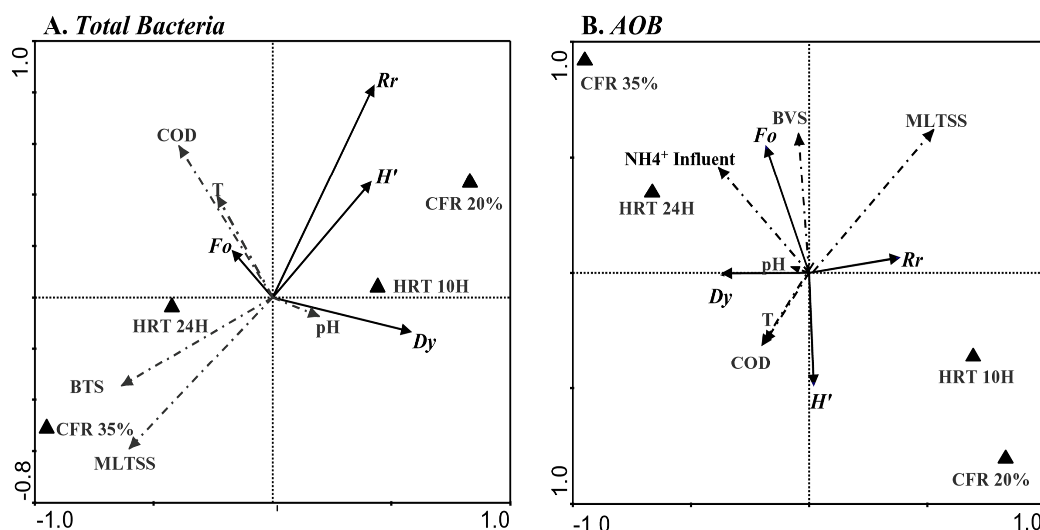


Figure IV.3. Redundancy analyses (RDA) of the ecological indices of *total Bacteria* community (**A**) and ammonium oxidizing β-Proteobacteria (*AOB*) community (**B**). The ordination diagrams (biplots) show operational conditions (dotted arrows and triangle) and ecological indices (straight arrows), measured in the eight experiments performed in the MBMBR system. Operational parameters: pH, Temperature (T), Mixed Liquor Volatile Suspended Solids (MLVSS), Biofilm Total Solids (BTS), Biofilm Volatile Solids (BVS), Biological Oxygen Demand at 5 days in influent water (BOD_5), Chemical Oxygen Demand in influent water (COD), Hydraulic Retention time (HRT: 10 and 24 h), ammonium influent concentration ($NH4^+$ Influent) and CFR (v/v) (20% or 35%). HRT and CFR are shown as nominal variables (triangle). Ecological indices: Range weighted richness index (*Rr*), Functional organization (*Fo*), Shannon–Wiener (*H'*) and Dynamics index (*Dy*) for suspended biomass and attached biofilm.

RDA analysis revealed strong positive correlation of *Rr* and *H'* indexes with a 20% (v/v) of CFR. In fact, the highest values of these indexes were recorder in experiments 1 and 2 conducted with 20% (v/v) CFR (**Fig. IV.2A**). Moreover, it is notable, the strong negative correlation between biomass concentrations (MLTSS and BTS) and *Rr* and *H'* indices, specifically when the system operated with 20% (v/v) of CFR, indicating that high richness and diversity in the MBMBR were obtained at low biomass concentrations (c.a. 2,500 mg/L) and CFR (20%, v/v) in both samples type (SB and AB) (Experiments 1 and 2). Similarly, a slightly negative correlation between

biomass concentrations and *Dy* index when the MBMBR was conducted with 20% (v/v) CFR was observed, so high stability of the community was obtained in the experiments conducted with high CFR (35%, v/v) (Experiment 5, 6, 7 and 8). The RDA analysis was in agreement with the trend observed in cluster analysis (**Fig. IV.1A**), in which experiments 1 and 2 clustered away from the rest of the samples, as well as the high stability observed within each experiment performed with a 35% (v/v) of CFR. As for *Fo* index, the direction and location of the *Fo* vector revealed little influence of CFR and solid concentrations (MLTSS and BTS), due to the minimal variations (non-significant) observed in the values of this index among the experiments (**Fig. IV.2A**), suggesting that changes in community structure did not entail significant changes in their functional organization. Similarly, Calderón *et al*. (2012b) using TGGE reported differences in bacterial community structure of biofilm developed in a MBBR system in function of CFR, despite of *Fo* index values were similar among the experiments performed with different CFR (20%, 35% and 50%). In agreement with these authors, Matos *et al*. (2012) also observed differences in bacterial community structure of SB and AB analyzed by DGGE, which were induced by an increase of CFR. However, these authors did not report data about functional organization and diversity of this community. In relation to the strong negative correlation of biomass concentration (MLTSS and BTS) with *Rr* and *H'* indexes observed in our study, similar results in a MBR system has been previously reported by Calderón *et al*. (2013).

In accordance with RDA analysis, COD concentration and temperature were the significant variables with minor influence on indices values, since they were correlated with the second COA, which only explained a 13.9% of total indices variations. Both physical-chemical parameters showed a slightly positive correlation with *Rr* and *H'* indices and a strong positive correlation with *Fo* index. However, as commented above,

Fo was fairly similar in all experiments (**Fig. IV.2A**), confirming the slight influence of these parameters on the indices. A slight increasing of diversity at higher COD concentrations was observed also by Cydzik-Kwiatkowska *et al.* (2012). In concordance with these authors, this correlation could be expected only when COD levels are in a restricted range, since at high COD concentrations the diversity tends to decrease. On the other hand, in studies based on TGGE or DGGE fingerprinting, temperature has been described as an important factor influencing the bacterial community structure in different WWTSs (Molina-Muñoz *et al.*, 2009; Siggins *et al.*, 2011; Cydzik-Kwiatkowska *et al.*, 2012; Calderón *et al.*, 2013). Concretely, Calderón *et al.* (2013) and Cydzik-Kwiatkowska *et al.* (2012) established a link between the diversity degree in the bacterial community and the temperature, reporting a negative correlation between both bacterial community and temperature. However, these studies were performed in systems based on suspended growth only (MBR and CAS, respectively). In this sense, it is necessary to consider that a minor influence of the temperature in attached biofilm has already been reported (Guelli de Souza, 2012). In addition, even if currently the information about this matter is limited, the coexistence of both types of biomass (SB and AB) seems to favour multiple interactions between them including the migration of microorganisms between the two types of biomass (Boltz *et al.*, 2011; Makowska *et al.*, 2013). In this context, although temperature in our study showed significant differences among some experiments (**Table IV.2A**), possibly, presence of attached biofilm and possible recurrence of interactions between SB and AB could have buffered the temperature effect in functional organization, richness, diversity and dynamic of the bacterial community developed in the MBMBR system.

Regarding the AOB community, the Monte Carlo permutations test revealed that only MLTSS (p = 0.048) and BVS (p = 0.048) were the significant parameters explaining the variation of the indices. Both parameters were mainly correlated with the second canonical ordination axis (vertical). The first and second canonical ordination axes (COA) described 13.2% and 1.5% of total variation of the indices values, respectively (83% of the indices-operational parameters relations explained by the first axis and 7.2% by the second one). This indicates that the total variation of the evenness of AOB community was poorly explained with the physico-chemical parameters measured. Accordingly to Wells *et al*. (2009) and Wang *et al*. (2012a), other parameters no included in our analysis (inhibitory chemicals compounds, dissolved oxygen concentration as well as stochastic influences and predation) could have an important effect on the dynamic of AOB community in WWTSs.

In contrast to the trend observed for total Bacteria community, AOB community showed significant differences in *Fo* levels among some experiments. RDA revealed slight positive correlation of this index with MLTSS concentration and a strong positive correlation with BVS (**Fig. IV.3B**), indicating that irrespective of CFR higher functional organization was obtained in experiments performed with higher MLTSS concentrations (4,500 mg/L) in which also higher BVS were obtained (**Table IV.2B**). However, this result must be considered with caution, since with a 35% (v/v) of CFR, the highest *Fo* values were obtained in experiment 5 performed with lower MLTSS concentration (2,500 mg/L) (**Fig. IV.2B**). This result is curious, since experiment 5 not only showed the highest values of *Fo* index, but also the lowest values of *Rr* and *H'* obtained in our study (**Fig. IV.2B**), suggesting that AOB community in this experiment could be more sensible to changes in environmental/operational conditions. In our view, these findings could explain the independent clustering of some samples from

experiment 5 observed in cluster analysis of AOB TGGE profile (**Fig. IV.1B**). As for *Dy*, despite of Student's test revealed no significant differences of this index among experiments (**Fig. IV.2B**), RDA analysis revealed negative correlation between *Dy* and MLTSS concentration. Similar result was obtained for total Bacteria community, confirming that in the experiments performed with high MLTSS concentration the stability of both communities was favoured. These RDA results can help to interpret the cluster analyses, since they were in agreement with the clustering trend observed (**Fig. IV.1**). In the other hand, for AOB community the RDA biplot showed an opposite trend for Rr values compared to total Bacteria community. Briefly, a slight positive correlation between Rr values of AOB community and MLTSS concentration was revealed, indicating that for both CFR (20% or 35%, v/v) higher species richness of AOB was obtained in experiments 3 and 4 (20% CFR) and in experiment 7 and 8 (35% CFR) performed with higher MLTSS concentrations (c.a. 4,500 mg/L). Finally, although it is noticeable that *H'* values were fairly similar among the experiments (**Fig. IV.2B**) the angle formed between the vectors of *H'* and BVS revealed a strong negative correlation of this operational parameter on the diversity index (**Fig. IV.3B**). It can be observed in **Fig. IV.2** (**B**) that the highest values of *H'* index were obtained in experiment 1 in which minimal BVS concentration was obtained.

The nitrifying bacteria have been described as highly sensitive to several environmental/operational parameters such as, temperature, dissolved oxygen and ammonium concentration in WWTPs (Lydmark *et al*., 2007; Wells *et al*., 2009; Pal *et al*., 2012). However, there are certain discrepancies among the results reported about the influence of environmental/operational conditions on the AOB communities and, accordingly to Wang *et al*. (2012a) the ecological principles underlying AOB community dynamics are poorly understood. As an example of these discrepancies and

concretely in studies based on DGGE; Lydmark *et al*. (2007) reported that ammonia concentration was an important factor to explain changes in AOB community structure. Conversely, Ziembińska *et al*. (2009) observed that low ammonia concentration in the influent did not have a significant influence on AOB community structure. On the other hand, all the studies, establishing a link between AOB community structure and temperature (using also DGGE), concluded that temperature did not have influence on the community structure (Limpiyakorn *et al*., 2005; Ziembińska *et al*., 2009; Cydzik-Kwiatkowska *et al*., 2012). However, it is curious that a strong influence of the temperature on AOB community dynamic studied by T-RFLP methodology has been reported by Wells *et al*. (2009) and Wang *et al*. (2012a). In addition, these authors also reported no significant influence of solid concentration and HRT on AOB community structure. According to these comments and considering also our results, we suggest that further investigation to elucidate the effect of environmental/operational parameters in AOB community would be necessary.

At this point we would like to remind that the changes in the structure of total Bacteria and AOB communities observed in cluster analysis among all the experiments, not always resulted in significant changes of the values of ecological indices (*Rr*, *Fo*, *H'* and *Dy*). Therefore, differences in microbial community structure "possibly due to changes in the operational conditions" not always entailed changes in the functional stability of the bacterial communities and consequently in the efficiency of depuration process. In this sense, our results suggest that, despite of the differences observed in the community structure of both (total Bacteria and AOB) among experiments with similar MLTSS and different HRT (1-2; 3-4; 5-6 and 7-8) in cluster analysis, the functional stability of dominant populations in terms of *Rr*, *Fo*, *H'* and *Dy* stability did not was affected by changes of the HRT. By contrast, multivariate analyses revealed that

functional stability of both communities was mainly affected by changes in solid concentration (MLTSS, BTS or BVS), highlighting that the variation of the evenness of AOB community was poorly explained.

3.1.3. Phylogenetic study of the prevalent TGGEs bands of total Bacteria and AOB

Predominant TGGE bands were successfully re-amplified and sequenced from TGGE gels. Regard to dominant total Bacteria populations, a total of 33 band classes were identified in cluster analysis, 18 of which were common to eighth experiments conducted in the MBMBR system and were present in 100% of the analyzed samples (SB and AB) (**Fig. IV.4A**) confirming the high stability of the dominant populations among the experiments. A total of 28 TGGE bands were sequenced, most of them (24) were related to the phylum *Protobacteria* with a main cluster related to α-*proteobacteria* and a group with less abundance related to β-*proteobacteria* (See **Fig. IV.5**). Noting that most of the band class associated to β-*proteobacteria* (B6, B12, B8, B14, B11) were related to the family *Comamonadaceae*. The remaining bands sequenced were grouped in two minority lineages: *Actinobacteria* (bands; B19 and B23) and *Firmicutes* (bands; B4 and B5) (**Fig. IV.5**), noting that these bands were presents in all the samples analyzed through the eight experiments (**Fig. IV.4A**).

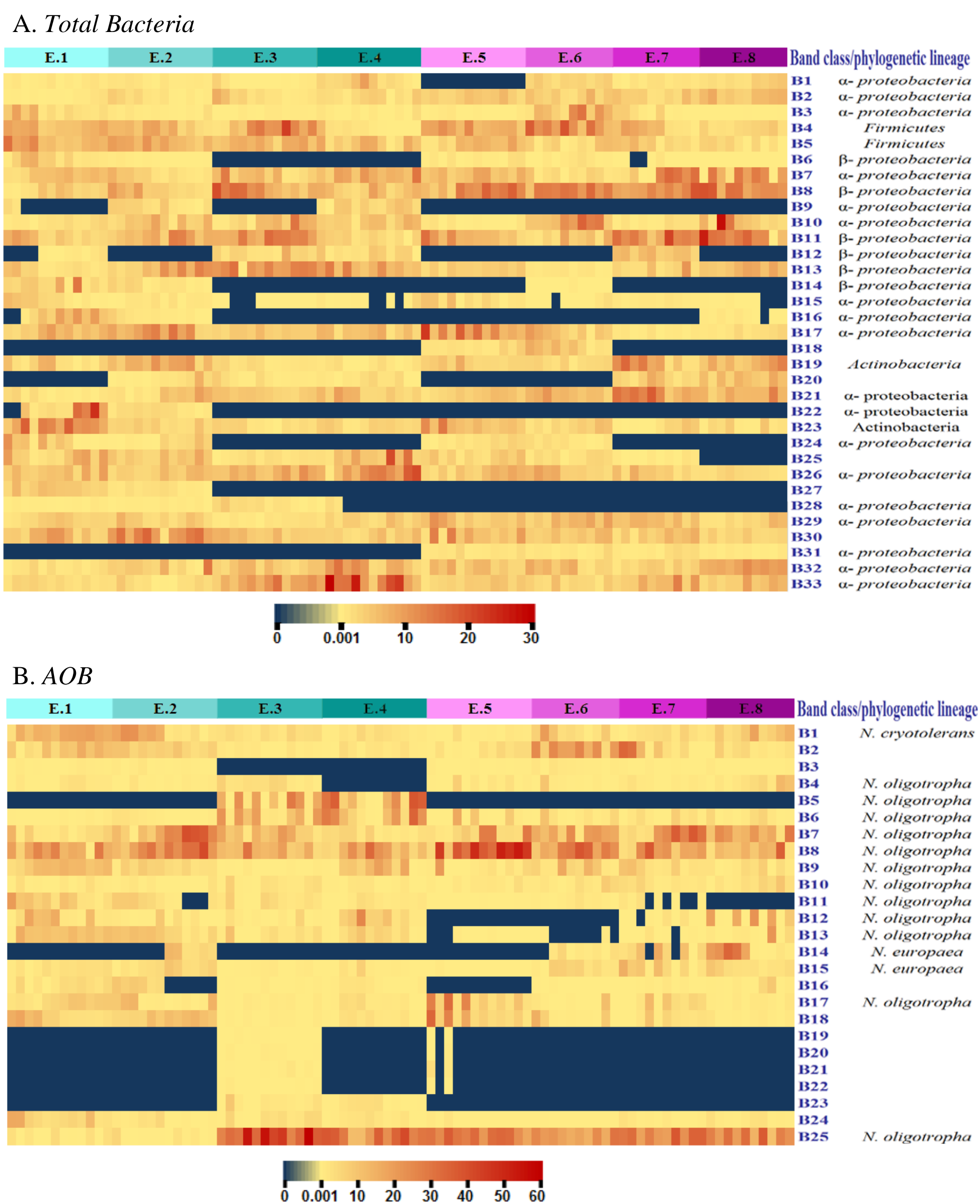


Figure IV.4. Heat maps representing the relative abundance of each TGGE band-class identified for *total Bacteria* community (**A**) and for *AOB* community (**B**) from suspended biomass (SB) and attached biofilm (AB) samples taken during the eight experiments (E.1-E.8) performed in the MBMBR system. Each rectangle panel represents one sample. The samples are arranged by sample type (SB-AB) and by sampling week, e. g. the first and second rectangle panels show the relative abundance of SB and AB, respectively in the first sampling week of the experiment 1. The phylogenetic lineages of the TGGE band-classes sequenced and identified are showed. The colour intensity of both scale bars indicates the different values of relative abundance in each TGGE band-class identified for *total Bacteria* community (**A**) and for *AOB* community (**B**). The maximum values of abundance among TGGE band-classes of *total Bacteria* and of *AOB* were 27.87% and 53.93%, respectively.

Numerous studies based on DGGE or TGGE showed that the phylum *Proteobacteria* is generally dominant in WWTPs, being β-*proteobacteria* the prevalence class (Xin-chun *et al*., 2007; Molina-Muñoz *et al*., 2009; Hesham *et al*., 2012; Li *et al*., 2012; Calderón *et al*., 2013). Moreover, the predominance of β-*proteobacteria* has been also observed (using DGGE/TGGE) in the biofilm community developed in MBBR systems (Fu *et al*., 2010; Calderón *et al*., 2012b). By contrast, in our study the dominance of α-*proteobacteria* appeared to be clear in SB and AB, since 84.8% of the total band class has been successfully re-amplified and sequenced. Our results are in concordance with recent studies conducted with real urban wastewater (Lopez-Lopez *et al*., 2012; Calderón *et al*., 2013), in which was also observed by re-amplification and sequencing of TGGE bands the prevalence of α-*proteobacteria* in a MBR and in the AB of a MBBR system, respectively. In addition, the family *Comamonadaceae* (β-proteobacteria), considered as part of the cosmopolitan freshwater cluster β1 (Glöckner *et al*. 2000) also has been described as prevalent in different WWTSs (Molina-Muñoz *et al*., 2009; Calderón *et al*., 2011; Calderón *et al*., 2012b). On the other hand, the low number of band classe related to Phyla *Actinobacteria* and *Firmicutes* in our study is not surprising, since the low abundance of both lineages in different WWTSs has been reported in several studies by TGGE/DGGE (Fernández *et al*., 2008; Molina-Muñoz *et al*., 2009; Kim *et al*., 2012; Calderón *et al*., 2013). Concretely, Hu *et al*. (2012) concluded that the low abundance of *Firmicutes* compared to *Proteobacteria* could be attributed to the weakness of the *Firmicutes* microcolonies under strong shear forces in the bioreactor.

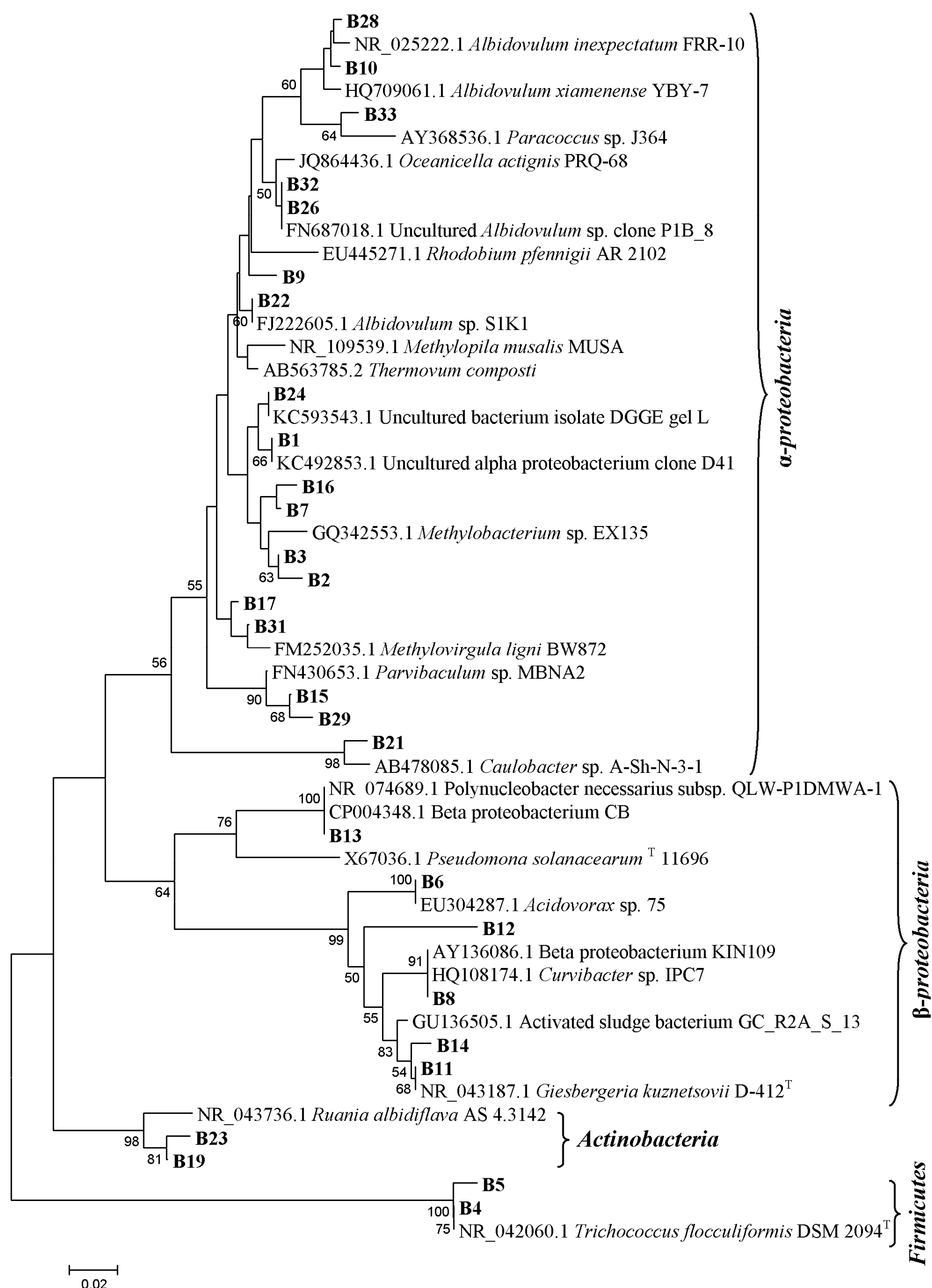


Figure IV.5. Neighbor-joining phylogenetic tree showing the positions of 28 total *Bacteria* sequences from re-amplified TGGE bands and the most similar sequences retrieved from the NCBI database, based on *ca*. 100 nt length of sequences. The scale bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes. T = type strain.

Regard to dominant AOB populations, a total of 25 band classes were identified in cluster analysis, 12 of which were common to eighth experiments conducted in the MBMBR system and were present in 100% of the analyzed samples (SB and AB) (**Fig. IV.4B**), confirming the lower stability of the dominant populations of AOB among experiments compared to dominant populations of total Bacteria. A total of 19 TGGE bands were successfully re-amplified and sequenced, however 4 of them (1, 2, 3 and 4) were not phylogenetic affiliated to ammonium oxidizing β-*proteobacteria*, but to the genus *Aquaspirillum* (**Fig. IV.6**); therefore these band classes were not considered in cluster analysis. **Figure IV.1** (**B**) shows the position of these bands in the TGGE gel (highlighted within a yellow rectangle). The 15 remaining bands sequenced belonged to ammonium oxidizing β-*proteobacteria* and were related to the genus *Nitrosomonas*, thus no sequences related to genus *Nitrosospira* were found. In accordance with the phylogenetic clusters of AOB belonging to the β-subclass of the *Proteobacteria* reported by Purkhold *et al*. (2003), the bands sequences were affiliated to *Nitrosomonas oligotropha/ureae* (B4, B7, B8, B25, B10, B11, B9, B12, B13, B17, B5 and B6), *Nitrosomonas Cryotolerans* (B1) and *Nitrosomonas europaea* (B14 and B15), see **Fig. IV.6.**

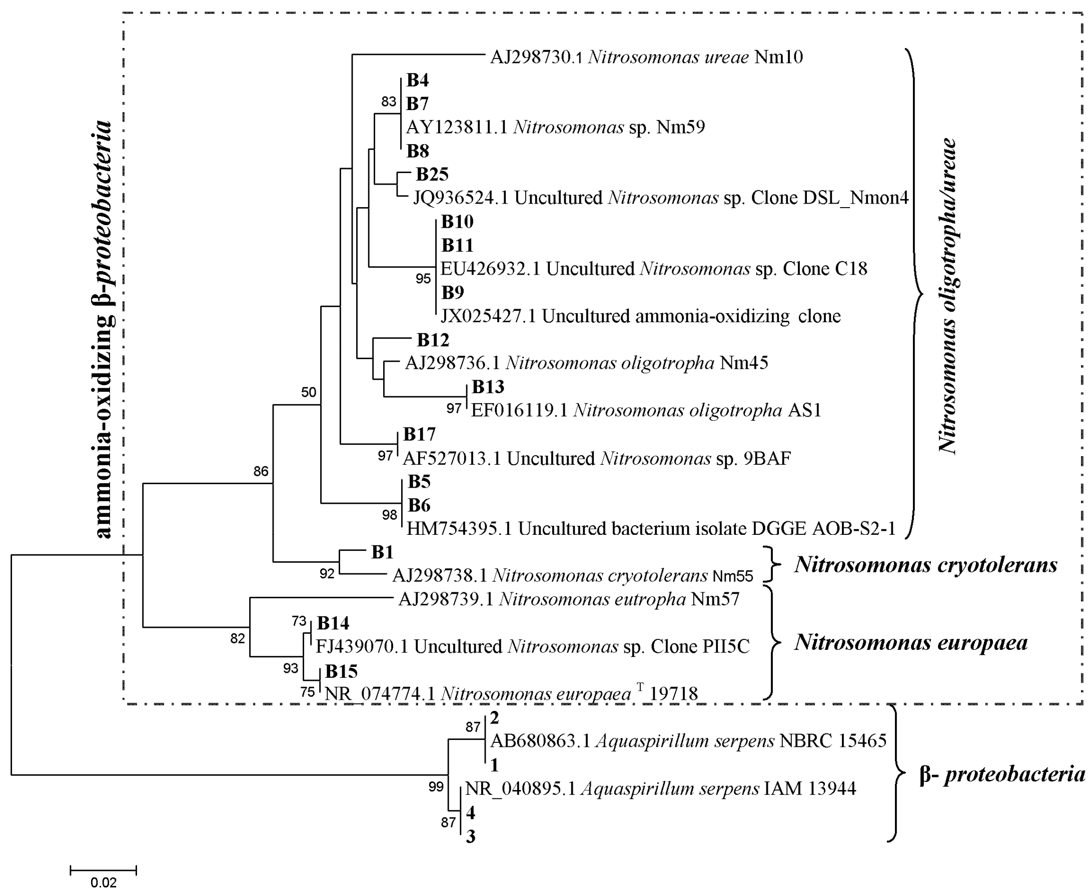


Figure IV.6. Neighbor-joining phylogenetic tree showing the positions of 15 *ammonium oxidizing β-proteobacteria* (AOB) sequences from re-amplified TGGE bands and the most similar sequences retrieved from the NCBI database, based on *ca*. 100 nt length of sequences. The bands (1, 2, 3 and 4) were re-amplified also from TGGE of AOB but they were not phylogenetic related to AOB. The scale bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes. T = type strain.

The presence of bands affiliated to *Aquaspirillum* in DGGE gels based on partial and specific amplification of the 16S rRNA gene belonging to the ammonium oxidizing β-*proteobacteria* (using CTO primers) has been previously reported (Cébron *et al*., 2004; Wittebolle *et al*., 2005). Indeed, accordingly to Purkhold *et al*. (2000) none of the different primers sets that exist to target ammonium oxidizing β-*proteobacteria* shows both 100% sensitivity (targeting all beta-subclass AOB) and 100% specificity (excluding all non beta-subclass AOB). Later, Purkhold *et al*. (2003) reported that although AOB phylogenetic profiles obtained from the amplification of the 16S rRNA gene and the *amoA* gene exhibit congruent topologies, the *amoA* primers miss some AOB populations and, consequently, these authors demonstrated that more resolution with 16S rRNA versus *amoA* gen is obtained. However, according to Mahmood *et al*. (2006) a disadvantage of the CTO primers is their non specific amplification of AOB sequences when AOB are present at low relative abundance. In the light of the above and in agreement with Wittebolle *et al*. (2005) we think that for a good study of AOB community by TGGE (using CTO primers) it would be important to verify the non specific affiliations by re-amplified and sequencing of the bands.

On the other hand, the predominance of bands related to genus *Nitrosomonas* over *Nitrosospira* has been commonly observed in both biofilm- and biomass-based technologies of wastewater treatment (Purkhol *et al*., 2000; Gómez-Villalba *et al*., 2006; Lydmark *et al*., 2007; Xia *et al*., 2010; Cerrone *et al*., 2013). Concretely, in our study similar result was observed, although some band classes assigned by fingerprinting remained unidentified, a 60% of the total band classes were successfully sequenced and affiliated to *Nitrosomonas* (**Fig. IV.4B**). Presence of *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans and Nitrosomonas europaea* in wastewater habitats has been reported by Purkhold *et al*. (2000) and (2003). In addition, the

prevalence of *N. oligotropha* and *N. europaea* in our MBMBR system and concretely the dominance of *N. oligotropha* also have been reported in previous studies analyzing AOB community by fingerprinting methodologies in different WWTSs (Lydmark *et al*., 2007; Wan *et al*., 2011; Wang *et al*., 2012a).

Physiological differences between members of *N. oligotropha* and *N. europaea* clusters, mainly due to their substrate (NH_3) affinity and urease activity have been reported by Koops and Pommerening-Röser (2001). Concretely, these authors described a low substrate affinity and positive urease activity of the members belonging to *N. oligotropha* cluster, while *N. europaea* showed a higher NH_3 affinity and negative urease activity. Accordingly to Bollmann *et al*. (2002), *N. oligotropha* consider as K-strategist are better adapted to growth al low ammonium concentration, being they better competitors under limiting ammonium concentrations compared to *N. europaea* (r-strategist). By contrast, *N. europaea* presents a relatively high growth rate and predominance in habitats with high N input and turnover. For these reasons, sometimes it is expected the predominance of *N. europaea* in WWTPs. However, we must not forget that in a MBMBR system the microorganisms developed in different types of aggregates (flocs and biofilm) and accordingly to Nicolella *et al*. (2000) the microorganism with lower growth rates (nitrifying bacteria) are located on the inner layers of aggregates. Also according with these authors, it is known that the diffusion of substrates through the aggregates not only depends to the substrate concentration in the surrounding medium, but also of the density and porosity of the aggregate. Therefore, although exists a high ammonium concentration in the influent, the availability of this substrate for member belong to *Nitrosomonas* might be reduced in the aggregates and consequently the developed of *N. oligotropha* could be favoured. In addition, has been reported that *N. oligotropha* are "*self-floculating*", in other words these bacteria tends to

development forming aggregates, while *N. europaea* are not typically self-flocculating in pure cultures (Koops and Pommerening-Röser, 2001; Bollmann *et al*., 2002). Therefore, other possible explanations for the higher abundance of *N. oligotropha* versus *N. europaea* in all the experiments performed in the MBMBR system could be the presence of support material (carriers) in the bioreactor, in which the growth of *N. oligotropha* could be favoured.

3.2. Study of total Bacteria community structure by 454 pyrosequencing

Despite the valuable information about the bacterial community structure provided by TGGE fingerprinting, nowadays it is undeniable that 454-pyrosequencing is one of the most successfully technology for deep exploration of microbial community in WWTPs. So far, this tool has been mainly applied to study the bacterial community structure in conventional activated sludge and MBRs systems (Hu *et al*., 2012; Zhang *et al*., 2012; Kim *et al*., 2013; Ye *and* Zhang, 2013), being poorly used to investigate the bacterial diversity in WWTSs based on both SB and AB (Kwon *et al*., 2010; Biswas *et al*., 2014).

Basing on the strong stability of the dominant total Bacteria community structure within of each experiment observed by TGGE fingerprinting, and considering the importance of the time in the biofilm development, the last sample of SB and AB of each experiment (1 SB6, 1 AB6, 2 SB6, 2 AB6, 3 SB6, 3 AB6, 4 SB6, 4 AB6, 5 SB6, 5 AB6, 6 SB5, 6 AB5, 7 SB5, 7 AB5, 8 SB5, 8 AB5) was selected to study the total Bacteria community structure by 454-pyrosequencing.

3.2.1. Richness and Diversity indices

After sequences processing, a total of 178,550 high quality reads for the 16 samples was obtained. The sequences reads were in a range from 7,050 to 23,627 per sample with an average read length of 431.3 bp. Subsequently, the number of reads of each sample was normalized to 7,050 sequences in order to perform the analyses of the different samples at similar sequencing depth as previously reported Zhang *et al.* (2012). **Table IV.3**. shows the numbers of OTUs, Chao 1 and Shannon-Wiener indices obtained for each sample (cut-off level of 3%). A total of 3,712 OTUs were detected at 97% sequences identity, being the number of OTUs per sample from 416 to 1026. The values obtained for Chao 1 and Shannon-Wiener indices ranged from 554.075 to 1632.7 and from 5.602 to 7.656, respectively. The values of Shannon-Wiener index for both sample types (SB and AB) in our MBMBR system were consistent with previously reported (cut-off level of 3%) in CAS and MBRs systems (Hu *et al.*, 2012; Ye *and* Zhang, 2013). However, our values were significantly higher compared with the values of Shannon-Wiener (cut-off level of 3%) described in a MBBR system by Biswas *et al.* (2014). The Shannon-Wiener values reported by these authors ranged from 1.42 to 4.16. This result could suggest that a higher bacterial diversity in SB and AB could be favoured in a MBMBR system compared with a system MBBR. A possible explanation for this significant difference between systems that combine suspended and attached growth could be the continuous sludge recirculation that takes place in our MBMBR system, unlike MBBR systems. However, these comparisons must be considered with caution, since that the values of OTUs, Chao 1 and Shannon-Wiener, largely depend on sequences processing.

Table IV.3. Number of unique OTUs, Chao 1 and Shannon- Wienner indices (cut-off level of 3%) measured in suspended biomass (SB) and attached biofilm (AB) samples taken from the MBMBR in the last week of each experiment. The samples nomenclature (from left to right) indicates the experiment, the samples type (SB or AB) and the sampling week. In black or grey colours are written the SB or AB samples,

Sample	OTUs	Chao 1	Shannon-Wiener
1 SB6	617	807.513	6.905
1 AB6	595	706.341	7.182
2 SB6	624	751.444	7.077
2 AB6	496	623.525	5.703
3 SB6	592	768.207	6.678
3 AB6	416	524.075	5.718
4 SB6	514	670.635	6.133
4 AB6	498	621.78	5.602
5 SB6	950	1447.1	7.365
5 AB6	1026	1632.7	7.568
6 SB5	809	1188.6	6.613
6 AB5	864	1217	6.928
7 SB5	848	1283	7.656
7 AB5	834	1267.2	7.053
8 SB5	804	1331.3	7.009
8 AB5	806	1305.2	7.115

On the other hand, clear differences between SB and AB considering the OTUs richness and Chao 1 index have been previously reported (Kwon *et al*., 2010; Biswas *et al*., 2014). For this reason and taking into account also the possible influence of HRT (10h or 24h), MLTSS (c.a 2500mg/L or c.a. 4500mg/L) and CFR (20% or 35%, v/v) in our study, the rarefaction measures of the OTUs, the richness index (Chao 1) and the diversity index (Shannon-Wiener) were plotted in function of these parameters (**Fig. IV.7**). It can be observed that the number of OTUs, the species richness (Chao 1) and the taxonomic diversity (Shannon-Wiener) did not show significant differences

(Student's test, $p > 0.05$) in function of sample type (SB and AB), HRT (10h or 24h) and MLTSS concentration (c.a 2500mg/L or 4500mg/L). However, it is noticeable the significant increase (Student's test, $p < 0.05$) of the number of OTUs and the species richness when the system was conducted with a 35% (v/v) of CFR. This influence of the CFR, "although less evident in the figure", also was observed in the values of Shannon-Wiener index (Student's test, $p < 0.05$). These results indicate that an increase in the CFR favoured the richness and the diversity in the MBMBR system. It is worth highlighting that in our study of the total Bacteria community carried out by TGGE the diversity index (Shannon-Wienner, H') showed the higher values in the experiments 1 and 2, conducted with a 20% (v/v) of CFR (**Fig. IV.2A**). This conflicting result between both methodologies (TGGE and 454-pyrosequencing) could be expected since, in agreement with Marzorati *et al*. (2008), the resolution of the DGGE/TGGE techniques is normally not satisfactory to describe the "full" microbial diversity of complex environments. Consequently, we consider that the result obtained by 454-pyrosequencing is more reliable and consistent.

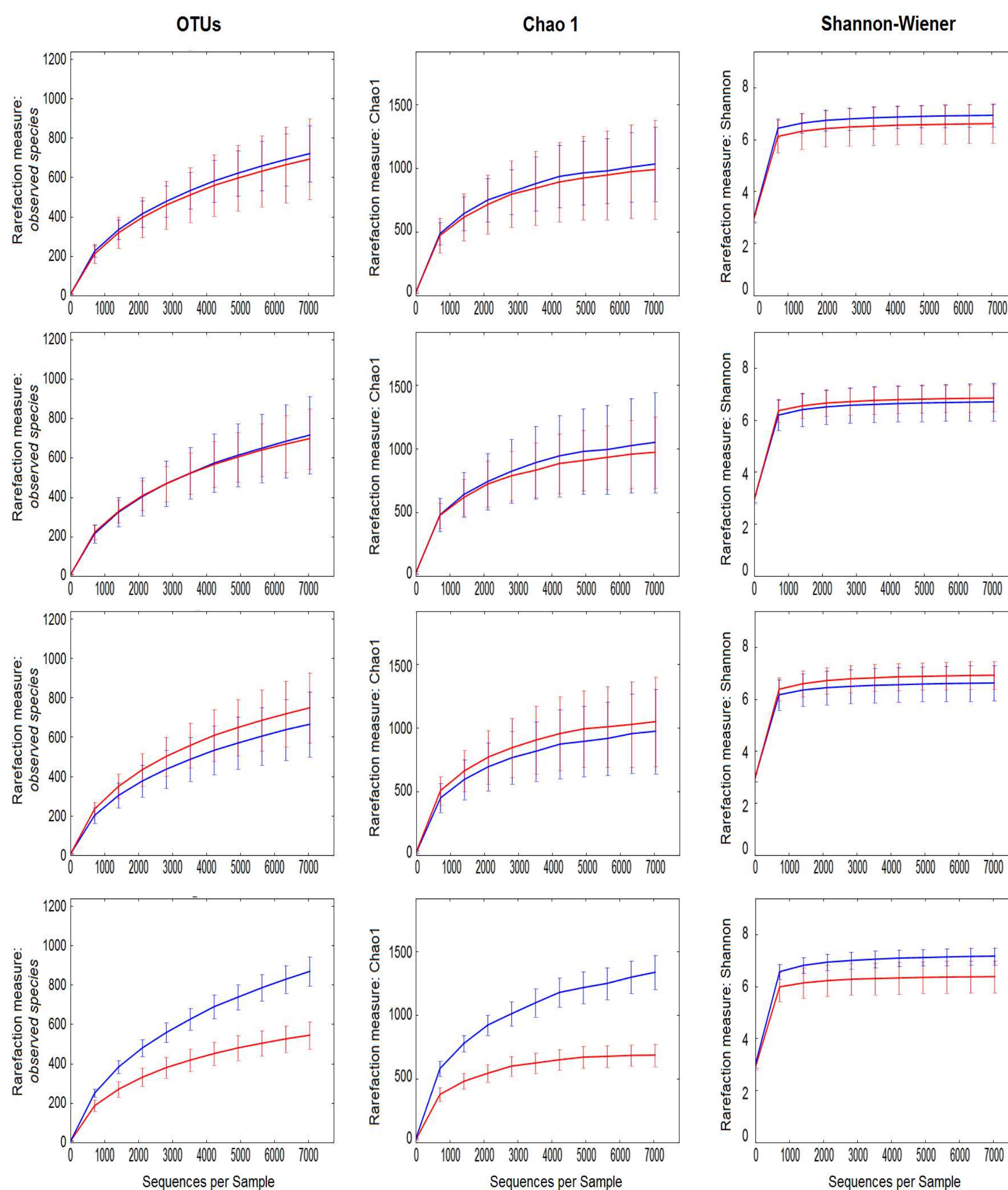


Figure IV.7. Rarefaction curves of OTUs, Chao 1 and Shannon-Wiener indices clustered at 97% sequence identity. The rarefaction curves are plotted in function of the sample type (SB: suspended biomass and AB: attached biomass), Hydraulic retention Time (HRT), Mixed Liquor Total Suspended Solid (MLTSS) and Carrier Filling Ratio (CFR). Error bars show standard deviation.

Higher species richness in the MBMBR system with a 35% (v/v) of CFR could be expected, since according to Wang *et al*. (2005) when the CFR is increased, the total surface area of the carriers also increases, and as a direct consequence of this, more places are available for bacteria to attach. Moreover, also according to these authors, there is a steady relationship between SB and AB based on attachment and detachment process. Therefore, it is possible that the increase of the species number in AB could contribute to increase the species number in SB and consequently in the MBMBR system. In addition, the available studies concerning the microbial community structure under different CFR came to the conclusion that the CFR is an important factor influencing the total Bacteria community structure (Calderón *et al*., 2012b) and nitrifying community's structure (Bernet *et al*., 2004). Specifically, Calderón *et al*. (2012b) using TGGE, studied the bacterial community structure of the AB in a MBBR system under 20%, 35% and 50% (v/v) of CFRs, confirming by RDA analysis differences in the composition of the biofilm bacterial community among the three CFR tested.

3.2.2. Total Bacteria community composition

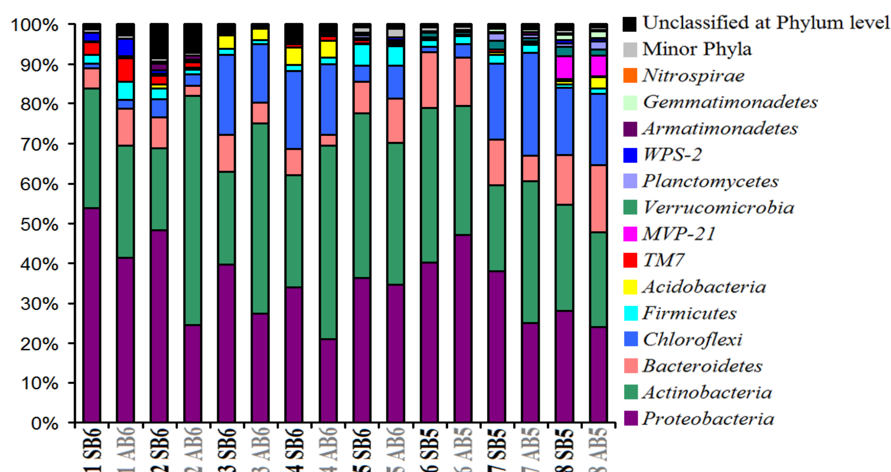
Taxonomic study at phylum level. **Fig. IV.8.** Summarize the taxonomic composition of the total Bacteria community at phylum level for each sample type (SB and AB) in the eight experiments. Thirty bacterial phyla were identified across the entire data set, 16 of which denominated as "minor phyla" constituted by less than 1% of the total sequences in all the samples, being *Chlorobi*, WYO and *Synergistetes* the most abundant among them (**Fig. IV.8B**). Within the major phyla (sequences abundance ≥1% in at least one sample) *Proteobacteria*, ranging from 20.9 to 53.8% of the total bacterial sequences, and *Actinobacteria* (20.6-57.6%) were the predominant

phyla in both sample types (SB and AB) and in all the experiments, followed by *Bacterioidetes* (2.4-16.9%), *Chloroflexi* (1.2-25.8%) and *Firmicutes* (0.8-4.9%). Others major phyla, *Acidobacteria*, TM7, MVP-2, *Verrucomicrobia*, *Planctomycetes*, WPS-2, *Armatimonadetes*, *Gemmatimonadetes* and *Nitrospirae* showed abundance ≥1% in only a few samples. A percentage of sequences between 0.2 and 8.5% were unclassified at phylum level. As can be observed in **Figure IV.8**, the taxonomic composition at phylum level of SB and AB in the same experiment was very similar. In fact, one-way analysis (ANOVA and ANOSIM) revealed no significant differences ($p>0.05$) between sample types (SB and AB) at phylum level.

In general, the phyla identified in our study have been found previously in SB or AB from different WWTSs, such as, CAS, MBRs, MBBRs and IFAS (Kwon *et al*., 2010; Wells *et al*., 2011; Hu *et al*., 2012; Zhang *et al*., 2012; Biswas *et al*., 2014). Specifically, the predominance of phyla *Proteobacteria*, *Actinobacteria*, *Bacterioidetes*, *Chloroflexi* and *Firmicutes* in our MBMBR system also is consistent with the results obtained in other studies (Kwon *et al*., 2010; Ye *et al*., 2011; Zhang *et al*., 2012; Ye *and* Zhang, 2013). However, it is curious that the predominant phylum in SB samples was *Proteobacteria* in all the experiments (except experiment 5) while the predominant phylum in AB was *Actinobacteria* in most of the experiments (2, 3, 4, 5, and 7) (**Fig. IV.8A**). Kwon *et al*. (2010) also observed higher abundance of *Actinobacteria* in the AB compared to the SB present in an IFAS system. According to these authors, the incorporation of support material in the bioreactor could provide a good environment to the preferential growth of members belonging to *Actinobacteria*. In addition, this phylum has been described as the second predominant phylum in different WWTSs based only in SB (McLellan *et al*., 2010; Lim *et al*., 2012; Ye *and* Zhang, 2013). Taking into account these considerations, the high abundance of *Actinobacteria* in our

system could be due to the presence of carriers, which not only could favour the development of member belonging to the phylum *Actinobacteria*, but also their retention in the MBMBR system.

A. % of Sequences in each major Phylum



B. % of Sequences in each minor Phylum

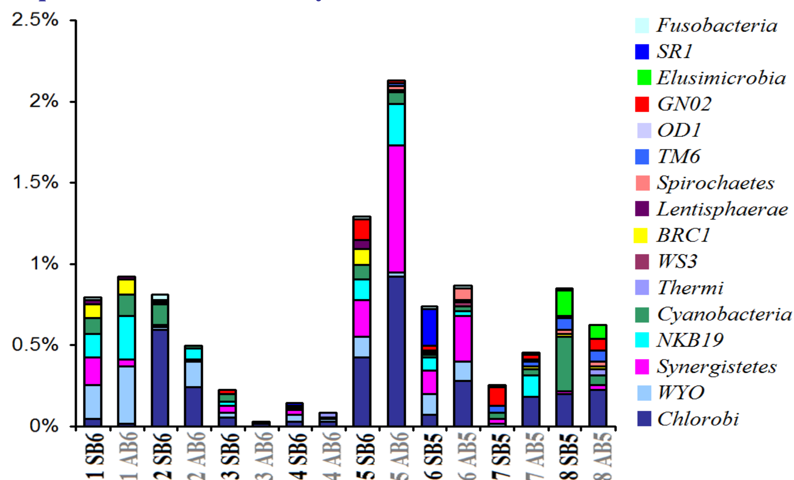


Figure IV.8. Relative abundances of each major (**A**) and minor phylum (**B**) in suspended biomass (SB) and attached biofilm (AB) samples taken from the MBMBR in the last week of each experiment. The relative abundance is presented in terms of percentages of the total high quality sequences in a sample. Major phyla occurred at ≥1% abundance in at least in one sample. Minor phyla occurred at <1% abundance in all the samples. The samples nomenclature (from left to right) indicates the experiment, the samples type (SB or AB) and the sampling week. In black or grey colours are written the SB or AB samples, respectively.

In general, the phyla identified in our study have been found previously in SB or AB from different WWTSs, such as, CAS, MBRs, MBBRs and IFAS (Kwon *et al*., 2010; Wells *et al*., 2011; Hu *et al*., 2012; Zhang *et al*., 2012; Biswas *et al*., 2014). Specifically, the predominance of phyla *Proteobacteria*, *Actinobacteria*, *Bacterioidetes*, *Chloroflexi* and *Firmicutes* in our MBMBR system also is consistent with the results obtained in other studies (Kwon *et al*., 2010; Ye *et al*., 2011; Zhang *et al*., 2012; Ye *and* Zhang, 2013). However, it is curious that the predominant phylum in SB samples was *Proteobacteria* in all the experiments (except experiment 5) while the predominant phylum in AB was *Actinobacteria* in most of the experiments (2, 3, 4, 5, and 7) (**Fig. IV.8A**). Kwon *et al*. (2010) also observed higher abundance of *Actinobacteria* in the AB compared to the SB present in an IFAS system. According to these authors, the incorporation of support material in the bioreactor could provide a good environment to the preferential growth of members belonging to *Actinobacteria*. In addition, this phylum has been described as the second predominant phylum in different WWTSs based only in SB (McLellan *et al*., 2010; Lim *et al*., 2012; Ye *and* Zhang, 2013). Taking into account these considerations, the high abundance of *Actinobacteria* in our system could be due to the presence of carriers, which not only could favour the development of member belonging to the phylum *Actinobacteria*, but also their retention in the MBMBR system.

Another interesting result was the abundance values of *Chloroflexi* phylum in the different experiments. For both samples type (SB and AB), the abundance of *Chloroflexi* was significantly higher in the experiments 3, 4, 7 and 8 (abundance range 14.5-25.8%) performed with higher MLTSS concentration (c.a. 4,500 mg/L) compared with the experiments 1, 2, 5 and 6 (abundance range 1.1-8%) conducted with lower MLTSS concentration (c.a. 2,500mg/L). It suggests the possible influence of solid

concentration in the members of *Chloroflexi* developed in the MBMBR. This result is consistent with the results reported by Miura *et al*. (2007b). These authors, using FISH methodology, observed that the abundance of *Chloroflexi* in a MBR was higher when the system was conducted with higher MLTSS concentration, demonstrating also that the members of *Chloroflexi* are abundant in MBRs. Although, the metabolic functions of these bacteria are still unclear, because only few members of this phylum have been isolated, several studies reported that the members of *Chloroflexi* phylum play an important ecological role in the degradation of carbohydrates in WWTSs (Miura *et al* 2007a; Miura *et al*., 2007b; Rivière *et al*., 2009). Therefore, a higher abundance of this phylum could favour the biodegradation process, being probably the solid concentration a key operational parameter to promote an increase in the abundance of this phylum.

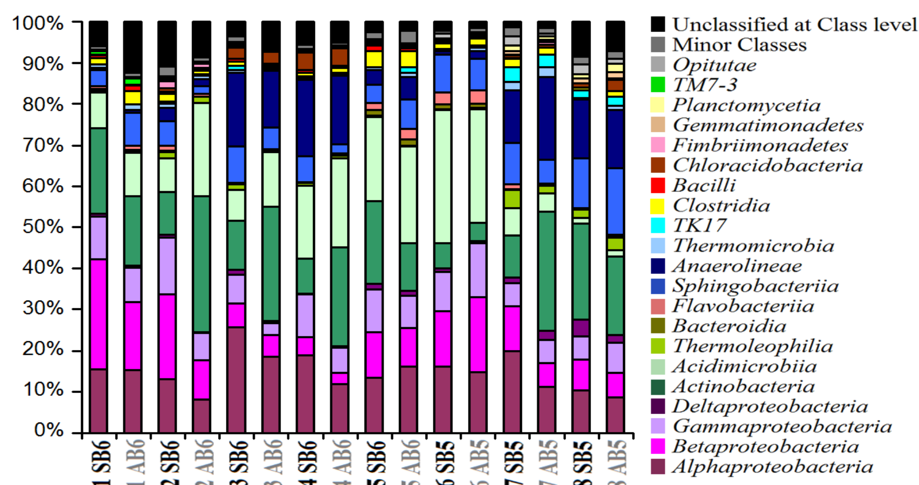
Taxonomic study at class and order levels. In concordance with the results observed in the study of richness and diversity indices, as well as in the taxonomic study at phylum level, no significant differences were observed between SB and AB at class and order level (ANOVA and ANOSIM, $p > 0.05$). A total of 71 class and 117 orders were identify in the MBMBR system, being only a 29.58% (21) and 23.1% (27) considered as major classes and orders, respectively (abundance ≥1% in at least one sample) (**Fig. IV.9 A** and **B**). A percentage of sequences between 1.4 and 12.4% at class level and between 4.8-16.8% at order level were unclassified. Within the phylum *Proteobacteria*, α-, β- and γ-*proteobacteria* were the most predominant classes (abundance >1% in all samples), noting that ε-*proteobacteria* was considered as minor class, since occurred at very low levels (abundance <1% in all the samples) in the MBMBR, being this result consistent with those previously reported in others WWTSs (Kwon *et al*., 2010; Zhang *et al*., 2012; Hu *et al*., 2012). Among these classes, α-*proteobacteria* was the most abundant (8.1-25.6%), followed by β-*proteobacteria* (2.7-

26.8%) with the exceptions of the samples 1 SB6, 1 AB6, 2 SB6, 2 AB6 and 6 AB5 in which β-*proteobacteria* was the predominant class. It is remarkable the trend of β-*proteobacteria* to decrease in the experiments (3, 4, 7 and 8) conducted with higher MLTSS concentration (4,500 mg/L) compared to the experiments (1, 2, 5 and 6) performed with lower MLTSS (**Fig. IV.9A**), suggesting a possible influence of the solid concentration in the β-subdivision. Among the major orders belonging to phylum *Proteobacteria*, *Rhizobiales* (2.75-12.8%), *Burkholderiales* (2.4-24.2%) and *Xanthomonadales* (1.56-12.3%) were the most abundant orders related to α-, β- and γ-*proteobacteria*, respectively and were present in all the samples (**Fig. IV.9B**). These orders also were found as dominant and commonly shared in all the WWTPs studied by Zhang *et al*. (2012).

The globally held belief that the β-subdivision is the predominant class of *Proteobacteria* in WWTSs appears to be currently questioned by the results obtained by 454-pyrosequencing and phylochip. In various studies based on these methodologies the class α-*proteobacteria* versus β-*proteobacteria* has been reported as predominant in different WWTSs (Xia *et al*., 2010; Hu *et al*., 2012; Ye *and* Zhang, 2013). Nevertheless, also using 454-pyrosequencing it has been reported the predominance of β-*proteobacteria* versus α-*proteobacteria* (Kwon *et al*., 2010; Zhang *et al*., 2012; Kim *et al*., 2013). A preliminary hypothesis to explain predominance of α-*proteobacteria* versus β-*proteobacteria* in WWTSs has been related to high salt concentration in the influent, since the predominance of α-*proteobacteria* was observed in a treatment system of saline sewage (Zhang *et al*., 2012; Ye *and* Zhang, 2013). However, others studies carried out with domestic or industrial wastewater in which the predominance of α-proteobacteria versus β-*proteobacteria* was clear, no solid hypothesis to explain this fact were reported (Xia *et al*., 2010; Hu *et al*., 2012). Consequently, so far the possible

factors that could explain the prevalence of α- or β-*proteobacteria* in WWTSs remain still unknown.

A. % of Sequences in each major Classes



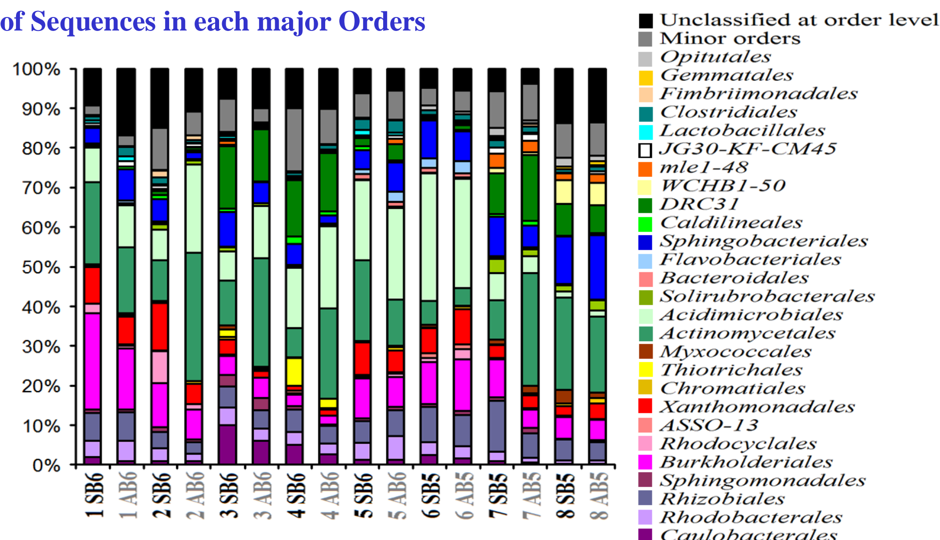


Figure IV.9. Relative abundances of each major class (**A**) and of each major order (**B**) in suspended biomass (SB) and attached biofilm (AB) samples taken from the MBMBR in the last week of each experiment. The relative abundance is presented in terms of percentages of the total high quality sequences in a sample. Major classes and orders occurred at ≥1% abundance at least in one sample. Minor classes occurred at <1% abundance in all the samples. The samples nomenclature (from left to right) indicates the experiment, the samples type (SB or AB) and the sampling week. In black or grey colours are written the SB or AB samples, respectively.

On the other hand, two predominant classes (abundance >1% in all samples) were related to phylum *Actinobacteria*: *Acidimicrobiia* (1.5-32.35%) composed by members of the order *Acidimicrobiales* (1.5-32.35%); and *Actinobacteria* class (4.5-33.04%) in which all the sequences belonged to the order *Actinomycetales* (4.5-33-05%). It is worth noting that in most of the samples *Acidimicrobiia* or *Actinobacteria* were predominant versus α-, β-, γ- and δ-*proteobacteria* (**Fig. IV.9 A** and **B**), confirming also at class and order levels the predominance of members belonging to *Actinobacteria* in various samples.

Regarding the classes and orders related to phylum *Bacterioidetes*, only *Sphingobacteriia* class (1.91-16.24%) and *Sphingobacteriales* (4-16.24%) showed an abundance >1% in all the samples. In accordance with Zhang *et al*. (2012) and Hu *et al*. (2012) within *Bacterioidetes*, *Sphingobacteriales* order appears to be predominant in WWTPs. Members of this order are thought to degrade macromolecules such as polysaccharides and proteins (Weissbrodt *et al*., 2014).

As for *Chloroflexi* phylum, *Anaerolineae* (0.54-20%), *Thermomicrobia* (0.27-2.5%) and *TK17* (0-3.5%) were the three major classes. As it can be observed in **Fig. IV.9** (**A**), the predominant class in most samples was *Anaerolineae* which was mainly composed of members related to *DRC 31* order. According to the results reported by Zhang *et al*. (2012) and Ye *and* Zhang (2013), *Caldilineales* appears to be predominant order within the class *Anaerolineae* in different WWTPs. However, in our system lower number of sequences were related to *Caldilineales* (0.14-1.98%) compared with *DRC 31* (0-16.9%), emphasizing that in the sample 1 SB6 any sequence was related to DRC 31. The presence of various uncultured (*DRC 31*, *JG30-KF-CM45*, *mle1-48* and *WCHB1-50*) as major orders within the phylum *Chloroflexi* in our MBMBR system is

curious in comparison with the results reported by Zhang *et al*. (2012). These authors also considered as major orders those with abundance ≥1% at least in one sample, but they didn't find any uncultured as major order. Taking into account that *Anaerolineae* class as well as *Caldilineales*, *DRC 31* and *WCHB1-50* orders belonging to *Chloroflexi* tended to increase their abundance in the experiments (3, 4, 7 and 8) conducted with higher MLTSS concentration (4,500 mg/L), it can be speculated that an increase of the MLTSS concentration in the MBMBR could favour the development of these uncultured microorganisms related to *Chloroflexi*.

Regarding *Firmicutes*, two major classes were related to this phylum; *Bacilli* (0.01-1.4%) and *Clostridia* (0.55-3.9%), being *Lactobacillales* (0-1.3%) and *Clostridiales* (0.54-3.2%) the major orders related to these classes, respectively. According to the model of the microbial function in MBBRs described by Biswas *et al*. (2014) "*the early AB community is succeeded by one that contains a greater abundance of putatively anaerobic microorganisms*", confirming a gradual increase of the members belonging to *Clostridia* over time. By contrast, our pyrosequencing-derived results did not show this trend over time for experiments performed with 20% CFR (from experiment 1 to experiment 4, ten months) and with 35% CFR (from experiment 5 to experiment 8, eight months) in any member related to *Firmicutes*. However, this discrepancy could be due to the fact that in our MBMBR over time with a 20% (v/v) of CFR or 35% of CFR different operational conditions were conducted. Consequently, the changes in operational conditions could affect the development of microorganisms belonging to *Firmicutes*, since as above commented in the sub-paragraph 3.1.3 the microcolonies of *Firmicutes* appears to be weak under shear forces in the bioreactor.

The remaining major classes, *Chloracidobacteria*, *Fimbriimonadetes*, *Gemmatimonadetes*, *Planctomycetia*, *TM7-3* and *Opitutae* as well as some major classes (δ-*proteobacteria*, *Thermoleophilia*, *Bacteroidia*, *Flavobacteriia*, *Thermomicrobia*, *TK17* and *Bacilli*) related to the five relevant phyla (*Proteobacteria*, *Actinobacteria*, *Bateroidetes*, *Chloroflexi* and *Firmicutes*) showed abundance ≥1% in only a few samples.

Taxonomic study at family and genus levels. According to the two studies that approach the analysis of bacterial community structure in SB and AB by 454-pyrosequencing (Kwon *et al*., 2010; Biswas *et al*., 2014) the differences between both samples type (SB and AB) are more evident at lower taxonomic levels such as, family and genus as well as at OTUs level. Nevertheless, in concordance with all the results obtained in the present study, no significant differences between SB and AB were observed at family, genus and OTUs level (ANOVA and ANOSIM; $p > 0.05$). It is a strong reason to emphasise once more the clear similarity of the total Bacteria community structure between samples type in the MBMBR. This inconsistency of our results with previous reports (Kwon *et al*., 2010; Biswas *et al*., 2014) could be explained considering the follow hypothesis: **1.** Biswas *et al*. (2014) in a MBBR only found differences between SB and "*mature biofilm*" as above discussed in sub-paragraph (3.1.1); **2.** Kwon *et al*. (2010) reported differences between both samples type in IFAS system in which the biofilm was developed in a fixed synthetic mesh (no freely moving carriers), so a minor interaction between samples type and consequently higher differences between SB and AB could be expected in this system compared with our MBMBR; **3.** It is necessary bearing in mind that the three systems (MBBR, IFAS and MBMBR) differ from each other on some aspects of the operational design, being

important to consider that in our MBMBR a continuous sludge recirculation takes place.

A total of 156 families and 217 genera were found in the entire date set. Same as in previous studies, that approached the bacterial diversity in WWTSs by 454 pyrosequencing (Kim *et al*., 2013; Zhang *et al*., 2012), a large proportion of high quality sequences were not assigned to any phylogenetic lineage at family and genus levels. Concretely, the averages values of unclassified sequences were 28.65±10.35 and 55.66±11.76% at family and genus level, respectively. Thirty-three major families (abundance ≥ 1% in at least one sample) were observed in the entire data set (**Fig. IV.10A**). Among these, *Chitinophagaceae*, *Microthixaceae*, *Gordoniaceae*, *Intrasporangiaceae*, *Mycobacteriaceae*, *Xanthomonadaceae*, *Comamonadaceae*, *Caulobacteraceae*, *Bradyrhizobiaceae*, *Hyphomicrobiaceae* and *Rhodobacteriaceae* were commonly shared by all samples and the most predominant (abundance >1% in more than half of the samples) in the MBMBR system (**Fig. IV.10A**). The families detected in our MBMBR have been also found in the different WWTSs studied by Wang *et al*. (2012b) and Zhang *et al*. (2012). It would be useful to highlight that among our major families, *Chitinofagaceae*, *Xanthomonadaceae, Bradyrhizobiaceae* and *Sphingomonadaceae* were also reported by Pal *et al*. (2012) as the major families (using T-RFLP) in the AB from a MBBR system. Accordingly to these authors, the members belonging to *Sphingomonadaceae* and *Xanthomonadaceae* could have an important role in the biofilm development, since they excrete exopolymeric substance (EPS), which are essential for biofilm formation. In addition, also these authors reported that, although some members of *Bradyrhizobiaceae* are symbiotic nitrogen fixer, they can form-biofilms outside the host where nitrogen fixation is not a priority.

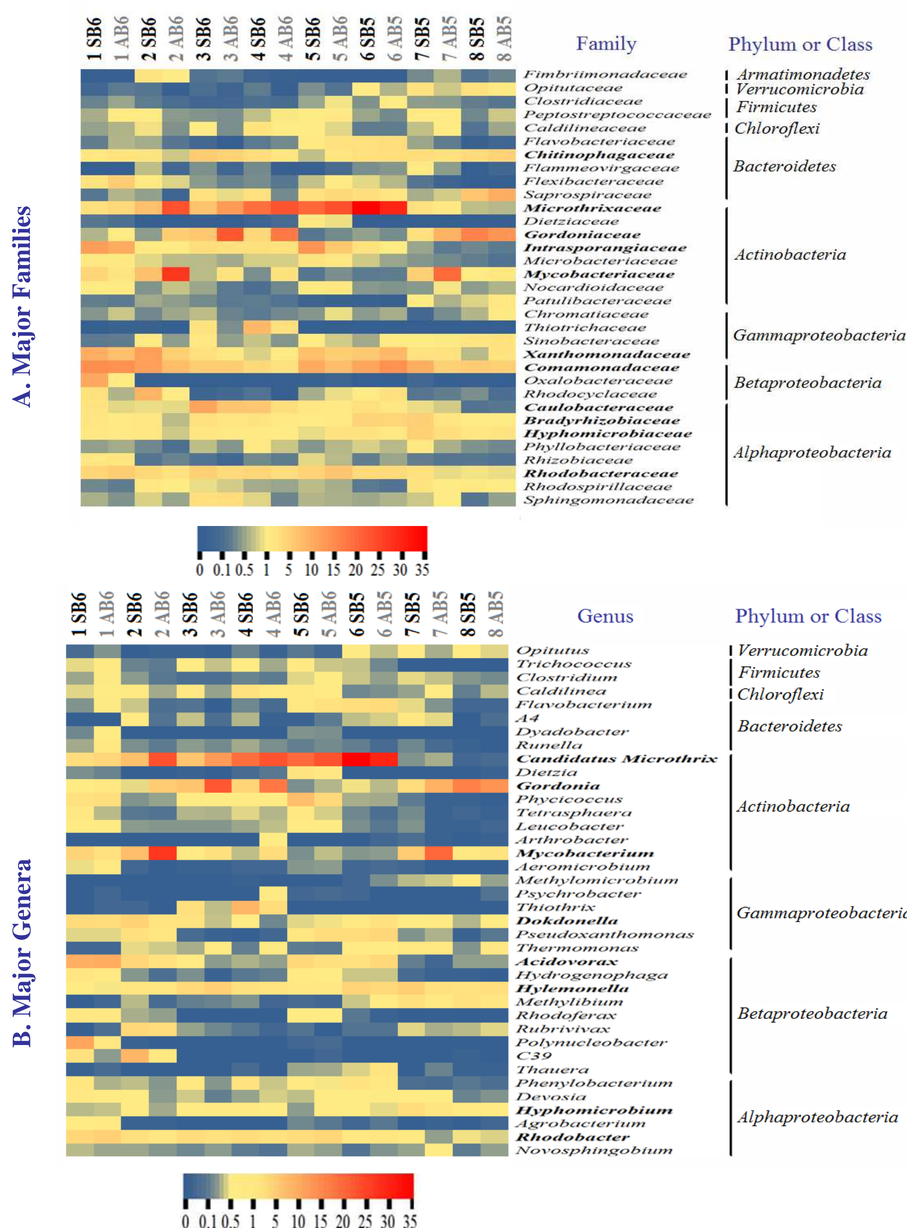


Figure IV.10. Heat maps representing the relative abundance of each major family (**A**) and of each major genus (**B**) in suspended biomass (SB) and attached biofilm (AB) samples taken in the last sampling week of each experiment (1, 2, 3, 4, 5, 6, 7 and 8) performed in the MBMBR system. The relative abundance is presented in terms of percentages of the total high quality sequences in a sample. Major families occurred at ≥1% abundance in at least one sample. Major generous occurred at ≥0.5% abundance in at least one sample. Both scale bars indicates the different values of relative abundance associated to the different intensities colours. The maximum values of abundance among major families and generous were 32.18% and 32.1%, respectively. The samples nomenclature (from left to right) indicates the experiment, the samples type (SB or AB) and the sampling week. In black or grey colours are written the SB or AB samples, respectively.

Regarding to the core microbiota at genus level, thirty-eight major genera (abundance > 0.5% in at least one sample) were identified, being eight of them commonly shared by all samples and most predominant (abundance >0.5% in more than half of the samples) in the MBMBR system (**Fig. IV.10B**). Five of the predominant genera; *Rhodobacter*, *Hyphomicrobium*, *Hylemonella*, *Acidovorax* and *Dokdonella* belonged to *Proteobacteria* and three of them, *Mycobacterium*, *Gordonia* and *Candidatus Microthrix* belonged to *Actinobacteria* (**Fig. IV.10B**). In accordance with previous reports (Zhang *et al*., 2012; Biswas *et al*., 2014), the major families and genera detected in our MBMBR system represent the core of the microbiota in the different WWTPs. However, the high abundance of the genera *Candidatus Microthrix* and *Gordonia* in most of the experiments performed in the MBMBR is remarkable (**Fig. IV.10B**). Both genera are commonly found in biological WWTPs, where they have been described as the most common filamentous bacteria causing bulking and/or foaming problems (Nielsen *et al*., 2009; Muller *et al*., 2012). For these reasons, it should be emphasized that no bulking and/or foaming events were detected in the MBMBR system, suggesting that the incorporation of carriers could mitigate the negative effects of these microorganisms in biological WWTSs. In addition, the abundance of filamentous bacteria has been positive correlated to the EPS concentration in WWTSs (Meng *et al*., 2006; Jamal Khan *et al*., 2012). Therefore, considering that EPS are essential for bacterial adhesion and biofilm development (Flemming, 2009), the abundance of the genera *Candidatus Microthrix* and *Gordonia* in our system could have contributed to the biofilm development. Additionally, most of the species belonging to *Gordonia* appears to have an important role in the depuration process, since, according to Arenskötter *et al*. (2004) they have abilities to degrade xenobiotics, environmental pollutant and slowly biodegradable natural polymers.

Finally, it is important to say that the order *Nitrosomonadales* and the family *Nitrosomonadaceae* were detected by 454-pyrosequencing, but, since the values of relative abundance were ranged from 0.02 to 0.6%, they were considered as a minor order and family. In addition, the members related to this family were unclassified at genus level. In order to identify possible differences in the relative abundance values of *Nitrosomonadaceae* between both sample types (SB and AB), ANOVA and ANOSIM were performed. The result of the Student's test and ANOSIM confirmed no significant differences ($p > 0.05$) between the two samples.

3.2.3. Statistical multivariate analysis: Influence of physico-chemical parameters on total Bacteria community structure at the different taxonomic levels and at OTUs level

It is known, that in engineered ecosystems, the microbial community structure is influenced by deterministic (species traits, interspecies interactions and environmental and/or operational parameters) and/or stochastic factors (Curtis *and* Sloan, 2006; van der Gast *et al*., 2008; Ofiţeru *et al*., 2010; Zhou *et al*., 2013). It is also widely known, that the efficiency of the depuration process largely dependent on the microbial community structure. For these reasons, various reports have emphasized the need to know the environmental and/or operational parameters that could affect the microbial community structure and therefore, the efficiency of the depuration process (Yuan *and* Blackall, 2002; Wells *et al*., 2011; Cydzik-Kwiatkowska *et al*., 2012; Valentín-Vargas *et al*., 2012; Hai *et al*., 2014).

In our study, to assess the relationships between total Bacteria community structure and the operational parameters, multivariate analyses were performed. In order

to obtain comprehensive and detailed information about the patterns of total Bacteria community structure in the MBMBR, RDA analysis was performed for each taxonomic level and for the OTUs level. For that, the operational parameters (MLVSS, BTS, COD, BOD_5, pH, temperature, HRT) and CFR (20% or 35%, v/v) were correlated to the values of relative abundance of each phylum, class, order, family, genus and OTU. In **Figure IV.11** are showed the biplot diagrams obtained at each taxonomic level and at OTUs level for both sample types (SB and AB) in the eight experiments. Although all the dataset have been used to perform the RDA analysis, to gain a more understandable figure, only the most relevant members, within each taxonomic unit, are showed. The family *Nitrosomonadaceae* also is showed.

According to the results of the Monte Carlo permutation test, MLVSS, BTSS, Temperature, BOD_5 and CFR were the significant factors ($p < 0.05$) explaining the variations in the structure of the total Bacteria community, being the solids concentration the most significantly linked to bacterial community variability (minor p-value) in all the RDA analyses. The first canonical ordination axis (horizontal) for each RDA analysis (phylum, class, order, family, genus and OTUs) described 36.4, 41.5, 38.4, 40.9, 48.5 and 40.8% of the variation of the relative abundance data, respectively (65.7, 56.6, 51.7, 50.8, 60.6 and 48.4% of the relative abundance-operational parameters relations explained by the first axis, respectively). The second one (vertical) for each RDA analysis (phylum, class, order, family, genus and OTUs) described 11.1, 19.9, 20.2, 19.4, 16.8 and 18.5% of the variation of the relative abundance data, respectively (19.9, 27.1, 27.2, 24.2, 20.9 and 21.9% of the relative abundance-operational parameters relations explained by the second axis, respectively).

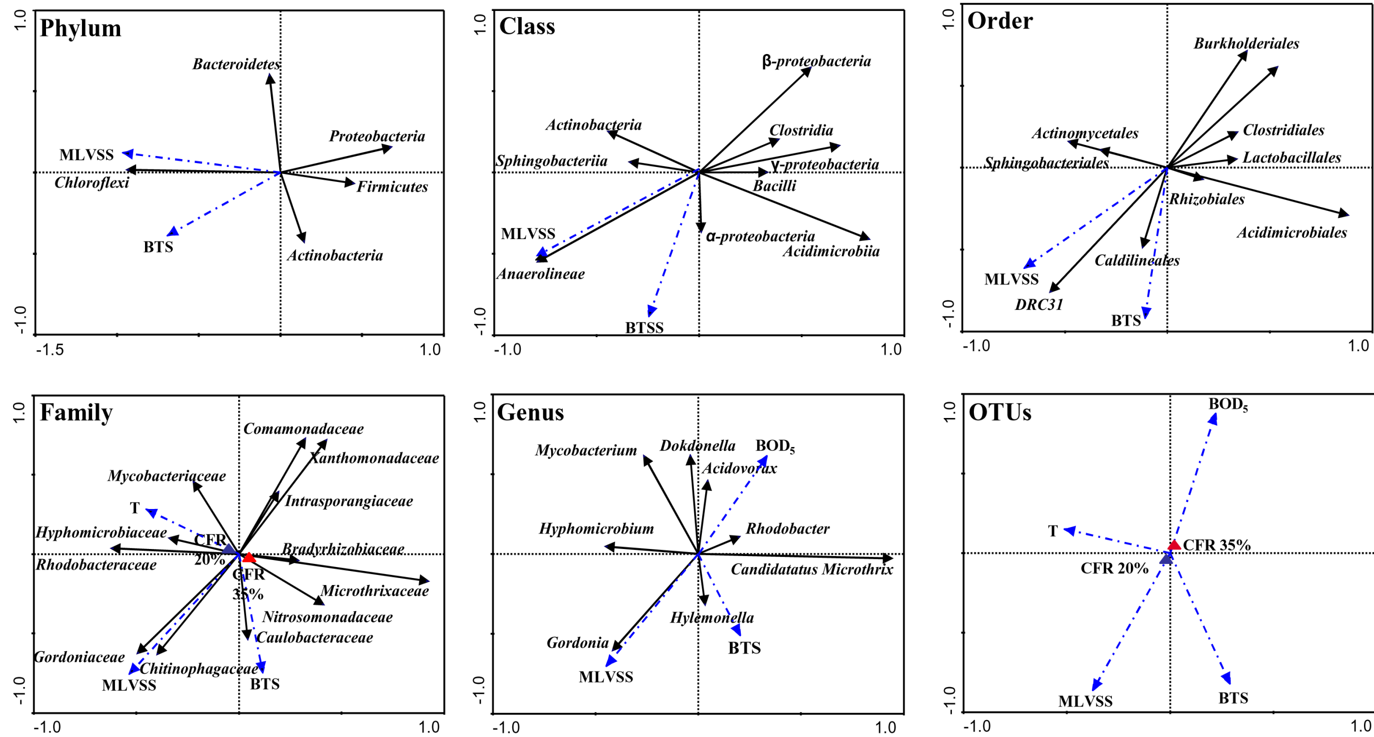


Figure IV.11. Redundancy analyses (RDA) of the relative abundance at Phylum, Class, Order, Family, Genus and OTUs level. The ordination diagrams (biplots) show operational conditions (dotted blue arrows and triangle) that have a significant influence on bacterial community and most prevalent phylogenetic lineages (straight black arrows), measured in the eight experiments performed in the MBMBR system. Operational parameters: Temperature (T), Mixed Liquor Volatile Suspended Solids (MLVSS), Biofilm Total Solids (BTS), Biological Oxygen Demand at 5 days in influent water (BOD_5) and CFR (v/v) 20% (blue triangle) or 35% (red triangle). CFR are shown as nominal variables (triangle).

RDA analysis at phylum level revealed a negative correlation of *Proteobacteria* and *Firmicutes* with the solid concentration (MLVSS) in the MBMBR, indicating that the relative abundance of these phyla tended to decrease in the experiments 3, 4, 7 and 8 conducted with higher solid concentration (**Table IV.2**). Furthermore, RDA confirmed the strong positive correlation between relative abundance of *Chloroflexi* phylum and MLVSS concentration discussed in the above sub-paragraph (3.2.1). The relative abundance of phylum *Bacteroidetes* showed a slight positive correlation with MLVSS. However, the angle formed between the vectors of *Actinobacteria* and MLVSS revealed a trivial influence of this parameter on the variations of relative abundance of *Actinobacteria* among experiments.

Concerning the influence of the operational parameters on the relative abundance of the members belonging to *Proteobacteria*, the RDA analysis at class level showed a strong negative correlation of the members belonging to β- and γ-*proteobacteria* with the solid concentration (MLVSS and BTS). This trend was also observed in the relative abundance of predominant orders (*Burkholderiales* and *Xanthomonadales*), families (*Commamonadaceae* and *Xantomonadaceae*) and genera (*Acidovorax* and *Dokdonella*) related to β- and γ-*proteobacteria*, respectively, with the exception of *Hylemonella* genus (β-*proteobacteria*). Furthermore, as it can be observed in the biplot at genus level (**Fig. IV.11**), *Acidovorax* and *Dokdonella* also were positively influenced by the BOD_5 influent concentration. By contrast, the relative abundance of the predominant members related to α-*proteobacteria* showed a slightly positive correlation with MLVSS and BTS. These results suggest that the clear predominance of α-*proteobacteria* versus β-*proteobacteria* in most of the experiments conducted in the MBMBR could be due to the strong influence of the solid concentration on the members belonging to β-*proteobacteria*.

As for the variations of relative abundance of the members belonging to *Actinobacteria,* RDA analysis at class and order level showed the same trend observed at the phylum level (trivial influence of MLVSS). Nevertheless, it is interesting that the four predominant families (*Microthrixaceae*, *Gordoniaceae*, *Intrasporangiaceae* and *Mycobacteriaceae*) and the predominant genera (*Candidatus Microtrhix*, *Gordonia* and *Mycobacterium*) related to *Actinobacteria* showed different trends in function of operational parameters (**Fig. IV.11**). *Gordoniaceae* and *Gordonia* showed a strong positive correlation with MLVSS concentration, indicating that their higher relative abundance was observed in the experiments 3, 4, 7 and 8 (see **Fig. IV.10A**) in which higher values of MLVSS were recorded (**Table IV.2**), while *Intrasporangiaceae* showed an opposite correlation (**Fig. IV.11**). However, *Mycobacteriaceae* and *Microthrixaceae* were mainly influenced by temperature. *Mycobacteriaceae* and *Mycobacterium* was positively correlated with this parameter, confirming that the higher values of relative abundance were obtained in experiments 2 and 7 (**Fig. IV.10A**) in which the higher values of temperature were recorded (**Table IV.2**). On the contrary, *Microthrixaceae* and *Candidatus Microtrhix* showed negative correlation with the temperature. Due to the fact that, in the entire data set, the temperature was not a significant operational parameter explaining the variations of the relative abundance at genus level, temperature vector was not showed in the RDA at genus level. However, in **Figure IV.10** it can be observed that relative abundance of *Mycobacterium* and *Candidatus Microtrhix* showed the same trend in function of temperature observed for *Mycobacteriaceae* and *Microthrixaceae*.

Marrengane *et al*. (2011), using qPCR, observed that temperature was an important parameter to explain the quantitative variations of the members belonging to *Gordonia* and *Candidatus Microtrhix*. More specifically, these authors reported an

increase of the members of *Gordonia* as result of an increase in temperature, while *Candidatus Microtrhix* showed the opposite trend (negative correlation with temperature). In the present study a clear negative correlation between members related to *Candidatus Microtrhix* and the temperature also was observed. However, the genus *Gordonia* showed an obvious and strong correlation with the solid concentration. Accordingly to Asvapathanagul *et al*. (2012), conflicting reports make difficult to predict the occurrence of these filamentous bacteria. Indeed, these authors, using qPCR, reported a negative correlation between temperature and *Gordonia amarae*.

Regarding to *Nitrosomonadaceae*, RDA revealed a negative influence of temperature and positive influence of solid concentration on relative abundance of the members related to this family. As it was commented in sub-paragraph 3.2.1, the influence of temperature on AOB community dynamic has been previously reported by Wells *et al*. (2009) and Wang *et al*. (2012a). Specifically, Wells *et al*. (2009) observed by RDA a strong negative correlation between the lineages of *Nitrosospira* and *Nitrosomonas*-like and the temperature, while *N. europaea* showed a positive correlation with this parameter.

In 2003, Briones *and* Raskin written in a review the phrase "*we are only now beginning to link community dynamics with processes and functions in engineered systems*", since then, several researches were focused on to establish a link between operational/environmental conditions and the bacterial community structure in different WWTSs. Consequently, nowadays it is know that among the different operational/environmental factors, the composition of the influent wastewater, temperature, solid concentration, HRT, organic matter concentration (COD and BOD_5), pH, salinity, CFR, etc. have an important influence on the variability of the bacterial

community structure and, thus, on the efficiency of the depuration process (Miura *et al*., 2007a; Molina-Muñoz *et al*., 2009; Siggins *et al*., 2011; Wells *et al*., 2011; Calderón *et al*., 2012b; Pal *et al*., 2012; Valentin-Vargas *et al*., 2012; Wang *et al*., 2012a; Gómez-Silván *et al*., 2014). In our study, the results obtained by TGGE and 454-pyrosequencing indicate that the dynamic and the structure of total Bacteria community in both samples types (SB and AB) were mainly affected by changes in the solid concentration, temperature, CFR and organic matter concentration in the influent. Emphasizing that, the solid concentration was the most relevant parameter explaining the dynamics of bacterial population in the MBMBR system. Others parameters such as, pH and HRT were not significant factors explaining the variations of the total Bacteria community structures developed in SB and AB in the MBMBR system.

Overall, the results of the present study demonstrated that the structure of the total Bacteria community in both samples types (SB and AB) was very similar, being characterized by a high degree of diversity and an optimal functional organization. These characteristics described a bacterial community able to maintain its functional stability under all the experimental conditions tested in the MBMBR system. Consequently, an optimal depuration process was guaranteed and obtained in the eight experiments. On the other hand, our results also confirmed that the AOB community developed in both samples types was very similar. In concordance with previous reports (Boon *et al*., 2002; Wan *et al*., 2011), lower levels of diversity in AOB community compared to total Bacteria community were obtained. The AOB community was characterized as more specialized and probably more fragile to environmental/operational conditions changes, being this community mainly affected by changes in the solid concentration and temperature.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST, a new generation of protein database search programs. Nucleic Acids Research, 25, 3389-3402.

APHA, AWWA, WEF, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, DC.

Arenskötter, M., Bröker, D., Steinbüchel, A., 2004. Biology of the Metabolically Diverse Genus *Gordonia*. Applied and Environmental Microbiology, 70, 3195-3204.

Asvapathanagul, P., Huang, Z., Gedalanga, P.B., Baylor, A., Olson, B.H., 2012. Interaction of Operational and Physicochemical Factors Leading to *Gordonia amarae*-Like Foaming in an Incompletely Nitrifying Activated Sludge Plant. Applied and Environmental Microbiology, 78, 8165-8175.

Ayarza, J.M., Guerrero, L.D., Erijman, L., 2010. Nonrandom Assembly of Bacterial Populations in Activated Sludge Flocs. Microbial Ecology, 59, 436-444.

Bassin, J.P., Kleerebezem, R., Rosado, A.S., van Loosdrecht, M-C.M., Dezotti, M., 2012. Effect of Different Operational Conditions on Biofilm Development, Nitrification, and Nitrifying Microbial Population in Moving-Bed Biofilm Reactors. Environmental Science and Technology, 46, 1546-1555.

Bernet, N., Sánchez, O., Dabert, P., Olaïzola, A., Godon, J.J., Delgenès, J.P., 2004. Effect of solid hold-up on nitrite accumulation in a biofilm reactor-molecular characterization of nitrifying communities. Water Science and Technology, 49, 123-130.

Biswas, K., Turner, S.J., 2012. Microbial community composition and dynamics ofmoving bed biofilm reactor systems treating municipal sewage. Applied Environmental Microbiology, 78, 855-864.

Biswas, K., Taylor, M.W., Turner, S.J., 2014. Successional development of biofilms in moving bed biofilm reactor (MBBR) systems treating municipal wastewater. Applied Microbiology and Biotechnology, 98, 1429-1440.

Boltz, J.P., Morgenroth, E., Brockmann, D., Bott, C., Gellner, W.J., Vanrolleghem, P.A., 2011. Systematic evaluation of biofilm models for engineering practice: components and critical assumptions. Water Science and Technology, 64, 930-944.

Boon, N., De Windt, W., Verstraete, W., Top, E.M., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from diferent wastewater treatment plants. FEMS Microbiology Ecology, 39, 101-112.

Briones, A., and Raskin, L., 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology, 14, 270-276.

Calderón, K., Rodelas, B., Cabirol, N., González-López, J., Noyola, A., 2012. Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment. Bioresource Technology, 102, 4618-4627.

Calderón, K., González-Martínez, A., Montero-Puente, C., Reboleiro-Rivas, P., Poy-atos, J.M., Juárez-Jiménez, B., Martínez-Toledo, M.V., Rodelas, B., 2012a. Bacterial community structure and enzyme activities in a membrane bireactor (MBR) using pure oxygen as an aeration source. Bioresource Technology, 103, 87-94.

Calderón, K., Martín-Pascual, J., Poyatos, J.M., Rodelas, B., 2012b. Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. Bioresource Technology, 121, 119-126.

Calderón, K., Reboleiro-Rivas, P., Rodríguez, F.A., Poyatos, J.M., González-López, J., Rodelas, B., 2013. Comparative analysis of the enzyme activities and the bacterial community structure based on the aeration source supplied to an MBR to treat urban wastewater. Journal of Environmental Management, 128, 471-479.

Caporaso J.G., Kuczynski J., Stombaugh J., *et al.*, 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods, 7, 335-336.

Cébron, A., Coci, M., Garnier, J., Laanbroek, H.J.L., 2004. Denaturing Gradient Gel Electrophoretic Analysis of Ammonia-Oxidizing Bacterial Community Structure in the Lower Seine River: Impact of Paris Wastewater Effluents. Applied and Environmental Microbiology, 70, 6726-6737.

Cerrone, F., Poyatos, J.M., Molina-Muñoz, M., Cortés-Lorenzo, C., González-López, J., Rodelas, B., 2013. Prevalence of Nitrosomonas cluster 7 populations in the ammonia-oxidizing community of a submerged membrane bioreactor treating urban wastewater under different operation conditions. Bioprocess and Biosystems Engineering, 36, 901-910.

Chan, Y.J., Chong, M-F., Law, C.L., Hassell, D.G., 2009. A review on anaerobic-aerobic treatment of industrial and municipal wastewater. Chemical Engineering Journal, 155, 1-18.

Cortés-Lorenzo, C., Molina-Muñoz, M.L., Gómez-Villalba, B., Vilchez, R., Ramos, A., Rodelas, B., Hontoria, E., González-López, J., 2006. Analysis of community composition of biofilms in a submerged filter system for the removal of ammonia and phenol from industrial wastewater. Biochemical Society Transactions, 34 part 1.

Curtis, T.P. and Sloan, W.T., 2006. Towards the design of diversity, stochastic models for community assembly in wastewater treatment plants. Water Science and Technology, 54, 227-236.

Cydzik-Kwiatkowska, A., Zielińska, M., Wojnowska-Baryla, I., 2012. Impact of Operational Parameters on Bacterial Community in a Full-Scale Municipal Wastewater Treatment Plant. Polish Journal of Microbiology, 61, 41-49.

de los Reyes, F.L., 2010. Challenges in determining causation in structure-function studies using molecular biological techniques. Water Research, 4, 4948-4957.

DeSantis TZ, Hugenholtz P, Keller K, *et al.,* 2006a. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Research, 34, W394-W399.

DeSantis TZ, Hugenholtz P, Larsen N, *et al.* 2006b. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied Environmental Microbiology, 72, 5069-5072.

De Vrieze, J., Verstraete, W., Boon, N., 2013. Repeated pulse feeding induces functional stability in anaerobic digestion. Microbial Biotechnology, 6, 414-424.

Edgar R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 26, 2460-2461.

Edgar R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics, 27, 2194-2200.

Falk, M.W., Songa, K.G., Matiasek, M.G., Wuertz, S., 2009. Microbial community dynamics in replicate membrane bioreactors - natural reproducible fluctuations. Water Research, 43, 842-852.

Fernández, N., Diaz, E.E., Amils, R., Sanz, J.L., 2008. Analysis of Microbial Community during Biofilm Development in an Anaerobic Wastewater Treatment Reactor. Microbial Ecology, 56, 121-132.

Flemming, H.C., 2009. Why Microorganisms Live in Biofilms and the Problem of Biofouling. Marine and Industrial Biofouling, 4, 3-12.

Fu, B., Liao, X., Ding, L., Ren, H., 2010. Characterization of microbial community in an aerobic moving bed biofilm reactor applied for simultaneous nitrification and denitrification. World Journal of Microbiology and Biotechnology, 26, 1981-1990.

Gavrilescu, M., Macoveanu, M., 2000. Attached-growth process engineering in wastewater treatment. Bioprocess Engineering, 23, 95-106.

Glöckner, F.O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A., Amann, R., 2000. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including and abundant group of actinobacteria. Applied Environmental Microbiology, 66, 5053-5065.

Gómez-Silván, C., Molina-Muñoz, M., Poyatos, J.M., Ramos, A., Hontoria, E., Rodelas, B., González-López, J., 2010. Structure of archaeal communities in membranebioreactor and submerged-biofilter wastewater treatment plants. Bioresource Technology, 101, 2096-2105.

Gómez-Silván, C., Arévalo, J., González-López, J., Rodelas, B., 2014. Exploring the links between population dynamics of total and active bacteria and the variables influencing a full-scale membrane bioreactor (MBR). Bioresource Technology, 162, 103-114.

Gómez-Villalba, B., Calvo, C., Vílchez. R., González-López, J., Rodelas, B., 2006. TGGE analysis of the diversity of ammonia oxidizing and denitrifying bacteria in submerged filter biofilms for the treatment of urban wastewater. Applied Microbiology and Biotechnology, 72, 393-400.

Guelli de Souza, S., 2012. Application of biofilm in the degradation of contaminants in industrial effluents—a review. Signpost Open Journal of Biochemistry and Biotechnology, 1, 1-10.

Hai, R., Wang, Y., Wang, X., Li, Y., Du, Z., 2014. Bacterial Community Dynamics and Taxa-Time Relationships within Two Activated Sludge Bioreactors. PLoS ONE 9(3), e90175.

Hesham, A.E.L., 2012. Molecular Genetics Tools to Understand Foaming and Bulking Filamentous Bacteria in Wastewater Treatment Plants. Journal Microbial and Biochemical Technology, 4, xvii-xviii.

Hu, M., Wang, X., Wen, X., Xia, Y., 2012. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresource Technology, 117, 72-79.

Ibarbalz, F.M., Figuerola, E.L.M., Erijman, L., 2013. Industrial activated sludge exhibit unique bacterial community composition at high taxonomic ranks. Water Research, 47, 3854-3864.

Ivanovic, I. and Leiknes, T.O., 2012. The biofilm membrane bioreactor (BF-MBR)—a review. Desalination and Water Treatment, 37, 288-295.

Jamal Khan, S., Rehman, Z.U., Visvanathan, C., Jegatheesan, V., 2012. Influence of biofilm carriers on membrane fouling propensity in moving biofilm membrane bioreactor. Bioresource Technology, 113, 161-164.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., Gibson, T.J., 1998. Multiple sequence alignment with Clustal X. Trends in Biochemical Sciences, 23, 403-405.

Kermani, M., Bina, B., Movahedian, H., Amin, M.M., Nikaein, M., 2008. Application of Moving Bed Biofilm Process for Biological Organics and Nutrients Removal from Municipal Wastewater. American Journal of Environmental Sciences, 4, 675-682.

Khan, S.J., Ilyas, S., Javid, S., Visvanathan, C., Jegatheesan, V., 2011. Performance of suspended and attached growth MBR systems in treatinghigh strength synthetic wastewater. Bioresource Technology, 102, 5331-5336.

Kim, Y.M., Chon, D.H., Kim, H.S., Park, C., 2012. Investigation of bacterial community in activated sludge with an anaerobic side-stream reactor (ASSR) to decrease the generation of excess sludge. Water Research, 46, 4292-4300.

Kim, T.S., Jeong, J.Y., Wells, G.F., Park, H.D., 2013. General and rare bacterial taxa demonstrating different temporal dynamic patterns in an activated sludge bioreactor. Applied Microbiology and Biotechnology, 97, 1755-1765.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M. and Woldendorp, J.W., 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Applied Environmental Microbiology, 63, 1489-1497.

Kraume, M., Drews. A. 2010. Membrane Bioreactors in Waste Water Treatment - Status and Trends. Chemical Engineering Technology, 33, 1251-1259.

Kwon, S., Taek-Seung, K., Gi Hyeon, Y., Joon-Hong, J., Hee-Deung, P., 2010. Bacterial Community Composition and Diversity of a Full-Scale Integrated Fixed-Film Activated Sludge System as Investigated by Pyrosequencing. Journal of Microbiology and Biotechnology, 20, 1717-1723.

Leiknes,T.O., Ødegaard, H., 2007. The development of a biofilm membrane bioreactor. Desalination, 202, 135-143.

Lepš, J., Šmilauer, P., 1999. Multivariate analysis of ecological data. Faculty of Biological Sciences, University of South Bohemia, Ceské Budejovice.

Li, C., Li, X.L., Ji, M., Liu, J., 2012. Performance and microbial characteristics of integrated fixed-film activated sludge system treating industrial wastewater. Water Science and Technology, 66, 2785-2792.

Liang, Z., Das, A., Beerman, D., Hu, Z., 2010. Biomass characteristics of two types of submerged membrane bioreactors for nitrogen removal from wastewater. Water Research 44, 3313-3320.

Lim, S.Y., Kim, S., Yeon, K.M., Sang, B.I., Chun, J., Lee, C.H., 2012. Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater. Desalination 287, 209-215.

Limpiyakorn, T., Shinohara, Y., Kurisu, F., Yagi, O., 2005. Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. FEMS Microbiology Ecology, 54, 205-217.

Liu, X.C., Zhang, Y., Yang M., Wang, Z.Y., LV, W.Z., 2007. Analysis of bacterial community structures in two sewage treatment plants with different sludge properties and treatment performance by nested PCR-DGGE method. Journal of Environmental Sciences, 19, 60-66.

Loisel, P., Harmand, J., Zemb, O., Latrille, E., Lobry, C., Delgenès, J.P., Godon, J.J., 2006. Denaturing gradient electrophoresis (DGE) and singlestrand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environmental Microbiology, 4, 720-731.

Lopez-Lopez, C., Martín-Pascual, J., González-Martínez, A., Calderón, K., González-López, J., Hontoria, E., Poyatos, J.M., 2012. Influence of filling ratio and carrier type on organic matter removal in a moving bed biofilm reactor with pretreatment of electrocoagulation in wastewater treatment. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 47, 1759-1767.

Lydmark, P., Almstrand, R., Samuelsson, K., Mattsson, A., Sörensson, F., Lindgren, P.E., Hermansson, M., 2007. Effects of environmental conditions on the nitrifying population dynamics in a pilot wastewater treatment plant. Environmental Microbiology, 9, 2220-2233.

Mahmood, S., Freitag, T.E., Prosser, J.I., 2006. Comparison of PCR primer ‐ based strategies for characterization of ammonia oxidizer communities in environmental samples. FEMS Microbiology Ecology, 56, 482-493.

Makowska, M., Spychała, M,. Mazur, R., 2013. Removal of Carbon and Nitrogen Compounds in Hybrid Bioreactors. Biomass Now - Cultivation and Utilization, Dr. Miodrag Darko Matovic (Ed.), ISBN: 978-953-51-1106-1, InTech, DOI: 10.5772/53582. Available from: http://www.intechopen.com/books/biomass-now-cultivation-and-utilization/removal-of-carbon-and-nitrogen-compounds-in-hybrid-bioreactors

Mannina, G., Viviani, G., 2009. Hybrid moving bed biofilm reactors, an effective solution for upgrading a large wastewater treatment plant. Water Science and Technology, 60, 1103-1116.

Marrengane, Z., Kumar, S.K.S., Pillay, L., Bux, F., 2011. Rapid quantification and analysis of genetic diversity among *Gordonia* populations in foaming activated sludge plants. Journal of Basic Microbiology, 51, 415-423.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints, practical tools for microbial ecology. Environmental Microbiology, 10, 1571-1581.

Matos, M., Pereira, M.A., Nicolau, A., Rodrigues, A.L., Brito, A.G., Nogueira, R., 2012. Influence of carrier concentration on the control of Galactomyces geotrichum bulking and bacterial community of biofilm reactors. Desalination and Water Treatment, 41, 325-334.

McLellan, S.L., Huse, S.M., Mueller-Spitz, S.R., Andreishcheva, E.N., Sogin, M.L., 2010. Diversity and population structure of sewage-derived microorganisms in wastewater treatment plant influent. Environmental Microbiology, 12, 378-392.

Meng, F., Zhang, H., Yang, F., Li, Y., Xiao, J., Zhang, X., 2006. Effect of filamentous bacteria on membrane fouling in submerged membrane bioreactor. Journal of Membrane Science, 272, 161-168.

Miura, Y., Noriko Hiraiwa, M., Ito, T., Itonaga, T., Watanabe, Y., Okabe, S., 2007a. Bacterial community structures in MBRs treating municipal wastewater, Relationship between community stability and reactor performance. Water Research, 41, 627-637.

Miura, Y., Watanabe, Y., Okabe, S., 2007b. Significance of *Chloroflexi* in Performance of Submerged Membrane Bioreactors (MBR) Treating Municipal Wastewater. Environmental Science and Technology, 41, 7787-7794.

Molina-Muñoz, M., Poyatos, J.M., Sánchez-Peinado, M., Hontoria, E., González-López, J., Rodelas, B., 2009. Microbial community structure and dynamics in a pilot-scale submerged membrane bioreactor aerobically treating domestic wastewater under real operation conditions. Science of the Total Environment, 407, 3994-4003.

Muller, E.E.L., Pinel, N., Gillece, J.D., Schupp, J.M., Price, L.B., Engelthaler, D.M., Levantesi, C., Tandoi, V., Luong, K., Baliga, N.S., Korlach, J., Keim, P.S., Wilmesa, P., 2012. Genome Sequence of "*Candidatus* Microthrix parvicella" Bio17-1, a Long-Chain-Fatty-Acid-Accumulating Filamentous Actinobacterium from a Biological Wastewater Treatment Plant. Journal of Bacteriology, 194, 6670-6671.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59, 695-700.

Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. Journal of Biotechnology, 80, 1-33.

Nielsen, P.H., Kragelund, C., Seviour, R.J., Nielsen, J.L., 2009. Identity and ecophysiology of filamentous bacteria in activated sludge. FEMS Microbiology reviews, 33, 969-998.

Ofiţeru, I.D., Lunn, M., Curtis, T.P., Wells, G.F., Criddle, C.S., Francis, C.A., Sloan, W.T., 2010. Combined niche and neutral effects in a microbial wastewater treatment community. Proceedings of the National Academy of Sciences, 107, 15345-15350.

Pal, L., Kraigher, B., Brajer-Humar, B., Levstek, M., Mandic-Mulec, I., 2012. Total bacterial and ammonia-oxidizer community structure in moving bed biofilm reactorstreating municipal wastewater and inorganic synthetic wastewater. Bioresource Technology, 110, 135-143.

Pholchan, M. K., Baptista, J.C., Davenport, R.J., Curtis, T.P., 2010. Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge reactors. Water Research 44, 1341-1352.

Plattes, M., Henry, E., Schosseler, P.M., Weidenhaupt, A., 2006. Modelling and dynamic simulation of a moving bed bioreactor for the treatment of municipal wastewater. Biochemical Engineering Journal, 32, 61-68.

Poyatos, J.M., Molina-Mu˜noz, M., González-López, J., Hontoria, E., 2010. Particle sizedistribution in permeate from a pilot-scale membrane bioreactor system under actual operating conditions. Particulate Science and Technology: An International Journal, 28, 309-318.

Purkhold, U., Pommerening-Röser, A., Juretschko, S.,Schmid, M.C., Koops, H.P., Wagner, M., 2000. Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amoA* Sequence Analysis: Implications for Molecular Diversity Surveys. Applied Environmental Microbiology, 66, 5368-5382.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Röser, A., Koops, H.P., 2003. 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates, extension of the dataset and proposal of a new lineage within the nitrosomonads. International Journal of Systematic and Evolutionary Microbiology, 53, 1485-1494.

Rahimi, Y., Torabiana, A., Mehrdadia, N., Habibi-Rezaieb, M., Pezeshkc, H., Nabi-Bidhendi, G-R., 2011. Optimizing aeration rates for minimizing membrane fouling and its effect on sludge characteristics in a moving bed membrane bioreactor. Journal of Hazardous Materials, 186, 1097-1102.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. The ISME Journal, 3, 700-714.

Saitou, N., Nei, M., 1987. The Neighbor-joining method, a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406-425.

Sánchez, O., Ferrera, I., González, J.M., Mas, J., 2013. Assessing bacterial diversity in a seawater-processing wastewater treatment plant by 454-pyrosequencing of the 16S rRNA and amoA genes. Microbial Biotechnology, 6, 435-442.

Sánchez, O., Garrido, L., Forn, I., Massana, R., Maldonado, M.I., Mas, J., 2011. Molecular characterization of activated sludge from a seawater-processing wastewater treatment plant. Microbial Biotechnology, 4, 628-642.

Sanz, J.L., Köchling, T., 2007. Molecular biology techniques used in wastewater treatment, An overview. Process Biochemistry, 42, 119-133.

Shannon, C.E., Weaver, W., 1963. The Mathematical Theory of Communication. University of Illinois Press, Urbana, IL.

Shokralla, S., Spall, J.L., Gibson, J.F. and Hajibabaei, M., 2012. Next-generation sequencing Technologies for environmental DNA research. Molecular Ecology, 21, 1794-1805.

Short, M.D., Abell, G.C.J., Bodrossy, L., van den Akker, B., 2013. Application of a Novel Functional Gene Microarray to Probe the Functional Ecology of Ammonia Oxidation in Nitrifying Activated Sludge. PLoS ONE, 8(10), e77139.

Siggins, A., Enright, A.M., O'Flaherty, V., 2011. Temperature dependent (37-15 °C) anaerobic digestion of a trichloroethylene-contaminated wastewater. Bioresource Technology, 102, 7645-7656.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4, molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24, 1596-1599.

Thomas, T., Gilbert, J. and Meyer, F., 2012. Metagenomics - a guide from sampling to data analysis. Microbial Informatics and Experimentation, 2, 1-12.

Valentín-Vargas, A., Toro-Labrador, G., Massol-Deyá, A.A., 2012. Bacterial Community Dynamics in Full-Scale Activated Sludge Bioreactors, Operational and Ecological Factors Driving Community Assembly and Performance. PLoS ONE, 7(8), 12.

van der Gast, C.J., Ager, D., Lilley, A.K., 2008. Temporal scaling of bacterial taxa is influenced by both stochastic and deterministic ecological factors. Environmental Microbiology, 10, 1411-1418.

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W., 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Current Opinion in Biotechnology, 27, 55-64.

Wagner, M. and Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology, 13, 218-227.

Wan, C.Y., De Wever, H., Diels, L., Thoeye, C., Liang, J.B., Huang, L.N., 2011. Biodiversity and population dynamics of microorganisms in a full-scale membrane bioreactor for municipal wastewater treatment. Water Research, 45, 1129-1138.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Applied Environmental Microbiology, 73, 5261-5267.

Wang, R.C., Wen, X.H., Qian, Y., 2005. Influence of carrier concentration on the per-formance and microbial characteristics of a suspended carrier biofilm reactor. Process Biochemistry, 40, 2992-3001.

Wang, X., Wen, X., Xia, Y., Hu, M., Zhao, F., Ding, K., 2012a. Ammonia Oxidizing Bacteria Community Dynamics in a Pilot-Scale Wastewater Treatment Plant. PLoS ONE, 7(4), e36272.

Wang, X., Hu, M., Xia, Y., Wen, X., Ding, K., 2012b. Pyrosequencing Analysis of Bacterial Diversity in 14 Wastewater Treatment Systems in China. Applied and Environmental Microbiology, 78, 7042-7047.

Wang, X.C., Liu, Q., Liu, Y.J., 2010. Membrane Fouling Control of Hybrid Membrane Bioreactor, Effect of Extracellular Polymeric Substances. Separation Science and Technology, 45, 928-934.

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

Weissbrodt, D.G., Shani, N., Holliger, C., 2014. Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment. FEMS Microbiology Ecology, doi: 10.1111/1574-6941.12326.

Wells, G.F., Park, H.D., Yeung, C.H., Eggleston, B., Francis, C.A., Criddle, C.S., 2009. Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: betaproteobacterial dynamics and low relative abundance of Crenarchaea. Environmental Microbiology, 11, 2310-2328.

Wells, G.F., Park, H.D., Eggleston, B., Francis, C.A., Criddle, C.S., 2011. Fine-scale bacterial community dynamics and the taxa-time relationship within a full-scale activated sludge bioreactor. Water Research, 45, 5476-5488.

Wittebolle, L., Boon, N., Vanparys, B., Heylen, K., De Vos, P., Verstraete, W., 2005. Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. Journal of Applied Microbiology, 99, 997-1006.

Wittebolle, L., Vervaeren, H., Verstraete, W., Boon, N., 2008. Quantifying community dynamics of nitrifiers in functionally stable reactors. Appied Envronmental Microbiology, 74, 286-293.

Wojnowska-Baryła, I., Cydzik-Kwiatkowska, A., Zielińska, M., 2010. The Application of Molecular Techniques to the Study of Wastewater Treatment Systems. Methods in Molecular Biology, 599, 157-183.

Xia, S., Duan, L., Song, Y., Li, J., Piceno, Y.M., Andersen, G.L., Alvarez-Cohen, L., Moreno-Andrade, I., Huang, C.L., Hermanowicz, S.W., 2010. Bacterial Community Structure in Geographically Distributed Biological Wastewater Treatment Reactors. Environmental Science and Technology, 44, 7391-7396.

Xin-chun, L.,Yu, Z., Min, Y., Zhen-yu, W., Wen-zhou, L.V., 2007. Analysis of bacterial community structures in two sewage treatment plants with diferent sludge properties and treatment performance by nested PCR-DGGE method. Journal of Environmental Sciences, 19, 60-66.

Ye, L., Shao, M.F., Zhang, T., Tong, A.H.Y., Lok, S., 2011. Analysis of the bacterial community in a laboratory-scale nitrification reactor and a wastewater treatment plant by 454-pyrosequencing. Water Research, 45, 4390-4398.

Ye, L., Zhang, T., 2013. Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. Applied Microbiology and Biotechnology, 97, 2681-2690.

Yuan, Z., Blackall, L.L., 2002. Sludge population optimisation: a new dimension for the control of biological wastewater treatment systems. Water Research, 36, 482-490.

Zhang, T., Shao, M. F., Ye, L., 2012. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME Journal, 6, 1137-1147.

Zhou, J., Liu, W., Deng, Y., Jiang, Y.H., Xue, K., He, Z.,Van Nostrand, J.D., Wu, L., Yang, Y., Wang, A., 2013. Stochastic Assembly Leads to Alternative Communities with Distinct Functions in a Bioreactor Microbial Community. mBio 4(2), e00584-12.

Ziembińska, A., Ciesielski, S., Miksch, K., 2009. Ammonia oxidizing bacteria community in activated sludge monitored by denaturing gradient gel electrophoresis (DGGE). Journal of General and Applied Microbiology, 55, 373-380.

V. CHAPTER 3

Quantitative study of the nitrifying and denitrifying communities, involved in the depuration process of urban wastewater in moving bed membrane bioreactor: Effect of operational conditions.

1. Introduction

Nitrogen compounds are a significant threat to natural aquatic ecosystems, mainly because they play an important role in the eutrophication process. These compounds contribute to oxygen depletion and some of them, such as ammonia and nitrite, are toxic to aquatic invertebrate and vertebrate species, including human beings (Paredes *et al*., 2007; Wang *et al*., 2010; Bin *et al*., 2011). Taking into account that the effluents from wastewater treatment plants (WWTPs) are one of the major sources of nitrogen compounds (Ahmed *et al*., 2012), it seems logical that the environmental legislation is becoming increasingly stringent concerning nitrogen concentration in wastewater effluents (Pal *et al*., 2012). Consequently, the reduction of nitrogen levels from discharges constitutes a prime objective of the wastewater treatment systems (WWTSs) and, according to Pal *et al*. (2012), to achieve this objective a better knowledge of the processes responsible for nitrogen removal is necessary to improve treatment performance and control.

While it is true that nitrogen compounds can be removed from wastewater by physicochemical processes, biological nitrogen removal is most commonly used, since this natural process is more effective, inexpensive and environmentally friendly (Ahn, 2006; Xia *et al*., 2008; Fu *et al*., 2010; Kumar *and* Lin, 2010; Seifi *and* Fazaelipoor, 2012; Vannecke *et al*., 2014). Traditionally, biological nitrogen elimination from wastewater has been accomplished using nitrification and denitrification. Bacterial nitrification comprises two-steps based on the sequential oxidation of ammonia via nitrite to nitrate, being these conversions catalyzed by aerobic chemolithoautotrophic bacteria: ammonia-oxidizing (AOB) and nitrite-oxidizing bacteria (NOB) (Lücker *et*

al., 2010; Bin *et al*., 2011; Lücker *et al*., 2013). In denitrification, nitrite and/or nitrate are reduced via nitric oxide and nitrous oxide to nitrogen gas by heterotrophic bacteria (Vlaeminck *et al*., 2011). Contrary to nitrifiers, denitrifiers are facultative anaerobes; they can utilize oxygen or nitrate/nitrite as electron acceptors, but they prefer to use oxygen. Therefore, bacterial denitrification takes place only under anoxic conditions (Francis *et al*., 2007; Seifi *and* Fazaelipoor, 2012). For these reasons, the conventional processes for biological nitrogen removal consist of two separate bioreactors to provide an enabling environment for each kind of bacteria, nitrifiers and denitrifiers. However, it is also known that in a single aerobic bioreactor, simultaneous nitrification and denitrification (SND) can occur as result of the oxygen concentration gradient created within microbial aggregates (flocs and/or biofilms), so that anoxic microenvironments take place in their inner layers (Hibiya *et al*., 2003; Yang *et al*., 2009; Khan *et al*., 2011).

In this context, moving bed membrane bioreactor (MBMBR), which consist in the addition of freely moving carriers (support material) to the conventional membrane bioreactor (MBR) (Leiknes *and* Ødegaard, 2007), have been shown to be successful for the enhancement of nitrification and denitrification process by SND under aerobic conditions (Yang *et al*., 2009; Khan *et al*., 2011). In MBMBR systems, the biodegradation is carried out by microorganism that growth both as suspended biomass (SB) and attached biofilm (AB). The immobilisation of microorganisms on the carriers enables uncoupling of the growth rate of nitrifying populations and the solid retention time (SRT) of the suspended mixed liquor phase, since in the AB a long SRT is maintained (Onnis-Hayden *et al*., 2011). This feature is crucial, because it is widely recognized that long SRT is required for the optimal development of nitrifying bacteria in a bioreactor (Zhang *et al*., 2009; Le-Clech, 2010; Onnis-Hayden *et al*., 2011; Zhang

et al., 2011). This is certainly due to the slow growth rate of nitrifiers. In addition, nitrifies and particularly AOB are highly sensitive to several environmental/operational conditions, such as low temperature, extreme pH, low dissolved oxygen concentrations, and toxic compounds (Siripong *and* Rittmann, 2007; Wang *et al*., 2010; Pal *et al*., 2012). In this sense, another advantage of the incorporation of biofilm process are the higher resistance of AB to toxic substances (Ivanovic *and* Leiknes, 2012) and the fact that they are less affected than suspended cultures by environmental changes such as nutrient concentration, temperature, pH and metabolic products (Guelli de Souza, 2012).

In WWTPs the ability to nitrify is apparently limited to a few bacteria lineages (Wagner *et al*., 2006). Specifically, the prevalent AOB are evolutionally related to the genera *Nitrosomonas* and *Nitrosospira*, both belonging to a monophyletic group within the β-*proteobacteria* (Purkhold *et al*., 2003). Among the bacteria responsible for the second step of nitrification, the genera *Nitrobacter* and *Nitrospira* have been reported as the most relevant NOB in biological nitrogen removal processes (Daims *et al*., 2001; Lücker *et al*., 2010). By contrast, the ability to reduce nitrate or nitrite under anoxic conditions is spread across several bacterial lineages. Due to this high taxonomic diversity of the denitrifiers, it has been difficult to identify the most relevant denitrifying bacteria in WWTPs (Wagner *et al*., 2006; Kim *et al*., 2013a).

There are many reasons to justify the importance of establishing links between microbiology and engineering aspects (de los Reyes, 2010; Short *et al*., 2013). For example, it is certain that nitrifiers, due to their fragility, are strongly affected by changes in operational parameters, with the consequent negative influence on nitrogen removal efficiency. Therefore, in agreement with previous reports (Siriponga *and*

Rittmann, 2007; Wang *et al*., 2010; Kim *et al*., 2011a), we think that, to improve the effectiveness of the biological nitrogen removal in WWTSs, a better understanding of the dynamic of N-cycle bacteria as well as the establishment of an adequate link between the abundance and diversity of N-cycle bacteria and environmental/operational conditions is essential.

To study the N-cycle bacteria in engineered environments, quantitative real-time PCR (qPCR) is one of the most popular, robust and highly reproducible methods (Smith *and* Osborn, 2009; Kim *et al*., 2013a). Additionally, this molecular technique is very useful for tracking changes across longitudinal and spatial scales in gene abundance, enabling to relate these changes with variations in environmental/operational conditions (Smith *and* Osborn, 2009). qPCR has been widely used to approach the study of N-cycle bacteria in conventional activated sludge (CAS) and MBR systems (Limpiyakorn *et al*., 2005; Zhang *et al*., 2009; Zhang *et al*., 2010; Limpiyakorn *et al*., 2011; Xia *et al*., 2012; Zeng *et al*., 2014). However, despite the great potential of the WWTSs that combine SB and AB to enhance total nitrogen removal (IFAS, integrated fixed-film activated sludge; MBBR, moving bed biofilm reactor, and MBMBR, Onnis-Hayden *et al*., 2011; Ivanovic *and* Leiknes, 2012), little quantitative information of N-cycle bacteria in both SB and AB fractions is available (Kim *et al*., 2011a).

In view of all the above mentioned, to provide greater knowledge of the dynamic of N-cycle bacteria in a MBMBR, the following goals were set up:

1. To determine the absolute and relative abundances of N-cycle bacteria (AOB, NOB and denitrifiers) in both SB and AB fractions, with a 20% and 35% (v/v) of CFR and under four different operational conditions.

2. To determine the AB contribution to the total number of copies of each targeted gene in the MBMBR system.

3. To determine the influence of physico-chemical parameters and CFR on the N-cycle bacteria abundance. For that, a multivariate statistical analysis was performed.

In order to gain comprehensive information about N-cycle bacteria developed in our MBMBR system, the quantitative study was assessed in both SB and AB samples. Substrate transfer within biofilms is usually limited, thus heterotrophic and nitrifying bacteria not only compete for the space inside the biofilm, but also for oxygen, their common electron acceptor (Elenter *et al*., 2007; Xia *et al*., 2008). According to Nogueira *et al*. (2002) due to this competition, a stratified structure is created within the biofilm. As a result, fast-growing heterotrophic bacteria are placed in the outer layers, where both substrate concentration and detachment rate are high, while nitrifiers are located in the inner layers (Elenter *et al*., 2007). However, inside the mixed liquor flocs, a less-stratified structure is expected, since the flocs have a more open structure and a larger relative surface area compared to biofilms (Morgenroth *et al*., 2002). Additionally, the biomass age is another important difference between both SB and AB fractions.

2. Material and Methods

2.1. Experimental design: *description of the pilot-scale MBMBR experimental plant and operating conditions*

The pilot-scale experimental plant used to perform this study and the operating conditions have been described in detail in chapter 1 (sub-paragraph 2.1.1). Briefly, the

MBMBR system consisted of two continuously aerated bioreactors: a moving bed bioreactor (MBBR) in which carriers moved freely by aeration in an operating volume of 358 L, and a membrane bioreactor (MBR) composed of three ultrafiltration membrane modules of hollow fibre (Zenon®) submerged in 87 L of operating volume. Continuous sludge recirculation was established to maintain the same concentration of total suspended solids in both reactors. The effluent was collected in a back-washing tank. The MBMBR was installed in the municipal WWTP "Puente de los Vados" (Emasagra S.A., Granada, Spain). The urban wastewater was pumped from the primary settling tank to the MBBR. The carrier K1, made from high density polyethylene, developed by AnoxKaldnes (Norway), was used as a support material. Characteristics of the carrier K1 are described in detail in chapter 1 (sub-paragraph 2.1.2). It is worth highlighting that carrier K1 provides a large protected surface for the bacteria, favouring the development and retention of slow growing microorganisms (Pal *et al*., 2012).

To gain a broad understanding about how the variations in operational conditions affect N-cycle bacteria developed in both SB and AB fractions, different Carrier Filling Ratios (CFRs, 20% or 35%, v/v), Hydraulic Retention Times (HRTs, 10h or 24h) and Mixed Liquor Total Suspended Solids (MLTSS, c.a. 2,500 mg/L or 4,500 mg/L) were tested. For that, eight experimental phases (designated as experiments 1-8) were conducted in the MBMBR system. The MBMBR system was continuously operated throughout the study with either 20% or 35% (v/v) CFR. Experiments 1-4 (conducted in 2011) were performed with 20% (v/v) CFR, while experiments 5-8 (conducted in 2012) were performed with 35% (v/v) CFR. The same combinations of HRT and MLTSS were established for both CFRs (20% and 35%, v/v).

Table III.1 (**Chapter 1**) displays the details of the sampling periods and the operational conditions established for each experiment.

Inflow rates of 45.5 and 18.96 L/h were used to maintain the same biomass concentration at a HRT of 10 h and 24 h, respectively. To maintain the selected MLTSS concentrations of the system in experiments 1–8, different purges of 35, 18, 20, 8, 24, 52, 25 and 8 L/day were required, respectively. After completing each experimental phase, the membranes were cleaned with sodium hypochlorite (1 g/L), as previously reported by Poyatos *et al*. (2010).

2.2. Physico-chemical analysis

The physico-chemical analysis and the biofilm recovery method required for the analysis of Biofilm Total Solids (BTS) and Biofilm Volatile Solids (BVS) have been described in detail in chapter 1 (sub-paragraph 2.3). In short, Biological Oxygen Demand at 5 days (BOD_5), Chemical Oxygen Demand (COD), MLTSS, Mixed Liquor Volatile Suspended Solids (MLVSS), BTS, BVS and ammonium ion (NH_4^+) were analyzed daily in influent, MBBR and effluent. All parameters were measured according to Standard Methods for the Examination of Waste and Wastewater (APHA, 2005). The BTS and BVS concentrations in the MBBR were calculated according to Plattes *et al*. (2006). NH_4^+ was determined by ionic chromatography using a conductivity detector (Methrom). Separation and dilution of the cation was carried out on a Metrosep C 4 column using as eluent a solution of dipicolinic acid, and distilled water as a regenerate. The pH was measured using a Crison pH 25 pH meter (Crison instruments S.A., Barcelona, Spain) and the temperature in the bioreactor was monitored with an automatic control device available in the MBBR.

The quality of the effluent under all the conditions tested was in agreement with the EU regulation for discharges (91/271/CEE, 1991) (see **Table III.2**; **Chapter 1**). In **Table III.2** (**Chapter 1**) are showed the mean values of MLTSS, MLVSS, BTS, BVS, BOD_5, COD, temperature and pH as well as the BOD_5 and COD removal rates measured in the bioreactor for each experiment. The mean values of NH_4^+ concentration measured in the influent and effluent of the MBMBR system as well as the NH_4^+removal rates are showed in **Table V.1**.

Table V.1. Average values ± standard deviation of the concentration of ammonia (NH_4^+) in the influent and effluent, and NH_4^+ removal rates (%) in the eight experiments carried out in the MBMBR. LSD: least significant difference (Student's test, $p < 0.05$). Data in columns with same superscript letter are not statistically different.

	Experiment	**NH_4^+ influent (mg/L)**	**NH_4^+ effluent (mg/L)**	**NH_4^+ removal (%)**
CFR 20%	1	69.89 ± 20.83^{a}	14.79 ± 5.21^{bc}	78.27 ± 5.86^{ab}
	2	88.03 ± 35.27^{ab}	4.77 ± 4.48^{a}	94.04 ± 5.88^{c}
	3	72.58 ± 16.06^{a}	6.73 ± 8.17^{ab}	91.46 ± 10.22^{c}
	4	84.48 ± 36.87^{ab}	4.92 ± 5.70^{a}	93.73 ± 9.48^{c}
CFR 35%	5	107.19 ± 29.70^{b}	32.37 ± 9.80^{d}	71.61 ± 4.00^{a}
	6	79.98 ± 33.44^{ab}	3.06 ± 3.56^{a}	96.05 ± 5.34^{c}
	7	77.44 ± 31.51^{ab}	7.14 ± 10.14^{abc}	90.35 ± 12.17^{c}
	8	77.83 ± 30.78^{ab}	14.70 ± 5.72^{c}	80.85 ± 4.84^{b}
	LSD	40.23	9.22	10.46

With regard to the biofilm recovery to calculate BTS and BVS concentrations, fifty units of carriers with adhered biofilm were collected in sterile conditions from different areas of the MBMBR, using a sieve-sampling device as recommended by Calderón *et al.* (2012). The carrier units were placed in flasks with 50 mL sterile saline solution (0.9% NaCl). These samples were vortexed for 1 min, sonicated for 3 min, and

the resultant biofilm suspensions were collected by centrifuging for 5 min at 3,000 × g. This process was repeated twice. The biofilm pellet obtained after the last centrifugation step was resuspended in 50 ml of sterile saline solution (0.9% NaCl).

2.3. Quantitative investigation of N-cycle bacteria by qPCR

To investigate the quantitative changes of N-cycle bacteria present in SB and AB developed in the MBMBR system by qPCR, two samples of SB and AB collected during the steady period of each experiment were selected. Specifically, samples of both AB and SB were retrieved from the system in the first and last weeks of each steady-state sampling period. The selected samples were also used for the study of the community structure of total Bacteria and ammonia oxidizing bacteria by TGGE and 454-pyrosequencing, in which these samples were denominated as: 1 SB4, 1 AB4, 1 SB6, 1 AB6, 2 SB4, 2 AB4, 2 SB6, 2 AB6, 3 SB4, 3 AB4, 3 SB6, 3 AB6, 4 SB4, 4 AB4, 4 SB6, 4 AB6, 5 SB4, 5 AB4, 5 SB6, 5 AB6, 6 SB3, 6 AB3, 6 SB5, 6 AB5, 7 SB3, 7 AB3, 7 SB5, 7 AB5, 8 SB3, 8 AB3, 8 SB5, 8 AB5. The name of each sample codes the experiment (1-8), the sample type (SB or AB) and the sampling week in each experiment. E.g. for experiment 1, the SB sample taken in the sixth week of this experiment was named 1 SB6.

For the quantitative study of N-cycle bacteria in the SB, 2 ml of mixed liquor were concentrated by centrifugation for 2 min at 14,000 × g. After discarding the supernatant, the pellet obtained (ca. 250 mg) was used for DNA extraction. In the case of AB, it was necessary to remove the biofilm from the supporting material. The method for biofilm recovery for DNA extraction has been described in detail in chapter 2 (sub-paragraph 2.2). In essence, five units of biofilm-colonized carriers were placed

in sterile flaks with 5 mL of sterile saline solution (0.9% NaCl), vortexed for 1 min, sonicated for 3 min, and the resultant biofilm suspensions were collected by centrifugation for 5 min at 3,000 × g. This process was repeated twice. The biofilm pellet obtained after the last centrifugation step (ca. 250 mg) was used for DNA extraction.

2.3.1. DNA extraction and qPCR assays

Genomic DNA from c.a. 250 mg of SB and AB samples was extracted using the FastDNA® SPIN Kit for Soil and the FastPrep®-24 instrument (MPBiomedicals, Germany), according to the manufacturer‘s indications. In order to measure DNA quality and quantity, the DNA samples were analyzed electrophoretically on 1% (w/v) agarose gels and spectrophotometrically by determination of the absorbance at 260 nm and the absorbance ratios at 260 nm and 280 nm, using NanoDrop ND-1000 (Thermo Scientific).

To determine the abundance of total Bacteria, nitrifiers and denitrifiers developed in SB and AB, five different qPCR assays were conducted to target the following genes: 16S rRNA (total Bacteria), 16S rRNA of AOB, *amoA* (AOB), 16S rRNA of *Nitrospira* (NOB), and *nosZ* (denitrifying bacteria). It should be mentioned that the quantification of the *nxrA* gene of *Nitrobacter* spp. was attempted using the primers nxrAF 1370 and nxrAR 2843 described by Wertz *et al*. (2008), but no amplification products were detected in any sample. The absence or low abundance of *Nitrobacter* in the MBMBR could be connected to the fact that, contrary to *Nitrospira*, *Nitrobacter* prefer to grow as suspended cells instead of attaching to mixed liquor flocs

or biofilms, so they are easily washed out of the bioreactors (Wang *et al*., 2012a; Zeng *et al*., 2014).

The qPCR assays were performed in a StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems). Primers and conditions for each qPCR reaction are summarized in **Tables V.2** and **V.3**, respectively. The qPCR conditions for each assay were adjusted from previously described protocols (Kowalchuk *et al*., 1997, Ovreas *et al.,* 1997, Rotthauwe *et al*., 1997; Scala *and* Kerkhof, 1998; Dionisi *et al*., 2002). Quantitative amplification of each target gene (16S rRNA, 16S rRNA of AOB, *amoA*, 16S rRNA of *Nitrospira* and *nosZ*) was performed in a total volume of 25 µl with TrueStart Hot Start DNA polymerase (Fermentas Thermo Fisher Scientific Inc, Waltham, MA, USA) and SYBR green I (Sigma–Aldrich, St. Louis, MO, USA). SYBR green I was diluted in dimethyl sulfoxide (DMSO). All samples were measured in triplicate and negative controls were included in all qPCR assays. Primers and DMSO were supplied by Sigma Aldrich (St. Louis, MO, USA), dNTPs by Promega (Madison, USA), and BSA by New England Biolabs (Ipswich, MA, USA).

Table V.2. Primer used for quantification of total bacteria, nitrifiers and denitrifiers by qPCR.

Bacterial group	Target	Sequences of primers (5'-3')		References
Bacteria	V3-16S rRNA	PRBA338f PRUN518r	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Ovreas *et al.,*1997
Ammonia oxidizing β-proteobacteria (AOB)	16S rRNA	CTO189fAB CTO189fC CTO654r	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	Kowalchuk *et al*., 1997
	amoA	*amoA*-1F *amoA*-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Rotthauwe *et al*., 1997
Nitrite oxidizing bactéria (NOB)	16S rRNA Group *Nitrospira*	NSR1113f NSR1264r	CCTGCTTTCAGTTGCTACCG GTTTGCAGCGCTTTGTACCG	Dionisi *et al*., 2002
Denitrifying bacteria	*nosZ*	Nos1527F Nos1773R	CGCTGTTCHTCGACAGYCA ATRTCGATCARCTGBTCGTT	Scala and Kerkhof, 1998

Table V.3. qPCR conditions for quantification of total bacteria, nitrifiers and denitrifiers.

	Bacterial group:	Bacteria	Ammonia oxidizing β-proteobacteria (AOB)		Nitrite oxidizing bacteria (NOB) Group *Nitrospira*	Denitrifying bacteria
	Target:	V3-16S rRNA	16S rRNA	*amoA*	16S rRNA	*nosZ*
qPCR master mix	Buffer (10X)	1X	1X	1X	1X	1X
	$MgCl_2$ (25 mM)	1.5mM	1.5mM	1.5mM	1.5mM	1.5mM
	dNTPs (10 mM)	0.2mM	0.2mM	0.2mM	0.2mM	0.2mM
	Albumin (BSA) (100X)	0.25X	0.25X	0.25X	0.25X	0.25X
	Primers (10 µM)	0.2 µM	0.08 µM	0.2 µM	0.2 µM	0.08 µM
	DMSO + 2X SYBR®GreenI	1.25 µl	1.25 µl	1.25 µl	1.25 µl	1.25 µl
	TrueStart™ Hot Start (5U/µl)	0.625U	0.625U	0.625U	0.625U	1U
qPCR conditions	**Holding Stage**	95˚C 3min	94˚C 5min	94˚C 5min	94˚C 5min	94˚C 7min
	Cycling Stage:	40 Cycles	40 Cycles	40 Cycles	40 Cycles	40 Cycles
	Denaturation	94˚C 1min	94˚C 30sec	94˚C 30sec	94˚C 30sec	94˚C 30sec
	Annealing	56˚C 40sec	57˚C 30sec	55˚C 30sec	65˚C 30sec	50˚C 1min
	Elongation	72˚C 40sec	60˚C 2min	60˚C 2min	72˚C 30sec	72˚C 1min
	Melt Curve Stage	60ºC - 95ºC + 0.3ºC/min Fluorescence measured each 15 sec				

Absolute quantifications for both SB and AB samples were achieved by constructing standard curves with serial dilutions of linearized plasmids harboring PCR-amplified inserts of the targeted genes. The TOPO® TA cloning vector kit (Invitrogen, Carlsbad, USA) was used for cloning into the pCR® 2.1 TOPO® plasmid vector and transformation into ONE SHOT *Escherichia coli*. Successful insertion of the target amplicons into the vector was confirmed by PCR using the M13 forward and reverse primers. Plasmid DNA was subsequently extracted using the Pure yield® Plasmid Miniprep System (Promega, Madison, USA) and linearized using the *Bam*HI restriction enzyme. The linearized plasmids were purified with the High Pure PCR product

purification kit (Roche Applied Science, Penzberg, Germany). Plasmid quality and concentration were measured on 1% (w/v) agarose gels and by spectrophotometry using NanoDrop ND-1000 (Thermo Scientific). The standard curves, generated for total Bacteria, 16S rRNA of AOB, *amoA*, 16S rRNA of *Nitrospira* and *nosZ* assays, showed a correlation coefficient $r^2 > 0.99$. The efficiency of PCR amplification for each gene was between 90% and 100%. The quality of all the qPCR amplifications was verified using both agarose gels and melting curve analysis. The specificity of the primers used in this study was confirmed by sequence analysis of a sample of the cloned amplicons used as standards.

Template copy number was estimated using the online software "Calculator for determining the number of copies of a template" (Staroscik, 2004), as was described by Jackson *et al.* (2013). The number of copies of each targeted gene estimated by qPCR in both fractions was expressed per L of mixed liquor and per L of carriers, respectively. To evaluate the percentage of contribution of each specific fraction of the biomass (SB or AB) to the total number of copies of each target gene per L of the MBMBR system, the effective mixed liquor volumes in the bioreactor were calculated by subtracting the amount displaced by the carrier media (3.8% for a CFR of 20%, 6.65% for a CFR of 35%). The number of copies of the target genes in the AB in 1 L of the MBMBR was calculated taking into account the number of carrier units per L of bioreactor (1030 carriers) and the CFR (20 or 35%, v/v), according to the formula described by Plattes *et al.* (2006) and detailed in chapter 1 (sub-paragraph 2.4). The number of carrier units (Kaldnes K1) per L of bioreactor has been previously reported by Gapes *and* Keller (2009).

2.4. Statistical analysis

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

Multivariate statistical analysis was performed using CANOCO for Windows v.4.5 software (ScientiaPro, Budapest, Hungary). Redundancy analysis (RDA), a linear constrained ordination method, was used for data analysis (Lepš *and* Šmilauer, 1999). RDA was chosen after a preliminary detrended correspondence analysis (DCA), which revealed a linear rather than unimodal response of the abundance of all the targeted genes. RDA was performed to search for patterns in the set of operational conditions and to assess their relationship with the abundance values of each targeted gene. The Monte Carlo permutation test was used to calculate the statistical significance of the contribution of each operational parameter to the canonical axes. RDA was performed separately for each sample type, in order to know whether the abundance values from SB and AB were affected by the same or different system variables. The following environmental variables were included in the RDA: HRTs (10 h or 24 h), CFR (20% or 35%, v/v), pH, temperature, COD, BOD_5 and NH_4^+ influent concentration. MLTSS and MLVSS were used in RDA for the SB, while BTS and BVS were used in RDA for the AB. Among these variables, MLTSS and BVS were removed from the final analyses due to their strong linear correlation with MLVSS and BTS, respectively. In addition, HRT and CFR were expressed as nominal variables. All the non-nominal environmental variables were transformed to $\log(x + 1)$, except pH.

3. Results and discussion

3.1. Absolute and relative abundances of total Bacteria, AOB, NOB (*Nitrospira*) and denitrifiers (*nosZ*) in both SB and AB fractions

In order to compare the abundances of N-cycle bacteria in the MBMBR with those reported in previous studies, the number of copies of the targeted genes in SB and AB fractions were expressed per L of mixed liquor or L of carriers (**Fig. V.1**) as well as per gram of MLVSS or BVS, respectively (**Fig. V.2**). For both SB and AB samples and regardless of how the number of targeted molecules was expressed, the average number of copies of the gene markers of bacteria (16S rRNA), AOB (16S rRNA), AOB (*amoA*), NOB (16S rRNA of *Nitrospira*) and denitrifiers (*nosZ*) were always inside the following ranges: 10^{11}-10^{12}, 10^{7}-10^{9}, 10^{7}-10^{9}, 10^{4}-10^{7} and 10^{8}-10^{9}, respectively (**Figs. V.1** and **V.2**). These orders of magnitude were in concordance with those previously reported in conventional activated sludge (CAS), MBR, sequencing biofilm batch reactors (SBBR), and IFAS systems (Geets *et al*., 2007; van den Akker *et al*., 2010; Zhang *et al*., 2010; Limpiyakorn *et al*., 2011; Xia *et al*., 2012; Wang *et al*., 2014). Similar results were also reported for a MBMBR by Liang *et al*. (2010), who quantified by qPCR the abundance of total Bacteria and AOB in the SB developed in a MBMBR, and in a MBR operated under anoxic/aerobic conditions. These authors found the same orders of magnitude for the abundance of total Bacteria (10^{12} copies 16S rRNA gene/L) and AOB (10^{8} copies 16SrRNA gene, *amoA* gene/L) in both types of WWTSs.

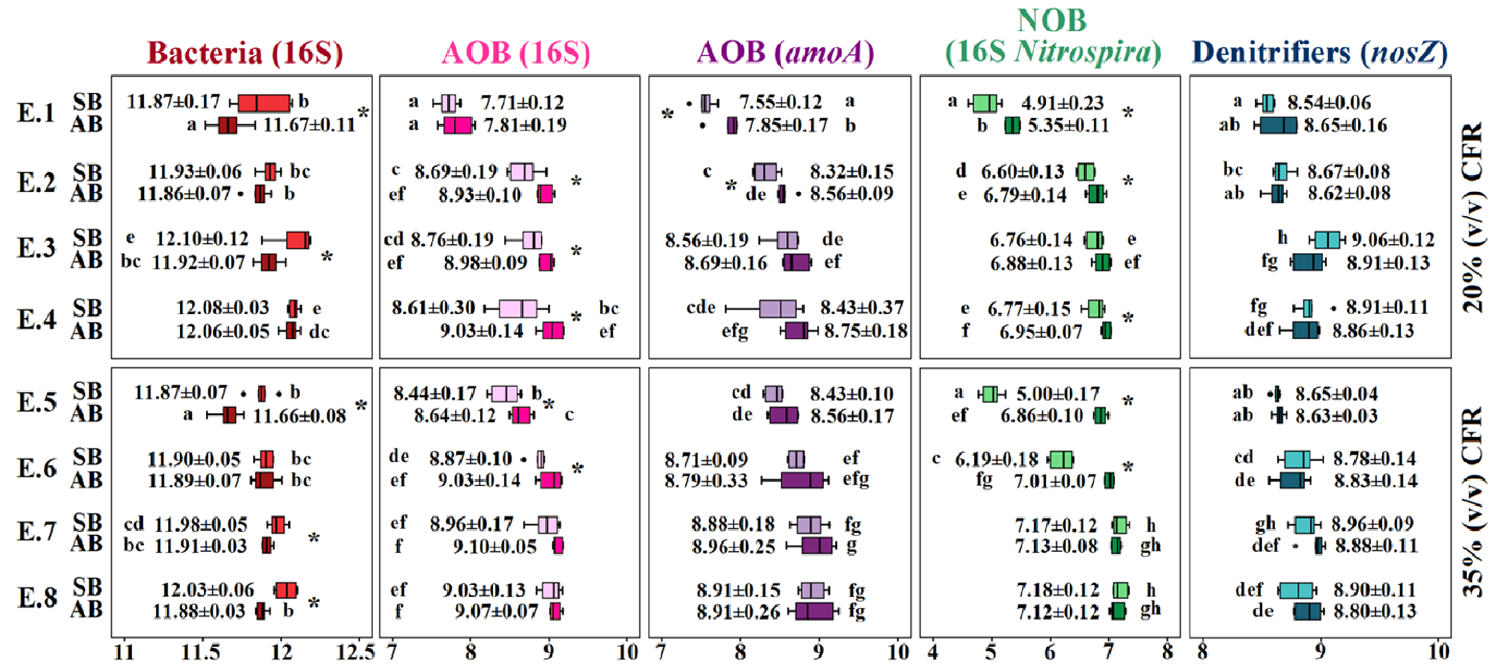


Figure V.1. Box-and-Whisker plots of the number of copies (in logarithmic scale) of targeted genes per liter of mixed liquor for suspended biomass (SB) or liter of carriers for attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR with a 20% or 35% (v/v) CFR. In plots, upper and lower bounds of the box denote the 75th and 25th percentiles, upper and lower bounds of bars represent the 90th and 10th percentiles, and outliers values are represented by circles. Average values ± standard deviations are shown near the boxes. Multifactor ANOVA showed significant differences in the number of copies between sample type (SB and AB). *Significant differences between SB and AB in the same experiment. Data followed by the same lower-case letter do not significantly differ according to the Student's test ($p < 0.05$).

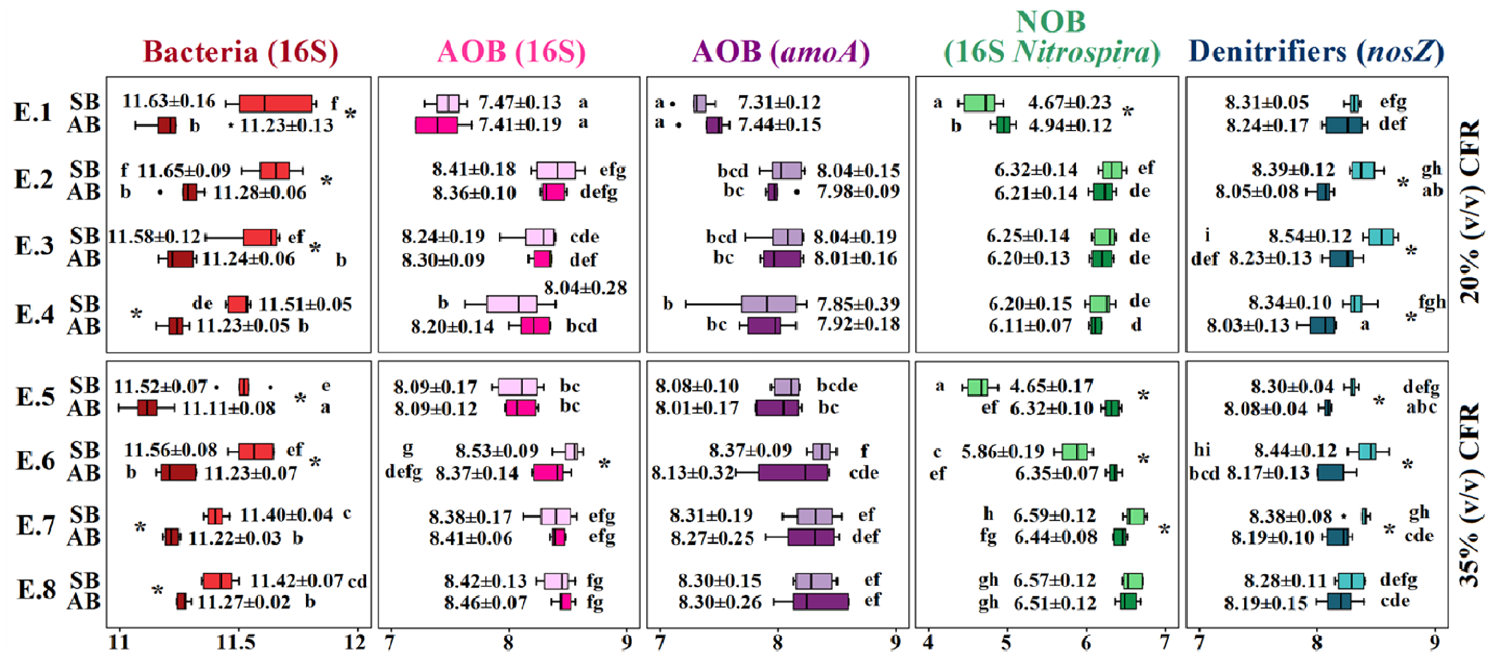


Figure V.2. Box-and-Whisker plots of the number of copies (in logarithmic scale) of targeted genes per gram of MLVSS for suspended biomass (SB) or gram of BVS for attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR with a 20% or 35% (v/v) CFR. In plots, upper and lower bounds of the box denote the 75th and 25th percentiles, upper and lower bounds of bars represent the 90th and 10th percentiles, and outliers values are represented by circles. Average values ± standard deviations are shown near the boxes. Multifactor ANOVA showed significant differences in the number of copies between sample type (SB and AB). *Significant differences between SB and AB in the same experiment. Data followed by the same lower-case letter do not significantly differ according to the Student's test ($p < 0.05$).

In addition, in concordance with the results reported by Liang *et al*. (2010), slightly differences (lower than one order of magnitude) were observed between the abundance of AOB measured using either the 16S rRNA gene or *amoA* as specific markers. Considering the purely quantitative nature of the qPCR results, these differences appear to be common, since it is well known that the amplification of the *amoA* gene misses some AOB populations, while the primers used to target the specific partial region of the 16S rRNA gene can also amplify some non-AOB targets (Purkhold *et al*., 2000; Purkhold *et al*., 2003; Mahmood *et al*., 2006).

In general, although the absolute abundance of all targeted molecules in both SB and AB fractions remained rather stable throughout the eight experiments, the number of copies of gene markers of total Bacteria and denitrifiers displayed a slightly higher stability (differences lower than 1 order of magnitude among all experiments) than those of nitrifying bacteria. Specifically, it is remarkable that the lower abundance of nitrifiers (AOB and NOB) in both SB and AB samples was observed in the first experiment carried out with either 20% or 35% (v/v) CFR (experiments 1 and 5, respectively). It seems to indicate a progressive development of the community of slow-growing nitrifying bacteria, which reached stability at the second experiment. This was probably due to the fact that the system was continuously operated throughout the study for each percentage of CFR, so the nitrifiers have enough time to increase their numbers between the first and the second experiment. This finding could be also related to the significantly lower removal efficiency of NH_4^+ in the MBMBR observed in experiments 1 and 5 (**Table V.1**). In this sense, Wang *et al*. (2014) found a positive correlation among the abundance of AOB and the removal efficiency of NH_4^+ in a SBBR.

The analysis of variance revealed small differences in the abundance of the targeted genes (lower than one order of magnitude) between SB and AB samples ($p < 0.05$) (**Figs. V.1** and **V.2**). These results suggest that both SB and AB fractions played a similar role in the nitrification-denitrification process under all the conditions tested. The only exception to the above mentioned trend is the large and significant difference in the abundance of NOB between SB and AB observed in experiment 5, being the higher number of copies associated to the AB fraction. At the same time, it was noticeable the higher abundance of NOB in the AB fraction (more than one order of magnitude) in experiment 5, compared to experiment 1. Taking into account that the operational differences between experiments 1 and 5 were the CFR (20 and 25% v/v, respectively) and the HRT (10 and 24h, respectively) (**Table III.2**; **Chapter 1**), it could be suggested that higher CFR and HRT promoted the initial development of NOB in the AB.

In general, nitrifiers showed a similar trend of abundance, regardless of how the number of targeted molecules was expressed (per g biomass or L of volume). However, for total Bacteria and denitrifiers there was a higher difference between SB and AB fractions when the abundances were expressed per g of biomass, being the higher average values associated to SB (**Fig. V.2**). This was mainly due to the fact that a higher biomass concentration was obtained in AB compared to SB in all experiments, with the exception of experiment 8, in which a decrease in biofilm solids (BTS and BVS) was observed due to the detachment process (**Table III.2**; **Chapter 1**). For this reason, a lower difference in the abundance of total Bacteria and denitrifiers between SB and AB was recorded in experiment 8.

As mentioned above (sub-paragraph 1), a better diffusion of substrates in the SB faction could be expected; hence, the substrate availability per gram of biomass for fast-

growing heterotrophic bacteria could be higher in SB than in AB. Furthermore, according to Nogueira *et al*. (2002), fast-growing heterotrophic microorganisms grow mainly in suspension, while the slow-growing nitrifiers form biofilms. These considerations could explain the higher abundance of heterotrophic bacteria (total Bacteria and denitrifiers) per gram of biomass observed in SB. As for nitrifying bacteria, conflicting results have been reported in the few works that approach the study of N-cycle bacteria in both SB and AB fractions in WWTSs. Onnis-Hayden *et al*. (2011), using fluorescence *in situ* hybridisation (FISH), demonstrated that in an IFAS system the abundance of AOB and NOB attached to carriers was clearly higher, suggesting that most nitrifiers occurring in SB were originated from biofilm detachment process. On the contrary, van den Akker *et al*. (2010) reported that the number of copies of AOB gene markers per gram of biomass detected by qPCR did not vary significantly ($p>0.05$) between SB and AB developed in IFAS system. In concordance with these authors, our results showed an equivalent abundance of AOB per g of biomass in both SB and AB samples in nearly all experiments.

Significant differences between the relative abundance of nitrifiers (% of AOB and NOB among the total Bacteria community) in SB and AB fractions have been previously reported in IFAS system by Kim *et al*. (2011a). Against this background, in order to calculate which fraction of the total bacterial community was represented by each N-cycle bacterial group studied, the number of copies detected of the targeted genes was expressed as the percentage of the number of copies of the 16S rRNA gene of bacteria, for both SB and AB fractions (**Fig. V.3**).

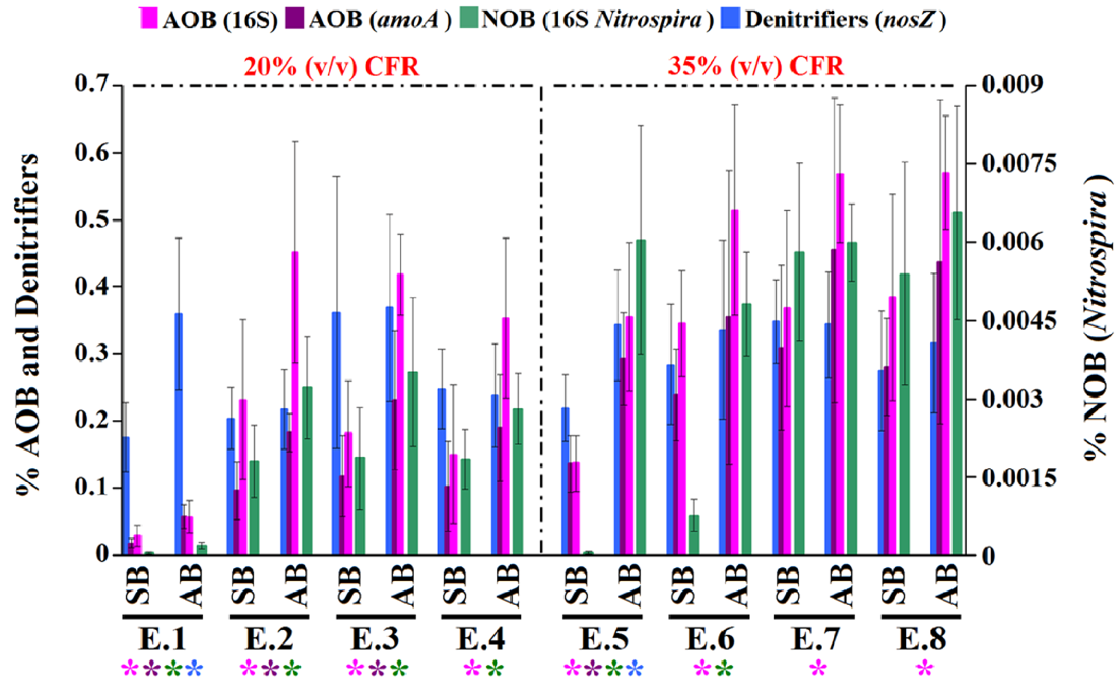


Figure V.3. Average values ± standard deviation of the relative abundance, of nitrifiers and denitrifiers measured in suspended biomass (SB) and attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR. Relative abundance is expressed as percentage of total bacterial community. *Significant differences between SB and AB in the same experiment, according to Student's test ($p > 0.05$). The color of asterisk indicates the group in which significant differences were found.

To evaluate the relative abundance of nitrifiers and denitrifiers gene markers, it was assumed that the average number of 16S rDNA copies per bacterial cell were 3.6 (Harms *et al*., 3003). For both AOB and *Nitrospira* one copy of the 16S rDNA per cell, while for AOB *amoA* gene two copies per cell were considered (Harms *et al*., 3003). In the case of denitrifiers, in concordance with Palmer *et al*. (2009) one copy of the *nosZ* gene was assumed. The relative abundance values obtained (less than 1%) must be considered with caution, since the number of 16S rRNA genes per operon varying dramatically between species (1-15 copies) (Smith *and* Osborn, 2009). Therefore, the relative abundance cannot be interpreted directly as representative of total bacterial concentration (Smith *and* Osborn, 2009; Kim *et al*., 2011a). Even so, it is worth noting that our results were in concordance with those reported by Kim *et al*. (2011a), who also found relative abundance values of AOB and NOB less than 1% in both SB and AB fractions.

Comparing the relative abundance of nitrifiers between SB and AB, ANOVA revealed small although significant differences ($p<0.05$) in all experiments (except for NOB in experiments 7 and 8), confirming a slightly higher proportion of AOB and NOB in the AB fraction. However, a similar relative abundance of denitrifiers was observed in SB and AB in most experiments, with the exception of the first experiments carried out with either 20% or 35% (v/v) CFR (experiments 1 and 5, respectively). In these experiments, a higher proportion of denitrifiers was associated to AB. These results were consistent with earlier studies which showed that the immobilisation of microorganisms on carriers favours the development of nitrifying bacteria (Kim *et al*., 2011a; Zielińska *et al*., 2012). Additionally, when our findings are compared with those previously reported, an interesting theory could be suggested. While Kim *et al*. (2011a) observed much higher proportion of AOB and NOB in AB than in SB in IFAS system,

van den Akker *et al*. (2010) did not record significant differences of the relative abundance of AOB between SB and AB, although they reported that the biomass attached to the carriers in the IFAS contained an overall slightly higher proportion of AOB than the SB fraction. Considering that in our study the significant differences observed between SB and AB were quite modest (lower than 0.1% in all experiments), our results were more similar to those reported by van den Akker *et al*. (2010), who used a 25% (v/v) of CFR. Taking into account that Kim *et al*. (2011a) used a 50% CFR, it could be suggested that a higher CFR could favour an increase in the proportion of nitrifies in AB. Additionally, slightly higher average relative abundances of AOB and NOB in AB were observed in experiments 5, 6, 7 and 8 (carried out with 35% (v/v) CFR) when each of them were compared with experiments 1, 2, 3 and 4 (20% CFR), respectively.

3.2. Absolute abundance of total Bacteria, AOB, NOB (*Nitrospira*) and denitrifiers in the MBMBR and biofilm contribution

The total number of copies of all targeted genes in the bioreactor (SB + AB fractions) and the specific contribution of AB were calculated for each experiment, taking into account the real volume of mixed liquor and biofilm-colonized carriers in the MBMBR (**Table V.4. A** and **B**, respectively). Overall, for both CFR (20 and 35%, v/v), the highest average numbers of copies of gene markers of total Bacteria, AOB, NOB and denitrifiers were recorded in experiments 3, 4, 7 and 8, carried out with higher MLTSS concentration (c.a. 4500 mg/L). These results suggest that a higher solid concentration favoured an increase in the abundance of the target populations in the MBMBR, irrespective of HRT (10 or 24h). Additionally, the abundance of nitrifiers was significantly higher in experiments 7 and 8 (20% CFR) compared with experiments

3 and 4 (35% CFR), indicating that a higher abundance of AOB and NOB was achieved when the MBMBR was operated with 35% CFR. By contrast, this trend was not observed for total Bacteria and denitrifiers, suggesting that changes in the CFR mainly affected the abundance of nitrifying populations developed in the MBMBR.

Table V.4. (A) Mean ± standard deviation of the number of copies (in logarithmic scale) of targeted genes measured in the MBMBR system (suspended biomass + attached biofilm) and (B) contribution of the biofilm (%) to the total number of copies of targeted genes, in the eight experiments conducted in a MBMBR system. LSD: least significant difference (Student' test, $p < 0.05$). Data followed by the same lower case-letter do not significantly differ among experiments.

A

Experiment	*Number of copies/L of MBMBR (SB+AB)*				
	Bacteria (16S)	**AOB (16S)**	**AOB (*amoA*)**	**NOB (16S*Nitrospira*)**	**Denitrifiers (*nosZ*)**
1	11.90±0.16[a]	7.80±0.12[a]	7.69±0.11[a]	5.10±0.16[a]	8.63±0.07[a]
2	11.98±0.05[abc]	8.82±0.12[c]	8.45±0.09[b]	6.71±0.09[c]	8.73±0.07[b]
3	12.14±0.10[d]	8.88±0.13[c]	8.66±0.15[c]	6.86±0.10[d]	9.10±0.11[e]
4	12.15±0.03[d]	8.81±0.20[c]	8.58±0.30[bc]	6.88±0.11[d]	8.97±0.09[cd]
5	11.94±0.07[ab]	8.62±0.14[b]	8.59±0.09[bc]	6.42±0.10[b]	8.75±0.03[b]
6	12.01±0.05[bc]	9.03±0.10[d]	8.87±0.06[d]	6.71±0.08[c]	8.91±0.09[c]
7	12.07±0.04[cd]	9.12±0.09[d]	9.02±0.17[de]	7.27±0.09[e]	9.05±0.08[de]
8	12.11±0.05[d]	9.16±0.08[d]	9.05±0.09[e]	7.28±0.08[e]	8.99±0.09[cd]
LSD	0.09	0,15	0.18	0.12	0.09

B

Experiment	*Biofilm contribution (%)*				
	Bacteria (16S)	**AOB (16S)**	**AOB (*amoA*)**	**NOB (16S*Nitrospira*)**	**Denitrifiers (*nosZ*)**
1	11.17±3.40 [a]	21.64±6.56 [a]	30.01±7.43 [a]	37.34±11.94 [b]	21.26±5.07 [bc]
2	15.23±2.53 [bc]	28.31±12.49 [ab]	27.46±10.23 [a]	25.30±10.10 [a]	15.91±2.45 [ab]
3	12.55±3.66 [ab]	26.87±10.11 [ab]	23.23±9.29 [a]	22.13±8.24 [a]	13.31±3.81 [a]
4	16.63±1.65 [cd]	36.19±16.68 [b]	31.64±12.40 [a]	24.44±8.45 [a]	16.44±5.04 [ab]
5	18.69±2.97 [de]	37.43±8.16 [b]	33.96±10.14 [a]	96.28±1.10 [c]	26.30±2.80 [cd]
6	26.85±2.49 [f]	35.35±6.10 [b]	33.98±17.33 [a]	70.44±8.64 [c]	30.53±11.12 [d]
7	24.41±2.53 [f]	35.05±11.08 [b]	31.91±12.50 [a]	25.99±6.60 [a]	24.17±3.93 [cd]
8	20.86±2.54 [e]	29.81±8.54 [ab]	29.53±16.70 [a]	25.56±9.24 [a]	23.83±6.96 [cd]
LSD	3.25	12.2	14.5	9.37	6.74

Regarding the biofilm contribution to the abundance of the target populations in the MBMBR, it is noticeable that the total abundance of each targeted gene was slightly

increased by the presence of AB under all the experimental conditions assayed in our study. Moreover, our results suggest that this beneficial effect was more evident for nitrifiers, since the biofilm contribution to the abundance of nitrifying bacteria was above 20% in all the experiments (**Table V.4B**). However, for total Bacteria and denitrifiers the biofilm contribution only showed percentages above 20% in experiments carrier out with 35% (v/v) CFR.

3.3. Statistical multivariate analysis: influence of operational parameters on the abundance of total Bacteria, AOB, NOB and denitrifiers

It is widely recognized that ammonium oxidation is the rate-limiting step for an optimal removal of nitrogen from WWTSs (Zhang *et al*., 2010; Bassin *et al*., 2012; Zielińska *et al*., 2012; Wang *et al*., 2014). Moreover, it is know that the stability of nitrification relies on several operating conditions, such as temperature, pH, dissolved oxygen, HRT and COD concentration of the influent (Bassin *et al*., 2012; Wang *et al*., 2012a). However, in agreement with Wang *et al*. (2012b), the relationships between operational conditions and the dynamics of nitrifying bacteria remain unresolved. The overall analysis of the quantification of N-cycle bacteria in the MBMBR described in section 3.2 indicated that higher biomass concentrations in the system and a higher CFR contributed to increase the abundance of all the genes targeted by qPCR. However, in order to better address the influence of these and other operational parameters on the biomass of SB and AB fractions, it seemed necessary to perform a statistical multivariate analysis for each sample type.

The influence of the simultaneous variation of physico-chemical parameters (BTS, MLVSS, COD, BOD_5, pH, temperature, HRT, NH_4^+ influent) and CFR (20% or

35%, v/v) on the number of copies of targeted molecules in the eight experiments was analysed by RDA. Additionally, to explore the correlation between NH_4^+ removal efficiency and the abundance of N-cycle bacteria in the MBMBR, this parameter was also introduced in the RDA. The biplot diagrams generated for SB and AB are shown in **Fig. V.4** (**A** and **B**, respectively). Dissolved oxygen was not included as variable, because its concentration was maintained stable in a range from 1.5 to 2 mg/L under all the experimental conditions assayed.

Regarding the SB fraction, the Monte Carlo permutation test revealed that MLVSS ($p = 0.002$), temperature ($p = 0.008$), COD ($p = 0.026$) and CFR ($p = 0.038$) were the significant factors explaining the variation in the number of copies of the gene markers of total Bacteria, AOB, NOB and denitrifiers. The angle formed between the arrows representing operational parameters in the plots and the canonical ordination axes (COAs) showed a strong correlation of MLVSS, COD and temperature with the first COA (horizontal), while the second COA (vertical) was mainly correlated with CFR. The first and second COA described 82.3% and 8.1% of the total variance of the number of copies of gene markers, respectively (89.1% of the variance of the biological data-environment relation by the first axis and 8.7% by the second one). As for the AB analysis and also according to the Monte Carlo permutation test, the number of copies of the targeted genes was significantly affected by BTS ($p = 0.002$), CFR ($p = 0.004$), temperature ($p = 0.016$) and COD ($p = 0.036$). The first COA was mainly correlated with BTS and COD, and described 91.4% of the variation of the total variance of the number of copies of gene markers (95.4% of the variance of the biological data-environment relation). The second axis was mainly correlated to CFR and temperature, and described 2.7% of the total variance of the number of copies (2.8% of the biological data-environment relation).

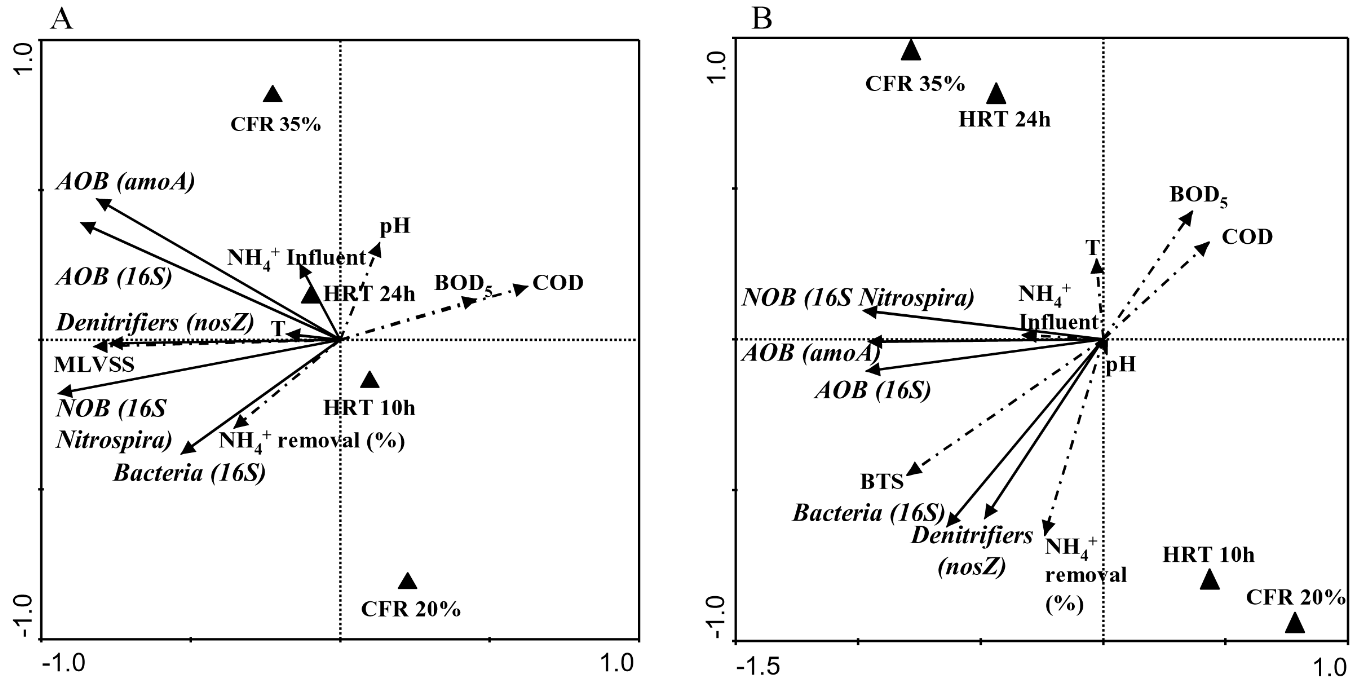


Figure V.4. Redundancy analyses (RDA) of suspended biomass (A) and attached biofilm (B). The ordination diagrams (biplots) show operational conditions (dotted arrows and ▲) and number of copies expressed per L (straight arrows), measured in the eight experiments performed in the MBMBR. Operational parameters: pH, Temperature (T), Mixed LiquorVolatile Suspended Solids (MLVSS), Biofilm Total Solids (BTS), Biological Oxygen Demand at 5 days in influent water (BOD5), Chemical Oxygen Demand in influent water (COD), Hydraulic Retention time (HRT: 10 and 24 h), CFR (v/v) (20% or 35%), NH_4^+ Influent and NH_4^+ removal (%). HRT and CFR are shown as nominal variables (▲).

Both RDAs (SB and AB) confirmed the positive correlation of the number of copies of all targeted genes with the concentration of solids in either the mixed liquor or the biofilm, and a negative correlation with COD in the influent. Additionally, in both analyses, a positive correlation was also found between the number of copies of gene markers of nitrifiers and an increase in the CFR (from 20% to 35%). MLVSS and BTS were the variables displaying the highest correlations to the first COA in the respective RDAs (**Fig. V.4**).

In WWTPs, MLVSS account for the particulate organic matter in the system, including substrates supporting growth as well as living microbial cells; thus, it is expected that an increase of MLVSS concentration favours an increase in the number of microorganisms and the biological activity of the sludge (Molina-Muñóz *et al.*, 2010; Gómez-Silván *et al.*, 2014). Indeed, Kim *et al.* (2011b), using qPCR, detected an increase of the number of copies of gene markers of total Bacteria, AOB and NOB in a full-scale CAS when the concentration of solids in the activated sludge was increased, suggesting that the selection of an adequate concentration by SRT control can be fundamental to achieve optimal efficiency of the nitrogen removal process. In concordance with these authors, RDA showed (**Fig. V.4**) that there were positive correlations between the concentration of solids and the removal efficiency of NH_4^+ in both fractions (SB and AB). On the other hand, the concentration of cells in biofilms is closely linked to the density and thickness of these aggregates, since these morphological characteristics play an important role in the diffusion of substrates (Nicolella *et al.*, 2000; Alves *et al.*, 2002; Celmer *et al.*, 2008). In addition, biofilms in WWTSs tend to change, in density, thickness and shape over the operating life cycle (Nicolella *et al.*, 2000). The study of such characteristics in the biofilms developed in the MBMBR was beyond the purpose of our study; however, the results showed an

increase of BTS over time with either 20 or 35% CFR, (except experiment 8. **Table III.2; Chapter 1**), which was positively correlated with the number of copies of the target genes as well as with the efficiency of NH_4^+ removal. These data as a whole illustrate that the substrate diffusion was not a limiting factor for the biofilm developed in our system.

Comparing the RDAs of data derived from SB and AB fractions, it was clear that the organic load of the influent (COD and DBO_5) and the concentration of solids inversely influenced the abundance of the targeted bacterial groups, since the arrow vectors pointed in opposite directions (**Fig. V.4 A** and 4 **B**). In fact, the higher COD values and the lowest concentrations of solids in the mixed liquor and biofilms were recorded simultaneously during experiments 1 and 2 (20% (v/v) CFR) and experiments 5 and 6 (35% (v/v) CFR) (**Table III.2**; **Chapter 1**). Therefore, lower numbers of copies of all the targeted molecules were observed in both SB and AB samples. Previous researchers have shown that fluctuations in organic matter content of the influent (COD or BOD_5) can have a large effect on the heterotrophic and nitrifying bacteria communities in WWTSs (Xia *et al*., 2008; Bassin *et al*., 2012; Whang *et al*., 2012; Wang *et al*., 2012b; Kim *et al*., 2013b). Specifically, an increase of COD concentration appears to be decisive for the competition among heterotrophic and autotrophic bacteria. As a result of this, the growth of nitrifying bacteria could be reduced (Bassin *et al*., 2012; Kim *et al*., 2013b; Hoang *et al*., 2014). In addition, the influence of COD concentration in the AB fraction was also reported by Bassin *et al*. (2012), who observed by FISH that a reduction of influent COD favoured and enrichment of nitrifiers in AB developed in a MBBR system. Therefore, the progressive development of nitrifying bacteria observed over time (which, as above mentioned, reached stability

at the second experiment with either 20 or 35% CFR) appears connected not only to the low-growth rate of nitrifiers, but also to COD concentration in the influent.

Regarding the influence of CFR on the number of copies of all targeted genes, the RDA analyses proved that by increasing the CFR (35% v/v) a higher number of copies of gene markers of nitrifiers were recorded in both SB and AB (with the only exception of NOB on SB), being the abundance of total Bacteria and denitrifiers less influenced by the increase of CFR. The type of carrier (material, shape and size) and the CFR are considered as two of the main operational variables in the WWTSs that combine SB and AB (Rodgers *and* Zhan, 2003). Several studies have shown the influence of these variables on the removal of C and N, biofilm morphology and composition, as well as on the diversity of total Bacteria total and nitrifying bacteria communities developed in the biofilm (Bernet *et al*., 2004; Wang *et al*., 2005; Levstek *and* Plazl, 2009; Chu *and* Wang, 2011; Martín-Pascual *et al*., 2012; Calderón *et al.,* 2012). In reference to the influence of the CFR, Calderón *et al*. (2012) confirmed by TGGE fingerprinting that this is one of the main factors explaining the variations of the total Bacteria community structure of the biofilms developed in a MBBR. In agreement with these authors, Bernet *et al*. (2004) found differences in diversity and composition of biofilm nitrifying bacteria as a function of the CFR (10% or 30%, v/v) in two inverse turbulent bed reactors. According to Wang *et al*. (2005), an increase of CFR promotes the increase of particle-particle and particle-wall collisions, which made the biofilm denser and thinner. It is expected that this type of biofilm, obtained under higher shear stress, is more stable and has a higher active biomass concentration and consequently a higher number of cells (Alves *et al*., 2002). Therefore, it could be suggested that the positive influence of an increase in the CFR on the abundance of nitrifiers might be correlated with changes in the morphological characteristics of the biofilm. In addition,

Wang *et al*. (2005) reported that, when the carrier concentration increases, the total surface area of carrier increases accordingly, and more positions are available for bacteria to attach, so this could be another reason to explain the higher abundance of nitrifiers with 35% CFR.

Concerning temperature, RDA of SB revealed a positive correlation between this parameter and the abundance of all targeted molecules. However, a minor influence of this parameter was recorded for AB, since temperature was mostly correlated to the second COA that only explains a 2.7% of the total variability. It is necessary to take into account that, although in the experiments 3 and 4 low temperatures were recorded (13.77ºC±1.91 and 10.62ºC±2.45), the abundance of all targeted genes did not decrease (**Fig. V.1**). This fact could be related to the concentration of solids, since in these experiments higher MLTSS and BTS were recorded (**Table III.2**; **Chapter 1**) and these variables were the most influential in our system.

Diverse molecular techniques, such as FISH, T-RFLP, DGGE and qPCR have allowed to know that nitrifying bacteria populations are influenced by temperature in different WWTSs (Park *et al*., 2008; Wells *et al*., 2009; Shore *et al*., 2012; Wang *et al*., 2012b; Zhang *et al*. 2014). In theory, the ideal range for nitrification is 25-30ºC (Bitton, 2010), although it has also been reported that temperatures above 25ºC lead to an increase of the specific growth rate of AOB (Xue *et al*., 2009). At the same time, denitrification takes place under a wide range of temperatures (5-50ºC), even though an increase in temperature have positive impact on both N_2O production and reduction (Holtan-Hartwig *et al*., 2002). Thus, the positive correlation between temperature and the abundance of nitrifiers and denitrifiers observed in SB appears to be in accordance to the before mentioned. With regard to the influence of temperature on AB, attached

growth processes have shown ability to achieve and maintain optimal nitrification under lower temperature, contrary to conventional suspended growth processes (Hoang *et al*., 2014). However, there are certain discrepancies among the few studies that approach the influence of temperature on N-cycle bacteria developed on AB. In this regard, Park *et al*. (2008) evaluated the influence of this variable (5, 10 and 25ºC) on the AOB community of the biofilm developed in an aerobic biofilm reactor (using DGGE and FISH). They observed a reduction of biomass concentration in the biofilm as well as changes in AOB community structure when the temperature was low, and consequently the nitrification was reduced. By contrast, Onnis-Hayden *et al*. (2011) reported efficient and complete nitrification at low temperatures (11-15ºC) in an IFAS system. Furthermore, these researches evaluated the abundance of nitrifiers (NOB and AOB) in SB and AB by FISH, concluding that nitrifiers can survive even at low temperatures in AB, being the nitrification process mainly associated to this fraction of the biomass. However, Hoang *et al*. (2014) reported that although a greater quantity of nitrifying bacteria and a greater quantify of biofilm attached to MBBR carriers was found at cold temperatures (1 ºC) compared to warm temperatures (20 ºC), the rates of nitrification decreased when the temperature was lower.

On the other hand, earlier studies based on DGGE and GeoChip, have demonstrated that ammonium influent concentration is an important structuring factor of AOB communities (Lydmark *et al*., 2007; Wang *et al*., 2014). Additionally, the ammonia content of the influent has been positive correlated with the AOB abundance by qPCR (Zhang *et al*., 2010). In our study, a positive correlation between the abundance of AOB and ammonium influent concentration was observed in both SB and AB samples (**Fig. V.4**). However, according to the Monte Carlo permutation test, this operational parameter did not show a significant influence on the abundance of targeted

molecules ($p<0.05$). One possible reason for this fact is that the ammonium influent concentration did not experience large variations among experiments (**Table V.1**).

To sum, the results presented in this study demonstrate that the abundance and dynamics of total Bacteria, AOB, NOB and denitrifiers were fairly similar in both SB and AB fractions. In fact, multivariate statistical analyses confirmed that SB and AB were influenced by the same variables (concentration of solids, COD, T and CFR) under the experimental conditions tested. Consequently, the total abundance (SB+AB) of all targeted bacterial groups as well as the biofilm contribution to the MBMBR were increased in the experiments carried out with higher MLTSS concentration (c.a. 4500 mg/L) and CFR (35%, v/v). The great similarity observed between both sample types suggested that SB and AB could play equally important roles in the overall nitrification process in our MBMBR.

References

Ahmed, Z., 2012. Microbial Communities in nutrient-removing membrane bioreactors, A review. Journal of Environmental Science and Technology, 5, 16-28.

Ahn, Y. H., 2006. Sustainable nitrogen elimination biotechnologies: a review. Process Biochemistry, 41, 1709-1721.

Alves, C.F., Melo, L.F., Vieira, M.J., 2002. Influence of medium composition on the characteristics of a denitrifying biofilm formed by *Alcaligenes denitrificans* in a fluidised bed reactor. Process Biochemistry, 37, 837-845.

APHA, AWWA, WEF, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, DC.

Bassin, J.P., Kleerebezem, R., Rosado, A.S., van Loosdrecht, M-C.M., Dezotti, M., 2012. Effect of Different Operational Conditions on Biofilm Development, Nitrification, and Nitrifying Microbial Population in Moving-Bed Biofilm Reactors. Environmental Science and Technology, 46, 1546-1555.

Bernet, N., Sánchez, O., Dabert, P., Olaïzola, A., Godon, J.J., Delgenès, J.P., 2004. Effect of solid hold-up on nitrite accumulation in a biofilm reactor-molecular characterization of nitrifying communities. Water Science and Technology, 49, 123-130.

Bin, Z., Zhe, C., Zhigang, Q., Min, J., Zhiqiang, C., Zhaoli, C., Junwen, L., Xuan, W., Jingfeng, W., 2011. Dynamic and distribution of ammonia-oxidizing bacteria communities during sludge granulation in an anaerobic–aerobic sequencing batch reactor. Water Research, 45, 6207-6216.

Bitton, G., 2010. Wastewater microbiology. Wiley-Lis, John Wiley and Sons, New Jersey, USA.

Calderón, K., Martín-Pascual, J., Poyatos, J.M., Rodelas, B., 2012. Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. Bioresource Technology, 121, 119–126.

Celmer, D., Oleszkiewicz, J.A., Cicek, N., 2008. Impact of shear force on the biofilm structure and performance of a membrane biofilm reactor for tertiary hydrogen-driven denitrification of municipal wastewater. Water Research, 42, 3057-3065.

Chu, L., Wang, J., 2011. Comparison of polyurethane foam and biodegradable polymer as carriers in moving bed biofilm reactor for treating wastewater with a low C/N ratio. Chemosphere, 83, 63-68.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.H., Wagner, M., 2001. In Situ Characterization ofNitrospira-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. Applied and Environmental Microbiology, 67, 5273-5284.

de los Reyes, F.L., 2010. Challenges in determining causation in structure-function studies using molecular biological techniques. Water Research, 4, 4948-4957.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of Nitrosomonas oligotropha-like ammonia-oxidizing bacteria and Nitrospira spp. from full-scale wastewater treatment plants by competitive PCR. Applied and Environmental Microbiology, 68, 245-253.

Elenter, D., Milferstedt, K., Zhang, W., Hausner, M., Morgenroth, E., 2007. Influence of detachment on substrate removal and microbial ecology in a heterotrophic/autotrophic biofilm. Water Research, 41, 4657-4671.

Francis, C.A., Beman, J.M., Kuypers, M.M., 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. The ISME journal, 1, 19-27.

Fu, B., Liao, X., Ding, L., Ren, H., 2010. Characterization of microbial community in an aerobic moving bed biofilm reactor applied for simultaneous nitrification and denitrification. World Journal of Microbiology and Biotechnology, 26, 1981-1990.

Gapes, D.J., Keller, J., 2009. Impact of oxygen mass transfer on nitrification reactions in suspended carrier reactor biofilms. Process Biochemistry, 44, 43-53.

Geets, J., De Cooman, M., Wittebolle, L., Heylen, K., Vanparys, B., De Vos, P., Verstraete, W., Boon, N., 2007. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. Applied Microbiology and Biotechnology, 75, 211-221.

Gómez-Silván, C., Arévalo, J., González-López, J., Rodelas, B., 2014. Exploring the links between population dynamics of total and active bacteria and the variables influencing a full-scale membrane bioreactor (MBR). Bioresource Technology, 162, 103-114.

Guelli de Souza, S., 2012. Application of biofilm in the degradation of contaminants in industrial effluents—a review. Signpost Open Journal of Biochemistry and Biotechnology, 1, 1-10.

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G., Sayler, G.S., 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environmental Science and Technology, 37, 343-351.

Hibiya, K., Terada, A., Tsuneda, S., Hirata, A., 2003. Simultaneous nitrification and denitrification by controlling vertical and horizontal microenvironment in a membrane-aerated biofilm reactor. Journal of Biotechnology, 100, 23-32.

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., Stintzi, A., 2014. Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1 °C. Water Research, 49, 215-224.

Holtan-Hartwig, L., Dörsch, P., Bakken, L.R., 2002. Low temperature control of soil denitrifying communities: kinetics of N2O production and reduction. Soil Biology and Biochemistry, 34, 1797-1806.

Ivanovic, I. and Leiknes, T.O., 2012. The biofilm membrane bioreactor (BF-MBR)—a review. Desalination and Water Treatment, 37, 288-295.

Jackson, G.A., Livingston, R.S., Riley, L.K., Livingston, B.A. and Franklin, G.L., 2013. Development of a PCR assay for the detection of *Spironucleus muris.* Journal of the American Association for Laboratory Animal Science, 52, 165-170.

Jamal Khan, S., Ilyas, S., Javid, S., Visvanathan, C., Jegatheesan, V., 2011. Performance of suspended and attached growth MBR systems in treating high strength synthetic wastewater. Bioresource Technology, 102, 5331-5336.

Kim, H.S., Schuler, A.J., Gunsch, C.K., Pei, R., Gellner, J., Boltz, J. P., Freudenberg, R.G., Dodson, R., 2011a. Comparison of conventional and integrated fixed-film activated sludge systems: attached-and suspended-growth functions and quantitative polymerase chain reaction measurements. Water Environment Research, 83, 627-635.

Kim, J., Lim, J., Lee, C., 2013a. Quantitative real-time PCR approaches for microbial community studies in wastewater treatment systems: Applications and considerations. Biotechnology Advances, 31, 1358-1373.

Kim, Y.M., Park, H., Cho, K.H., Park, J.M., 2013b. Long term assessment of factors affecting nitrifying bacteria communities and N-removal in a full-scale biological process treating high strength hazardous wastewater. Bioresource Technology, 134, 180-189.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M. and Woldendorp, J.W., 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Applied Environmental Microbiology, 63, 1489-1497.

Kumar, M., Lin, J.G. 2010., Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal—strategies and issues. Journal of Hazardous Materials, 178, 1-9.

Le-Clech, P., 2010. Membrane bioreactors and their uses in wastewater treatments. Applied Microbiology and Biotechnology, 88, 1253-60.

Leiknes,T.O., Ødegaard, H., 2007. The development of a biofilm membrane bioreactor. Desalination, 202, 135-143.

Lepš, J., Šmilauer, P., 1999. Multivariate analysis of ecological data. Faculty of Biological Sciences, University of South Bohemia, Ceské Budejovice.

Levstek, M., Platz, I., 2009. Influence of carrier type on nitrification in the moving bed biofilm process. Water Science and Technology, 59, 875-882.

Lydmark, P., Almstrand, R., Samuelsson, K., Mattsson, A., Sörensson, F., Lindgren, P. E., Hermansson, M., 2007. Effects of environmental conditions on the nitrifying population dynamics in a pilot wastewater treatment plant. Environmental microbiology, 9, 2220-2233.

Limpiyakorn, T., Shinohara, Y., Kurisu, F., Yagi, O., 2005. Communities of ammonia - oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. FEMS Microbiology Ecology, 54, 205-217.

Limpiyakorn, T., Sonthiphand, P., Rongsayamanont, C., Polprasert, C., 2011. Abundance of *amoA* genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. Bioresource Technology, 102, 3694-3701.

Lücker, S., Nowka, B., Rattei, T., Spieck, E., Daims, H., 2013. The genome of Nitrospina gracilis illuminates the metabolism and evolution of the major marine nitrite oxidizer. Frontiers in Microbiology, 4. 1-19.

Lücker, S., Wagner, M., Maixnera, F., Pelletierb, E., Kocha, H., Vacherie, B., Rattei, T., Damsté, J.S.S., Spieck, E., Paslierb, D.L., Daims, H., 2010. A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. Proceedings of the National Academy of Sciences, 107, 13479-13484.

Mahmood, S., Freitag, T.E., Prosser, J.I., 2006. Comparison of PCR primer‐based strategies for characterization of ammonia oxidizer communities in environmental samples. FEMS Microbiology Ecology, 56, 482-493.

Martín-Pascual, J., López-López, C., Cerdá, A., González-López, J., Hontoria, E., Poyatos, J.M., 2012. Comparative kinetic study of carrier type in a moving bed system applied to organic matter removal in urban wastewater treatment. Water Air Soil Pollut, 222, 1699-1712.

Molina-Muñoz, M., Poyatos, J. M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E., Gonzalez-Lopez, J., 2010. Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions. Bioresource Technology, 101, 696-704.

Morgenroth, E., Kommedal, R., Harremoës, P., 2002. Processes and modelling of hydrolysis of particulate organic matter in aerobic wastewater treatment—a review. Water Science and Technology, 45, 25-40.

Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. Journal of Biotechnology, 80, 1-33.

Nogueira, R., Melo, L. F., Purkhold, U., Wuertz, S., Wagner, M., 2002. Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. Water Research, 36, 469-481.

Onnis-Hayden, A., Majed, N., Schramm, A., Gu, A.Z., 2011. Process optimization by decoupled control of key microbial populations, Distribution of activity and abundance of polyphosphate-accumulating organisms and nitrifying populations in a full-scale IFAS-EBPR plant. Water Research, 45, 3845-3854.

Ovreås, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied and Environmental Microbiology, 63, 3367-3373.

Pal, L., Kraigher, B., Brajer-Humar, B., Levstek, M., Mandic-Mulec, I., 2012. Total bacterial and ammonia-oxidizer community structure in moving bed biofilm reactors treating municipal wastewater and inorganic synthetic wastewater. Bioresource Technology, 110, 135-143.

Palmer, K., Drake, H.L., Horn, M.A., 2009. Genome-derived criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units of nitrate reducers. Applied and Environmental Microbiology, 75, 5170-5174.

Paredes, D., Kuschk, P., Mbwette, T. S. A., Stange, F., Müller, R. A., Köser, H., 2007. New aspects of microbial nitrogen transformations in the context of wastewater treatment–a review. Engineering in Life Sciences, *7*, 13-25.

Park, J.J., Byun, I.G., Park, S.R., Park, T.J., 2008. Nitrifying bacterial communities and its activities in aerobic biofilm reactors under different temperature conditions. Korean Journal of Chemical Engineering, 25, 1448-1455.

Plattes, M., Henry, E., Schosseler, P.M., Weidenhaupt, A., 2006. Modelling and dynamic simulation of a moving bed bioreactor for the treatment of municipal wastewater. Biochemical Engineering Journal, 32, 61-68.

Poyatos, J.M., Molina-Mu˜noz, M., González-López, J., Hontoria, E., 2010. Particle sizedistribution in permeate from a pilot-scale membrane bioreactor system under actual operating conditions. Particulate Science and Technology: An International Journal, 28, 309-318.

Purkhold, U., Pommerening-Röser, A., Juretschko, S.,Schmid, M.C., Koops, H.P., Wagner, M., 2000. Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amoA* Sequence Analysis: Implications for Molecular Diversity Surveys. Applied Environmental Microbiology, 66, 5368-5382.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Röser, A., Koops, H.P., 2003. 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates, extension of the dataset and proposal of a new lineage within the nitrosomonads. International Journal of Systematic and Evolutionary Microbiology, 53, 1485-1494.

Rodgers, M., Zhan, X.M., 2003. Moving-medium biofilm reactors. Reviews in Environmental Science and Biotechnology, 2, 213-224.

Rotthauwe, J. H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology, 63, 4704-4712.

Scala, D.J., Kerkhof, L.J., 1998. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiology Letters, 162, 61-68.

Seifi, M., Fazaelipoor, M.H., 2012. Modeling simultaneous nitrification and denitrification (SND) in a fluidized bed biofilm reactor. Applied Mathematical Modelling, 36, 5603-5613.

Shore, J.L., M'Coy, W.S., Gunsch, C.K., Deshusses, M.A., 2012. Application of a moving bed biofilm reactor for tertiary ammonia treatment in high temperature industrial wastewater. Bioresource technology, 112, 51-60.

Short, M.D., Abell, G.C.J., Bodrossy, L., van den Akker, B., 2013. Application of a Novel Functional Gene Microarray to Probe the Functional Ecology of Ammonia Oxidation in Nitrifying Activated Sludge. PLoS ONE, 8(10), e77139.

Siripong, S., Rittmann, B.E., 2007. Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Research, 41, 1110-1120.

Smith, C.J., Osborn, A.M., 2009. Advantages and limitations of quantitative PCR (Q-PCR) ‐ based approaches in microbial ecology. FEMS Microbiology Ecology, 67, 6-20.

Staroscik A., 2004. URI Genomics and Sequencing Center: calculator for determining the number of copies of a template, Rhode Island, USA. Available at: http://cels.uri.edu/gsc/cndna.html

van den Akker, B., Beard, H., Kaeding, U., Giglio, S., Short, M.D., 2010. Exploring the relationship between viscous bulking and ammonia-oxidiser abundance in activated sludge: A comparison of conventional and IFAS systems. Water Research, 44, 2919-2929.

Vannecke, T.P.W., Bernet, N., Steyer, J-P-. Volcke, E.L.P., 2014. Modelling ammonium-oxidizing population shifts in a biofilm reactor. Water Science and Technology, 69.1, 208-216.

Vlaeminck, S.E., Hay, A.G., Maignien, L., Verstraete, W., 2011. In quest of the nitrogen oxidizing prokaryotes of the early Earth. Environmental Microbiology, 13, 283-295.

Wagner, M., Nielsen, P.H., Loy, A., Nielsen, J. L., Daims, H., 2006. Linking microbial community structure with function: fluorescence *in situ* hybridization-microautoradiography and isotope arrays. Current Opinion in Biotechnology, 17, 83-91.

Wang, R.C., Wen, X.H., Qian, Y., 2005. Influence of carrier concentration on the per-formance and microbial characteristics of a suspended carrier biofilm reactor. Process Biochemistry, 40, 2992-3001.

Wang, X., Wen, X., Criddle, C., Wells, G., Zhang, J., Zhao, Y., 2010. Community analysis of ammonia-oxidizing bacteria in activated sludge of eight wastewater treatment systems. Journal of Environmental Sciences, 22, 627-634.

Wang, F., Liu, Y., Wang, J., Zhang, Y., Yang, H., 2012a. Influence of growth manner on nitrifying bacterial communities and nitrification kinetics in three lab-scale bioreactors. Journal of Industrial Microbiology and Biotechnology, 39, 595-604.

Wang, X., Wen, X., Xia, Y., Hu, M., Zhao, F., Ding, K., 2012b. Ammonia oxidizing bacteria community dynamics in a pilot-scale wastewater treatment plant. PloS one, 7(4), e36272.

Wang, J., Zhang, C., Rong, H., 2014. Analysis and succession of nitrifying bacteria community structure in sequencing biofilm batch reactor. Applied Microbiology Biotechnology, 98, 4581-4587.

Wells, G.F., Park, H.D., Yeung, C.H., Eggleston, B., Francis, C.A., Criddle, C.S., 2009. Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: betaproteobacterial dynamics and low relative abundance of Crenarchaea. Environmental Microbiology, 11, 2310-2328.

Wertz, S., Poly, F., Le Roux, X., Degrange, V., 2008**.** Development and application of a PCR-denaturing gradient gel electrophoresis tool to study the diversity of *Nitrobacter*-like *nxrA* sequences in soil. FEMS Microbiology Ecology, 63, 261-271.

Whang, L.M., Wu, Y.J., Lee, Y.C., Chen, H.W., Fukushima, T., Chang, M.Y., Cheng, S.S., Hsu, S.F., Chang, C.H., Shen, W., Huang, C.K., Fu, R., Chang, B., 2012. Nitrification performance and microbial ecology of nitrifying bacteria in a full-scale membrane bioreactor treating TFT-LCD wastewater. Bioresource Technology, 122, 70-77.

Xia, S., Li, J., Wang, R., 2008. Nitrogen removal performance and microbial community structure dynamics response to carbon nitrogen ratio in a compact suspended carrier biofilm reactor. Ecological Engineering, 32, 256-262.

Xia, S., Jia, R., Feng, F., Xie, K., Li, H., Jing, D., Xu, X., 2012. Effect of solids retention time on antibiotics removal performance and microbial communities in an A/O-MBR process. Bioresource Technology, 106, 36-43.

Xue, Y., Yang, F., Liu, S., Fu, Z., 2009. The influence of controlling factors on the start-up and operation for partial nitrification in membrane bioreactor. Bioresource Technology, 100, 1055-1060.

Yang, S., Yang, F., Fu, Z., Lei, R., 2009. Comparison between a moving bed membrane bioreactor and a conventional membrane bioreactor on organic carbon and nitrogen removal. Bioresource Technology, 100, 2369-2374.

Zeng, W., Bai, X., Zhang, L., Wang, A., Peng, Y., 2014. Population dynamics of nitrifying bacteria for nitritation achieved in Johannesburg (JHB) process treating municipal wastewater. Bioresource Technology, 162, 30-37.

Zhang, B., Sun, B., Ji, M., and Liu, H., 2009. Population dynamic succession and quantification of ammonia-oxidizing bacteria in a membrane bioreactor treating municipal wastewater. Journal of Hazardous Materials, 165, 796-803.

Zhang, B., Sun, B., Ji, M., Liu, H., Liu, X., 2010. Quantification and comparison of ammonia-oxidizing bacterial communities in MBRs treating various types of wastewater. Bioresource Technology, 101, 3054-3059.

Zhang, S., Wanga, Y., He, W., Wua, M., Xing, M., Yang, J., Gao, N., Pan, M., 2014. Impacts of temperature and nitrifying community on nitrification kinetics in a moving-bed biofilm reactor treating polluted raw water. Chemical Engineering Journal, 236, 242-250.

Zielińska, M., Cydzik-Kwiatkowska, A., Wojnowska-Baryła, I., 2012. Changes in microbial communities of nitrifying immobilized biomass: the role of operacional conditions. Polish Journal of Environmental Studies, 21, 1107-1112.

VI. OVERALL DISCUSSION

Nowadays, there is certainly no doubt that improving of biological wastewater treatments is a key goal to get better quality of the effluents, which not only contribute to protect the environment, but also to enable their reuse. The biological wastewater treatments feed on the metabolic activity of microorganisms for the transformation of toxic substances, the degradation of organic pollutants and the removal of nutrients from urban and industrial effluents (Wells *et al*., 2011; Valentin - Vargas *et al*., 2012). In particular, the biodegradation process begins with the hydrolysis of macromolecules performed by extracellular microbial enzymes. This initial process is considered as the main rate-limiting step in organic matter degradation, since an important fraction of organic matter, present in the influent, must be hydrolysed by enzymes before it can be utilised by bacterial metabolism (Anupama *et al*., 2008; Burgess *and* Pletschke, 2008; Cadoret *et al*., 2002). For these reasons, the investigation of extracellular hydrolases as well as the study of structure and dynamics of microbial communities is crucial to understand the environmental or operational factors affecting efficiency and stability of the biological process (Wagner *and* Loy, 2002; Anupama *et al*., 2008; Cydzik-Kwiatkowska *et al*., 2012; Biswas *et al*., 2014; Gómez-Silván *et al*., 2013; Ibarbalz *et al*., 2013; Short *et al*., 2013; Vanwonterghem *et al*., 2014).

Traditionally, the biological wastewater treatments systems (WWTSs) have been based on suspended or attached growth processes. These two processes are selective for microorganisms, so that suspended growth processes favour the development of bacteria with shorter generation times, while in attached growth processes it is expected an enrichment of bacteria with long generation times (Wang *et al*., 2012). Currently, there are a variety of biological WWTSs that combine both suspended biomass (SB) and attached biofilm (AB) processes with one single objective: *to improve removal efficiency of organic matter and nutrients*. Specifically, moving bed

membrane bioreactor (MBMBR) based on the incorporation of biofilm process to the membrane bioreactor technology, has shown to be effective to improve organic substance and nutrients removal (Ivanovic *and* Leiknes, 2012). Despite a clear connection between microbial communities developed into the bioreactor and the effectiveness of the depuration process, little information is available related to bacterial populations and their associated enzymes grown on both SB and AB fractions (Kwon *et al*., 2010; van den Akker *et al*., 2010; Kim *et al*., 2011; Biswas *et al*., 2014).

On the basis of this previous knowledge, this study has focussed on the microbiology characterization of the SB and AB developed in MBMBR system as well as on the establishment of an adequate link between operational parameters and biological data. For this purpose, enzymatic activities (α-glucosidase, acid phosphatase and alkaline phosphatase), total Bacteria community and N-cycle bacteria were evaluated under different CFRs (20% or 35%, v/v), HRTs (10h or 24h) and MLTSS (c.a. 2,500 mg/L or 4,500 mg/L). Others operational parameters such as, BVS, BTS, BOD_5 influent, COD influent, temperature and pH varied also among experiments.

Comparing both samples type (SB and AB), the study of ***enzymatic activities*** revealed that with some exceptions (in which no significant differences between SB and AB were observed, $p<0.05$), the mean alkaline phosphatase, acid phosphatase and α-glucosidase were higher in SB samples. It is known that large parts of extracellular enzymes are retained in extracellular polymeric substances (EPS) in both attached biofilms and mixed liquor flocs (Burgess *and* Pletschke, 2008; Flemming *and* Wingender, 2010). However, it is must be considered that high molecular weight compounds have a limited diffusion through EPS (Cadoret*et al*., 2002; Dimock *and* Morgenroth, 2006). In addition, Morgenroth *et al*. (2002) suggested that the mixed

liquor flocs have a more open structure and a larger relative surface area compared to attached biofilms. In view of this, higher rates of hydrolytic activities in SB might relate to a better diffusion of substrate in the flocs, as well as to the contribution of suspended single cells to the hydrolytic activity. Multivariate statistical analyses confirmed that the levels of extracellular enzymatic activities were not influenced by the same variables in the SB and AB samples, under the operational conditions tested. Hydrolytic activities in the SB fraction were more sensitive to changes in MLVSS, COD, T and HRT, while they were mainly affected by BTS, CFR and COD in the AB fraction. Temporal evolution of the enzymatic activities, the biofilm contribution and the multivariate statistical analyses showed that the hydrolytic processes in the MBMBR system were enhanced in SB and AB when the experiments were performed with higher MLTSS concentration (ca. 4500 mg/L), HRT (24 h) and CFR (35%).

The study of ***total Bacteria and AOB communities*** by TGGE and 454-pyrosequencing, demonstrated that the structure of total Bacteria and AOB communities in both SB and AB samples was very similar. The clear similarity of AOB community structure between SB and AB in the MBMBR was in agreement with a previous work done in a Fixed-Film Activated Sludge System (IFAS) reported by Short *et al*. (2013). These authors (using Functional Gene Microarray) compared the AOB diversity in SB and AB during four months and over this time period they are not observed differences between SB and AB communities. Conversely, in the case of total Bacteria community structure our results showed inconsistency with those previously reported by (Kwon *et al*., 2010; Biswas *and* Turner 2012; Biswas *et al*., 2014). Biswas *and* Turner (2012) (using 16S rRNA gene clone libraries) observed differences between bacterial community of SB and AB in MBBR system. These authors reported that probably due to the low HRT used in the system, bacterial community in SB was

dominated by aerobic bacteria, whereas in AB anaerobic bacteria were dominants. Later, Biswas *et al*. (2014) also observed these differences by pyrosequencing. However, in both studies, the differences were observed between SB and "*mature biofilm*". This last concept was defined by Biswas *et al*. (2014) as "*biomass on carriers residing in a functional MBBR system for several years*". Nevertheless, similar community structure in SB and in "*early biofilm*", which were dominated by fast-growing aerobic bacteria, was also reported by Biswas *et al*. (2014). At the same time, Kwon *et al*. (2010) observed by 454-pyrosequencing clear differences between SB and AB developed in IFAS system. This inconsistency of our results with the reports above mentioned could be explained considering the follow hypothesis: **1.** Biswas *and* Turner (2012) and Biswas *et al*. (2014) in MBBR system, only found differences between SB and "*mature biofilm*", so the time seems to be an important factor to find differences between communities' structure of SB and AB. In our case, the BTS and BVS concentrations obtained over time for 20% CFR (from experiment 1 to experiment 4, ten months) and for 35% CFR (from experiment 5 to experiment 8, eight months) showed the evolution of biofilm (Chapter 2; Table D.2), but no differences between SB and AB were observed over time. Therefore, it could be suggested that these time periods were not large enough to observe differences in total Bacteria community structure between SB and AB; **2.** Kwon *et al*. (2010) reported differences between both samples type in IFAS system in which the biofilm was developed in a fixed synthetic mesh (no freely moving carriers), so a minor interaction between both SB and AB fractions and consequently higher differences between SB and AB could be expected in this system compared with our MBMBR; **3.** It is necessary bearing in mind that the three systems (MBBR, IFAS and MBMBR) differ from each other on some aspects of the operational design, being important to consider that in our MBMBR a continuous sludge recirculation takes place.

Total Bacteria community was characterized by a high degree of diversity and optimal functional organization. These characteristics described a bacterial community able to maintain its functional stability under all the experimental conditions tested in the MBMBR system (Marzorati *et al*., 2008). By contrast, AOB community was characterized as more specialized and probably more fragile to environmental/operational conditions changes. These results were in concordance with those previously reported by (Boon *et al*., 2002; Wan *et al*., 2011). Concerning to the taxonomic composition of total Bacteria community, *Proteobacteria* and *Actinobacteria* were the predominant phyla in both sample types (SB and AB) and in all the experiments, followed by *Bacterioidetes*, *Chloroflexi* and *Firmicutes*. However, it was curious that the predominant phylum in SB samples was *Proteobacteria* in all the experiments (except experiment 5) while the predominant phylum in AB was *Actinobacteria* in most of the experiments. Kwon *et al*. (2010) also observed higher abundance of *Actinobacteria* in the AB compared to the SB present in IFAS system. According to these authors, the incorporation of support material in the bioreactor could provide a good environment to the preferential growth of members belonging to *Actinobacteria*. Within the phylum *Proteobacteria*, α- and β- *proteobacteria* were the most predominant classes. *Acidimicrobiia* and *Actinobacteria* were the predominant classes related to phylum *Actinobacteria*. In general, the prevalence of these phyla and classes in our MBMBR system was consistent with the results previously reported in others WWTSs (Kwon *et al*., 2010; Ye *et al*., 2011; Zhang *et al*., 2012; Ye *and* Zhang, 2013; Hu *et al*., 2012). As for taxonomic composition of AOB community, all the TGGE-bands sequenced belong to ammonium oxidizing β-*proteobacteria* were related to genus *Nitrosomonas*, thus no sequences related to genus *Nitrosospira* were found. The prevalence of bands related to genus *Nitrosomonas* over *Nitrosospira* has been commonly observed in both biofilm-based and suspended biomass-based technologies

of wastewater treatment (Purkhol *et al*., 2000; Gómez-Villalba *et al*., 2006; Lydmark *et al*., 2007; Xia *et al*., 2010; Cerrone *et al*., 2013). In accordance with the phylogenetic clusters of AOB belonging to the β-subclass of the *Proteobacteria* reported by Purkhold *et al*. (2003), the bands sequences were affiliated to *Nitrosomonas oligotropha/ureae*, *N. cryotolerans* and *N. europaea*. The presence of these phylogenetic clusters in wastewater habitats has been reported by Purkhold *et al*. (2000) and (2003). The order *Nitrosomonadales* and the family *Nitrosomonadaceae* were detected by 454-pyrosequencing, but the members related to this family were unclassified at genus level.

On the other hand, the results obtained by TGGE and 454-pyrosequencing indicated that the dynamic and the structure of total Bacteria and AOB communities in both SB and AB fractions were mainly affected by changes in MLVSS, BTS, temperature, CFR and BOD_5 concentration in the influent.

Regarding the study of ***N-cycle bacteria*** by qPCR, the results obtained revealed that the abundance and dynamics of targeted genes of total Bacteria, AOB, NOB and denitrifiers was fairly similar in both SB and AB fractions. In concordance with van den Akker *et al*. (2010), the similarity observed between both biomass fractions suggest that SB and AB could play equally important roles in the overall nitrification process in our MBMBR. Additionally, multivariate statistical analyses confirmed that SB and AB were influenced by the same variables (MLVSS, BTS, COD, temperature and CFR) under the experimental conditions tested. Total abundance (SB+AB) of N-cycle bacterial groups as well as the biofilm contribution to the MBMBR was higher in the experiments carried out with higher MLTSS concentration (c.a. 4500 mg/L) and CFR (35%, v/v).

Overall, extracellular enzymatic activities and bacterial populations involved in the depuration process of urban wastewater in our MBMBR were mainly influenced by solid concentration, organic matter influent concentration, temperature and CFR. Emphasizing that, the solid concentration was the most relevant parameter explaining the variation in hydrolytic enzyme activity levels and the dynamics of bacterial populations in the MBMBR system. It is widely recognized that among the different operational/environmental factors, solid concentration, HRT, temperature, organic matter concentration (COD and BOD_5) and CFR have an important influence on the variability of the bacterial community and thus on the efficiency of the depuration process (Anupama *et al*., 2008; Molina-Muñoz *et al*., 2009; Siggins *et al*., 2011; Wells *et al*., 2011; Calderón *et al*., 2012; Pal *et al*., 2012; Valentin-Vargas *et al*., 2012; Wang *et al*., 2012; Gómez-Silván *et al*., 2014; Onnis-Hayden *et al*., 2011; Hoang *et al*., 2014; Bassin *et al*., 2012; Kim *et al*., 2011). Specifically, temperature has been described as one of the most important factor influencing the bacterial community structure in different WWTSs based on conventional suspended growth (Molina-Muñoz *et al*., 2009; Siggins *et al*., 2011; Cydzik-Kwiatkowska *et al*., 2012; Calderón *et al*., 2013). By contrast, a minor influence of the temperature has been reported in WWTSs that combine SB and AB (Hoang *et al*., 2014; Onnis-Hayden *et al*. 2011; Guelli de Souza, 2012). In concordance with these last mentioned authors, in our MBMBR system, temperature showed a slight influence on enzymatic activities as well as on structure and dynamic of total Bacteria community and N-cycle bacteria.

Finally, future trends in this research line should be focus on the study of activity, functions and interactions of microbial communities developed in SB and AB. The combination of DNA-based, mRNA-based, and protein-based analyses of microbial communities will be a good option to elucidate the compositions, functions,

and interactions of microbial communities, as well as to link these to operational/environmental parameters (Simon *and* Daniel, 2011). Specifically, to carry out mRNA-based and protein-based analyses, Metatranscriptomics and metaproteomics (the most recent sub-disciplines within the metagenomics field) have been shown to provide higher levels of resolution for functional analysis of microbial communities (Chistoserdova, 2009; Schneider *and* Riedel, 2010; Ju *et al.*, 2014).

References

Anupama, V.N., Amrutha, P.N., Chitra, G.S., Krishnakumar, B., 2008. Phosphatase activity in anaerobic bioreactors for wastewater treatment. Water Research, 42, 2796-2802.

Bassin, J.P., Kleerebezem, R., Rosado, A.S., van Loosdrecht, M-C.M., Dezotti, M., 2012. Effect of Different Operational Conditions on Biofilm Development, Nitrification, and Nitrifying Microbial Population in Moving-Bed Biofilm Reactors. Environmental Science and Technology, 46, 1546-1555.

Biswas, K., Taylor, M.W., Turner, S.J., 2014. Successional development of biofilms in moving bed biofilm reactor (MBBR) systems treating municipal wastewater. Applied Microbiology and Biotechnology, 98, 1429-1440.

Biswas, K., Turner, S.J., 2012. Microbial community composition and dynamics ofmoving bed biofilm reactor systems treating municipal sewage. Applied Environmental Microbiology, 78, 855-864.

Boon, N., De Windt, W., Verstraete, W., Top, E.M., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiology Ecology, 39, 101-112.

Burgess, J.E., Pletschke, B.I., 2008. Hydrolytic enzymes in sewage sludge treatment: a mini-review. Water SA, 34, 343-349.

Cadoret, A., Conrad, A., Block, J.C., 2002. Availability of low and high molecular weight substrate to extracellular enzymes in whole and dispersed activated sludges. Enzyme and Microbial Technology, 31, 179-186.

Calderón, K., Martín-Pascual, J., Poyatos, J.M., Rodelas, B., 2012. Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. Bioresource Technology, 121, 119-126.

Calderón, K., Reboleiro-Rivas, P., Rodríguez, F.A., Poyatos, J.M., González-López, J., Rodelas, B., 2013. Comparative analysis of the enzyme activities and the bacterial community structure based on the aeration source supplied to an MBR to treat urban wastewater. Journal of Environmental Management, 128, 471-479.

Cerrone, F., Poyatos, J.M., Molina-Muñoz, M., Cortés-Lorenzo, C., González-López, J., Rodelas, B., 2013. Prevalence of Nitrosomonas cluster 7 populations in the ammonia-oxidizing community of a submerged membrane bioreactor treating urban wastewater under different operation conditions. Bioprocess and Biosystems Engineering, 36, 901-910.

Chistoserdova, L., 2009. Functional Metagenomics: Recent Advances and Future Challenges. Biotechnology and Genetic Engineering Reviews, 26, 335-352.

Cydzik-Kwiatkowska, A., Zielińska, M., Wojnowska-Baryla, I., 2012. Impact of Operational Parameters on Bacterial Community in a Full-Scale Municipal Wastewater Treatment Plant. Polish Journal of Microbiology, 61, 41-49.

Dimock, R., Morgenroth, E., 2006. The influence of particle size on microbial hydrolysis of protein particles in activated sludge. Water Research, 40, 2064-2074.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nature Reviews Microbiology, 8, 623-633.

Gómez-Silván, C., Arévalo, J., González-López, J., Rodelas, B., 2014. Exploring the links between population dynamics of total and active bacteria and the variables influencing a full-scale membrane bioreactor (MBR). Bioresource Technology, 162, 103-114.

Gómez-Silván, C., Arévalo, J., Pérez, J., González-López, J., Rodelas, B., 2013. Linking hydrolytic activities to variables influencing a submerged membrane bioreactor (MBR) treating urban wastewater under real operating conditions. Water Research, 47, 66-78.

Gómez-Villalba, B., Calvo, C., Vílchez. R., González-López, J., Rodelas, B., 2006. TGGE analysis of the diversity of ammonia oxidizing and denitrifying bacteria in submerged filter biofilms for the treatment of urban wastewater. Applied Microbiology and Biotechnology, 72, 393-400.

Guelli de Souza, S., 2012. Application of biofilm in the degradation of contaminants in industrial effluents—a review. Signpost Open Journal of Biochemistry and Biotechnology, 1, 1-10.

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., Stintzi, A., 2014. Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1 °C. Water Research, 49, 215-224.

Hu, M., Wang, X., Wen, X., Xia, Y., 2012. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresource Technology, 117, 72-79.

Ibarbalz, F.M., Figuerola, E.L.M., Erijman, L., 2013. Industrial activated sludge exhibit unique bacterial community composition at high taxonomic ranks. Water Research, 47, 3854-3864.

Ivanovic, I. and Leiknes, T.O., 2012. The biofilm membrane bioreactor (BF-MBR)—a review. Desalination and Water Treatment, 37, 288-295.

Ju, F., Guo, F., Ye, L., Xia, Y., Zhang, T., 2014. Metagenomic analysis on seasonal microbiol variations of activated sludge from a full-scale wastewater treatment plant over 4 years. Environmental Microbiology Reports, 6, 80-89.

Kim, H.S., Schuler, A.J., Gunsch, C.K., Pei, R., Gellner, J., Boltz, J. P., Freudenberg, R.G., Dodson, R., 2011. Comparison of conventional and integrated fixed-film activated sludge systems: attached-and suspended-growth functions and quantitative polymerase chain reaction measurements. Water Environment Research, 83, 627-635.

Kim, Y.M., Park, H., Cho, K.H., Park, J.M., 2013. Long term assessment of factors affecting nitrifying bacteria communities and N-removal in a full-scale biological process treating high strength hazardous wastewater. Bioresource Technology, 134, 180-189.

Kwon, S., Taek-Seung, K., Gi Hyeon, Y., Joon-Hong, J., Hee-Deung, P., 2010. Bacterial Community Composition and Diversity of a Full-Scale Integrated Fixed-Film Activated Sludge System as Investigated by Pyrosequencing. Journal of Microbiology and Biotechnology, 20, 1717-1723.

Lydmark, P., Almstrand, R., Samuelsson, K., Mattsson, A., Sörensson, F., Lindgren, P. E., Hermansson, M., 2007. Effects of environmental conditions on the nitrifying population dynamics in a pilot wastewater treatment plant. Environmental microbiology, 9, 2220-2233.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints, practical tools for microbial ecology. Environmental Microbiology, 10, 1571-1581.

Molina-Muñoz, M., Poyatos, J.M., Sánchez-Peinado, M., Hontoria, E., González-López, J., Rodelas, B., 2009. Microbial community structure and dynamics in a pilot-scale submerged membrane bioreactor aerobically treating domestic wastewater under real operation conditions. Science of the Total Environment, 407, 3994-4003.

Morgenroth, E., Kommedal, R., Harremoës, P., 2002. Processes and modelling of hydrolysis of particulate organic matter in aerobic wastewater treatment—a review. Water Science and Technology, 45, 25-40.

Onnis-Hayden, A., Majed, N., Schramm, A., Gu, A.Z., 2011. Process optimization by decoupled control of key microbial populations, Distribution of activity and abundance of polyphosphate-accumulating organisms and nitrifying populations in a full-scale IFAS-EBPR plant. Water Research, 45, 3845-3854.

Pal, L., Kraigher, B., Brajer-Humar, B., Levstek, M., Mandic-Mulec, I., 2012. Total bacterial and ammonia-oxidizer community structure in moving bed biofilm reactorstreating municipal wastewater and inorganic synthetic wastewater. Bioresource Technology, 110, 135-143.

Purkhold, U., Pommerening-Röser, A., Juretschko, S.,Schmid, M.C., Koops, H.P., Wagner, M., 2000. Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amoA* Sequence Analysis: Implications for Molecular Diversity Surveys. Applied Environmental Microbiology, 66, 5368-5382.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Röser, A., Koops, H.P., 2003. 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates, extension of the dataset and proposal of a new lineage within the nitrosomonads. International Journal of Systematic and Evolutionary Microbiology, 53, 1485-1494.

Schneider, T., Riedel, K., 2010. Environmental proteomics: analysis of structure and function of microbial communities. Proteomics, 10, 785-798.

Shore, J.L., M'Coy, W.S., Gunsch, C.K., Deshusses, M.A., 2012. Application of a moving bed biofilm reactor for tertiary ammonia treatment in high temperature industrial wastewater. Bioresource technology, 112, 51-60.

Short, M.D., Abell, G.C.J., Bodrossy, L., van den Akker, B., 2013. Application of a Novel Functional Gene Microarray to Probe the Functional Ecology of Ammonia Oxidation in Nitrifying Activated Sludge. PLoS ONE, 8(10), e77139.

Siggins, A., Enright, A.M., O'Flaherty, V., 2011. Temperature dependent (37-15 °C) anaerobic digestion of a trichloroethylene-contaminated wastewater. Bioresource Technology, 102, 7645-7656.

Simon, C., Daniel, R., 2011. Metagenomic analyses: past and future trends. Applied and Environmental Microbiology, 77, 1153-1161.

Valentín-Vargas, A., Toro-Labrador, G., Massol-Deyá, A.A., 2012. Bacterial Community Dynamics in Full-Scale Activated Sludge Bioreactors, Operational and Ecological Factors Driving Community Assembly and Performance. PLoS ONE, 7(8), 12.

Valentín-Vargas, A., Toro-Labrador, G., Massol-Deyá, A.A., 2012. Bacterial Community Dynamics in Full-Scale Activated Sludge Bioreactors, Operational and Ecological Factors Driving Community Assembly and Performance. PLoS ONE, 7(8), 12.

van den Akker, B., Beard, H., Kaeding, U., Giglio, S., Short, M.D., 2010. Exploring the relationship between viscous bulking and ammonia-oxidiser abundance in activated sludge: A comparison of conventional and IFAS systems. Water Research, 44, 2919-2929.

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W., 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Current Opinion in Biotechnology, 27, 55-64.

Wagner, M. and Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology, 13, 218-227.

Wan, C.Y., De Wever, H., Diels, L., Thoeye, C., Liang, J.B., Huang, L.N., 2011. Biodiversity and population dynamics of microorganisms in a full-scale membrane bioreactor for municipal wastewater treatment. Water Research, 45, 1129-1138.

Wang, F., Liu, Y., Wang, J., Zhang, Y., Yang, H., 2012. Influence of growth manner on nitrifying bacterial communities and nitrification kinetics in three lab-scale bioreactors. Journal of Industrial Microbiology and Biotechnology, 39, 595-604.

Wang, X., Wen, X., Xia, Y., Hu, M., Zhao, F., Ding, K., 2012. Ammonia Oxidizing Bacteria Community Dynamics in a Pilot-Scale Wastewater Treatment Plant. PLoS ONE, 7(4), e36272.

Wells, G.F., Park, H.D., Eggleston, B., Francis, C.A., Criddle, C.S., 2011. Fine-scale bacterial community dynamics and the taxa-time relationship within a full-scale activated sludge bioreactor. Water Research, 45, 5476-5488.

Xia, S., Duan, L., Song, Y., Li, J., Piceno, Y.M., Andersen, G.L., Alvarez-Cohen, L., Moreno-Andrade, I., Huang, C.L., Hermanowicz, S.W., 2010. Bacterial Community Structure in Geographically Distributed Biological Wastewater Treatment Reactors. Environmental Science and Technology, 44, 7391-7396.

Ye, L., Shao, M.F., Zhang, T., Tong, A.H.Y., Lok, S., 2011. Analysis of the bacterial community in a laboratory-scale nitrification reactor and a wastewater treatment plant by 454-pyrosequencing. Water Research, 45, 4390-4398.

Ye, L., Zhang, T., 2013. Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. Applied Microbiology and Biotechnology, 97, 2681-2690.

Zhang, T., Shao, M. F., Ye, L., 2012. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME Journal, 6, 1137-1147.

VII. CONCLUSIONES / CONCLUSIONS

De acuerdo con los resultados obtenidos, así como con la revisión bibliográfica llevada a cabo, se presentan las siguientes conclusiones respecto al sistema MBMBR objeto del estudio:

1. Las actividades enzimáticas hidrolíticas fosfatasa ácida, fosfatasa alcalina y α-glucosidasa son mayores en la biomasa suspendida (BS) que en la biomasa adherida (biopelícula, BA), independientemente de las condiciones operacionales.

2. Los niveles de actividad enzimática extracelular de la BS y la BA no son influenciados por las mismas variables de operación. Las actividades enzimáticas hidrolíticas de la BS son influenciadas por la concentración de sólidos volátiles del licor mezcla (SVLM), la demanda química de oxígeno (DQO), la temperatura (T) y el tiempo de retención hidráulico (TRH), mientras que las actividades enzimáticas de la biopelícula son afectadas principalmente por la concentración de sólidos totales de la biopelícula (STB), el porcentaje de relleno (CFR) y la DQO.

3. Las condiciones de operación que determinan los niveles máximos de actividad enzimática hidrolitica son una concentración de STLM de 4.500 mg / L, un TRH de 24 h y un CFR de 35% (v/v).

4. La estructura y dinámica de la comunidad bacteriana total y la comunidad de bacterias oxidadoras de amonio (AOB) son muy similares tanto en la BS como en la BA. La comunidad bacteriana se caracterizó por un alto grado de diversidad, una óptima organización funcional y una dinámica estable, características de una comunidad capaz de mantener su estabilidad funcional bajo todas las condiciones experimentales ensayadas. La comunidad de bacterias oxidadoras de amonio

(AOB) se caracterizó por ser más especializada y probablemente más frágil frente a cambios en las condiciones ambientales y/o operacionales.

5. La comunidad bacteriana total está predominantemente compuesta por *Proteobacteria* y *Actinobacteria*, tanto en la BS como en la BA.

6. Las poblaciones de bacterias oxidadoras de amonio (clase β-*proteobacteria*) detectadas en el sistema se relacionan evolutivamente con bacterias del orden *Nitrosomonadales*, familia *Nitrosomonadaceae* y género *Nitrosomonas*.

7. Los factores operacionales que afectan de una forma más importante a la estructura y la dinámica de la comunidad bacteriana total, así como a la comunidad de bacterias oxidadoras de amonio (AOB), son la concentración de SVLM y STB, la T, el CFR y la demanda biológica de oxígeno (DBO_5).

8. La concentración de SVLM, STB, la DQO, la T y el CFR son las variables de operación que afectan de forma más significativa a la abundancia de AOB, NOB y desnitrificantes en la BS y BA.

9. Una concentración de STLM de 4.500 mg/L y un CFR de 35% (v/v) determinan los máximos niveles de abundancia de microorganismos nitrificantes y desnitrificantes en el sistema MBMBR.

Based on the results obtained, as well as the literature review carried out, the following conclusions are presented regarding the MBMBR system analyzed throughout the study:

1. Hydrolytic activities acid phosphatase, alkaline phosphatase and α-glucosidase in suspended biomass (SB) are higher than in attached biofilm (AB), regardless of the operational conditions.

2. The levels of extracellular enzymatic activities were not influenced by the same variables in the SB and AB samples. Hydrolytic activities in the SB fraction were more sensitive to changes in mixed liquor volatile solids concentration (MLVSS), chemical oxygen demand (COD), temperature (T) and hydraulic retention times (HRT), while they were mainly affected by biofilm total solids (BTS), carrier filling ratio (CFR) and COD in the AB fraction.

3. Operating conditions which determine the maximum levels of hydrolytic enzyme activity in MBMBR system are MLTSS concentration of 4,500 mg / L, HRT of 24 hours and CFR of 35% (v / v).

4. The structure and dynamics of total Bacteria and ammonia-oxidizing bacteria (AOB) communities are greatly similar in both SB and AB samples. Total Bacteria community is characterized by a high degree of diversity, optimal functional organization and strong stability. These characteristics described a bacterial community able to maintain its functional stability under all the experimental conditions tested. The AOB community is characterized as more specialized and probably more fragile to environmental/operational changes.

5. The total Bacteria community in thea MBMBR reactor is predominantly composed of *Proteobacteria* and *Actinobacteria* in both the SB and AB fractions.

6. Ammonia-oxidizing bacteria (class β-*proteobacteria*) populations in the MBMBR are evolutionarily related to order *Nitrosomonadales*, family *Nitrosomonadaceae* and genus *Nitrosomonas.*

7. The structure and dynamic of total Bacteria and AOB communities are mainly affected by changes in MLVSS, BTS, T, CFR and biological oxygen demand at 5 days (BOD_5).

8. The abundance of AOB, nitrite-oxidizing bacteria (NOB) and denitrifying bacteria in suspended biomass (SB) and attached biofilm (AB) are primarily affected by changes in MLVSS, BTS, COD, T and CFR.

9. Operating conditions which determine the maximum levels of abundance of AOB, nitrite-oxidizing bacteria (NOB) and denitrifying bacteria in MBMBR system are MLTSS concentration of 4,500 mg / L and CFR of 35% (v / v).

RELACIÓN DE TABLAS Y FIGURAS
TABLES AND FIGURES

I. INTRODUCTION

Figura I.1. Caricatura de la travesía en barco por el río Támesis del científico Michael Faraday y su carta publicada en el diario *The Times* en 1855, describiendo el mal estado del mismo. 37
Figura I.2. Evolución del mercado global de la tecnología MBR y su estimación para el año 2015. 42
Figura I.3. Nube de palabras que representa las principales "*Keywords*" utilizadas en los estudios realizados sobre la tecnología MBR. 42
Figura I.4. Representación de las dos configuraciones de la tecnología MBR. **A)** Biorreactores de membrana externa o de recirculación. **B)** Biorreactores de membrana integrada o sumergida. 43
Figura I.5. Proceso de colmatación reversible e irreversible y limpieza de membranas. 48
Figura I.6. Representación de la configuración aerobia y anaerobia de un sistema MBBR. 52
Figura I.7. Representación de la configuración de un sistema MBMBR. 55
Figura I.8. Soporte K1. 57
Figura I.9. Etapas de desarrollo de la biopelícula. 63
Figura I.10. Implicaciones de las enzimas extracelulares en la degradación biológica. 72
Figura I.11. Esquema global del ciclo del nitrógeno. 74
Figura I.12. Estructura y componentes del ribosoma procariota. 82
Figura I.13. Desarrollo y evolución histórica de las tecnologías NGS. 89
Figura I.14. **(A)** Esquema metodológico de la pirosecuenciación 454. **(B)** Reacción de pirosecuenciación tras la incorporación de nucleótidos (dNTPs). 91
Figura I.15. Creación de la librería de fragmentos de ADN para la secuenciación por la plataforma ilumina. 92

III. CHAPTER 1

Table III.1. Experimental design and operational conditions of the eight experiments conducted in the MBMBR system. 120
Table III.2. (A) Operational parameters: hydraulic retention time (HRT), mixed liquor total suspended solid and mixed liquor volatile suspended solid (MLTSS and MLVSS, respectively), biofilm total solid and biofilm volatile solid (BTS and BVS, respectively), temperature (T) and pH of the eight experiments conducted in a MBMBR system with a 20% (v/v) or 35% (v/v) CFR. **(B)** Organic matter: biological oxygen demand at 5 days (BOD5) and chemical oxygen demand (COD), in the influent and effluent, and BOD5 and COD removal rates (%) in the

eight experiments conducted in the MBMBR system with a 20% (v/v) or 35% (v/v) FRC. 126
Table III.3. Mean ± standard deviation of the enzymatic activities (mM/min L) alkaline phosphatase, acid phosphatase and α-glucosidase measured in suspended biomass (SB) and attached biofilm (AB) in the MBMBR. 132
Table III.4. Mean ± standard deviation (mM/min L) of the enzyme activities of alkaline phosphatase, acid phosphatase and α-glucosidase measured in the MBMBR system (suspended biomass + attached biofilm) and contribution of the biofilm (%) to the total hydrolytic activities, in the eight experiments conducted in a MBMBR system. 133

Figure III.1. Diagram of the Moving Bed Membrane Biofilm Reactor (MBMBR) used in this study. 118
Figure III.2. Alkaline phosphatase, acid phosphatase and α-glucosidase measured in suspended biomass (SB: mM/min L of mixed liquor) and attached biofilm (AB: mM/min L at 100% volumetric CFR) in experiments 1–4 conducted in the MBMBR system with a 20% (v/v) CFR. 129
Figure III.3. Alkaline phosphatase, acid phosphatase and α-glucosidase measured in suspended biomass (SB: mM/min L of mixed liquor) and attached biofilm (AB: mM/min L at 100% volumetric CFR) in experiments 5–8 conducted in the MBMBR with a 35% (v/v) CFR. 130
Figure III.4. Redundancy analyses (RDA) of suspended biomass (A) and attached biofilm (B). The ordination diagrams (biplots) show operational conditions and enzymatic activities, measured in the eight experiments performed in the MBMBR. 135

IV. CHAPTER 2

Table IV.1. Experimental design and operational conditions of the eight experiments conducted in the MBMBR system. 150
Table IV.2. (A) Operational parameters: Hydraulic Retention Time (HRT), Mixed Liquor Total Suspended Solid and Mixed Liquor Volatile Suspended Solid (MLTSS and MLVSS, respectively), Biofilm Total Solid and Biofilm Volatile Solid (BTS and BVS, respectively), Temperature (T) and pH of the eight experiments conducted in a MBMBR system with a 20% (v/v) or 35% (v/v) CFR. **(B)** Organic matter: Biological Oxygen Demand at 5 days (BOD_5) and Chemical Oxygen Demand (COD), in the influent and effluent, and BOD_5 and COD removal rates (%) in the eight experiments. **(C)** Ammonia (NH_4^+) concentration in the influent. 153
Table IV.3. Number of unique OTUs, Chao 1 and Shannon- Wienner indices (cut-

off level of 3%) measured in suspended biomass (SB) and attached biofilm (AB) samples taken from the MBMBR in the last week of each experiment. 189

Figure IV.1. Dendrograms generated by UPGMA clustering (Dice correlation coefficient) of 16S rRNA based TGGE patterns from suspended biomass (SB) and attached biofilm (AB) samples taken during the eight experiments performed in the MBMBR system, using Gel Compar II v. 4.601 (Applied Maths, Belgium). **A**. Profiles of *total Bacteria* community based on the amplification and separation of the V3 hypervariable region of the 16S rRNA gene. **B**. Profiles of *AOB* community based on the specific amplification of the 16S rRNA gene belonging to the ammonium oxidizing β-Proteobacteria (AOB) and subsequent amplification of the V3 hypervariable region of the 16S rRNA gene. 163

Figure IV.2. Box-and-Whisker plots of the ecological indices: Range weighted richness index (*Rr*), Functional organization (*Fo*), Shannon–Wiener (*H'*) and Dynamics index (*Dy*) calculated for suspended biomass (SB) and attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR with a 20% or 35% (v/v) CFR. **A**. Box-and-Whisker plots of the ecological indices for *total Bacteria* community and **B**. for *ammonium oxidizing β-Proteobacteria* (*AOB*) commmunity. 169

Figure IV.3. Redundancy analyses (RDA) of the ecological indices of *total Bacteria* community (**A**) and ammonium oxidizing β-Proteobacteria (*AOB*) community (**B**). The ordination diagrams (biplots) show operational conditions (dotted arrows and triangle) and ecological indices (straight arrows), measured in the eight experiments performed in the MBMBR system. 173

Figure IV.4. Heat maps representing the relative abundance of each TGGE band-class identified for *total Bacteria* community (**A**) and for *AOB* community (**B**) from suspended biomass (SB) and attached biofilm (AB) samples taken during the eight experiments (E.1-E.8) performed in the MBMBR system. 180

Figure IV.5. Neighbor-joining phylogenetic tree showing the positions of 28 total *Bacteria* sequences from re-amplified TGGE bands and the most similar sequences retrieved from the NCBI database, based on *ca.* 100 nt length of sequences. 182

Figure IV.6. Neighbor-joining phylogenetic tree showing the positions of 15 *ammonium oxidizing β-proteobacteria* (AOB) sequences from re-amplified TGGE bands and the most similar sequences retrieved from the NCBI database, based on *ca.* 100 nt length of sequences. 184

Figure IV.7. Rarefaction curves of OTUs, Chao 1 and Shannon-Wiener indices clustered at 97% sequence identity. 191

Figure IV.8. Relative abundances of each major (**A**) and minor phylum (**B**) in suspended biomass (SB) and attached biofilm (AB) samples taken from the MBMBR in the last week of each experiment. 194

Figure IV.9. Relative abundances of each major class (**A**) and of each major order (**B**) in suspended biomass (SB) and attached biofilm (AB) samples taken from the MBMBR in the last week of each experiment. 198

Figure IV.10. Heat maps representing the relative abundance of each major family (**A**) and of each major genus (**B**) in suspended biomass (SB) and attached biofilm (AB) samples taken in the last sampling week of each experiment (1, 2, 3, 4, 5, 6, 7 and 8) performed in the MBMBR system. 203

Figure IV.11. Redundancy analyses (RDA) of the relative abundance at Phylum, Class, Order, Family, Genus and OTUs level. The ordination diagrams (biplots) show operational conditions that have a significant influence on bacterial community and most prevalent phylogenetic lineages, measured in the eight experiments performed in the MBMBR system. 207

V. CHAPTER 3

Table V.1. Average values ± standard deviation of the concentration of ammonia (NH_4^+) in the influent and effluent, and NH_4^+ removal rates (%) in the eight experiments carried out in the MBMBR. 236

Table V.2. Primer used for quantification of total bacteria, nitrifiers and denitrifiers by qPCR. 239

Table V.3. qPCR conditions for quantification of total bacteria, nitrifiers and denitrifiers. 240

Table V.4. **(A)** Mean ± standard deviation of the number of copies (in logarithmic scale) of targeted genes measured in the MBMBR system (suspended biomass + attached biofilm) and **(B)** contribution of the biofilm (%) to the total number of copies of targeted genes, in the eight experiments conducted in a MBMBR system. 252

Figure V.1. Box-and-Whisker plots of the number of copies (in logarithmic scale) of targeted genes per liter of mixed liquor for suspended biomass (SB) or liter of carriers for attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR with a 20% or 35% (v/v) CFR. 244

Figure V.2. Box-and-Whisker plots of the number of copies (in logarithmic scale) of targeted genes per gram of MLVSS for suspended biomass (SB) or gram of BVS for attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR with a 20% or 35% (v/v) CFR. 245

Figure V.3. Average values ± standard deviation of the relative abundance, of nitrifiers and denitrifiers measured in suspended biomass (SB) and attached biofilm (AB) in the eight experiments (E.1-E.8) carried out in the MBMBR. Relative abundance is expressed as percentage of total bacterial community. 249

Figure V.4. Redundancy analyses (RDA) of suspended biomass (A) and attached biofilm (B). The ordination diagrams (biplots) show operational conditions and number of copies expressed per L, measured in the eight experiments performed in the MBMBR. 255