UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Ingeniería Química

Combined UASB-MBR system for the treatment of low-strength wastewater at ambient temperatures

Memoria presentada por

Dagmara Buntner

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Juan Manuel Garrido, Profesor Contratado Doctor de Ingeniería Química de la Universidad de Santiago de Compostela, y Jules van Lier, Professor at Delft University of Technology (The Netherlands), Faculty of Civil Engineering and Geosciences, Department of Water Management, Section Sanitary Engineering,

Informan:

Que la memoria titulada "Combined UASB-MBR system for the treatment of low-strength wastewater at ambient temperatures", que para optar al grado de Doctor en Ingeniería Química, Programa de Doctorado en Ingeniería Química y Ambiental, presenta Doña Dagmara Buntner, ha sido realizada bajo nuestra inmediata dirección en el Departamento de Ingeniería Química de la Universidad de Santiago de Compostela y Faculty of Civil Engineering and Geosciences, Department of Water Management, Section Sanitary Engineering, Technical University of Delft (The Netherlands).

Y para que así conste, firman el presente informe en Santiago de Compostela a 15 de marzo de 2013.

Juan Manuel Garrido

Jules van Lier

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Summary

Over the last 30–40 years, increasingly stringent environmental legislation relating to freshwater conservation and pollution reduction has driven technological development in the water sector. On the other hand, the rising consideration of wastewater treatment as non-negligible greenhouse gas (GHG) sources, have become a highly important issue. This, along with various governmental, institutional and organisational incentive, has encouraged problem holders to consider the application of more sophisticated technologies.

Anaerobic wastewater treatment process is well over 100 years old. It is a natural process in which a variety of different species from two entirely different biological kingdoms, the Bacteria and the Archaea, work together to convert organic wastes through a variety of intermediates into methane gas, an excellent source of energy. Apart from the significant reduction of organic matter content, pathogenic microorganisms are also eliminated. Additionally, the amount of excess sludge produced and nutrient requirements are far less than with aerobic treatment. However, although methane is a good renewable energy source, it is also a powerful greenhouse gas, and special attention should be paid especially if wastewater is treated at ambient temperatures.

A growing confidence in MBR technology has been demonstrated by the exponential increase in the number of installations worldwide, whilst cost remains the most significant barrier to the more widespread application. However, over the past 15 years, both capital (and particularly membrane) and operational costs of the MBR process have decreased dramatically, giving place for new opportunities, such as retrofitting of membranes into existing wastewater treatment plants (WWTP) for increasing capacity or water quality without detriment to footprint.

One of the possible combinations is the application of membrane technology as a post-treatment of anaerobic effluents. In this sense, UASB combined with MBR for the treatment of low strength wastewaters at ambient temperatures, as proposed in this Thesis, fits well into the state-of-the-art and market demand.

Taking the existent knowledge as starting point (**Chapter 1**), in this Thesis the start-up and the performance of combined UASB-MBR system employing two different types of wastewater were studied. Main focus was put on the organic matter elimination and its conversion to biogas, as well as nitrogen removal potential at ambient temperatures. Additionally, membrane performance and factors affecting it were evaluated. The wastewaters studied were semi-synthetic wastewater with a similar COD content that municipal wastewater (**Chapter 3**) or similar to dairy wastewater (**Chapter 4**), both prepared in the laboratory of the University of Santiago de Compostela (Spain).

Since most of the biological wastewater treatment processes are driven by either bacterial or archaeal microorganisms, their identification and quantification is crucial. Molecular techniques such as FISH (Fluorescent in situ hibrydization) and DGGE (denaturing gradient gel electrophoresis) allowed to describe and follow population dynamics during the operation of the combined UASB-MBR system (**Chapter 6**, **Chapter 7**), and helped to resolve some curiosities observed (**Chapter 7**).

Finally, it is a well known fact that one of the bottlenecks of membrane technology is fouling and filterability properties of sludge present in the bulk liquid. Since in this Thesis anaerobic effluent was treated in MBR stage, main contributors and their relation with sludge filterability and fouling were evaluated in short-term batch and long-term lab-scale reactor assays (**Chapter 5**).

The main content of each chapter and principal objectives that were achieved are gathered in the following sections.

In **Chapter 1**, starting with a brief historical background, the fundamentals of wastewater treatment are presented. The most crucial processes such as organic matter removal, nitrification, denitrification and anaerobic digestion will be explained, to give an overview on the complexity and interactions that might occur during wastewater treatment processes. In this sense, since organic matter and nutrient conversion are driven mostly by bacteria and archaea, different types of biomass forms will be described, focusing on attached, suspended and hybrid growth.

One of the crucial elements of combined UASB-MBR proposed in this Thesis is the Upflow Anaerobic Sludge Blanket (UASB) stage. Therefore, in **Chapter 1** the origin and worldwide applications of this technology will be resumed. Additionally, the

applicability of anaerobic digestion in wastewater treatment, especially concerning low-strength wastewater, will be evaluated, basing on the literature review.

On the other hand, since MBR stage was implemented in the combined UASB-MBR system studied in this Thesis, **Chapter 1** will also focus on MBR technology fundamentals, drawbacks and advantages over conventional activated sludge (CAS) systems. Different types of MBR reactors will be described, together with the most important factors influencing their operation, with critical flux and fouling mechanisms being of most importance. Moreover, anaerobic MBR (AnMBR) effectiveness in wastewater treatment in comparison with aerobic MBR will be discussed, will the emphasis on those systems working at ambient temperatures, which is also the case for the combined UASB-MBR proposed in this Thesis.

Finally, MBR technology as a UASB effluent post-treatment option will be evaluated. In this sense, this Thesis is a step forward into the development of combined UASB-MBR technology, since it is an attempt to resolve problems related to the main drawbacks of such a treatment, related with the need of post-treatment of anaerobic effluents, the operation of anaerobic MBR (fouling, low membrane fluxes) and aerobic MBR (high energy consumption and sludge production).

In **Chapter 2**, the analytical methods used in this Thesis will be described. The methodology was divided into liquid phase, solid phase, gaseous phase, biomass characterization and membrane performance. In order to characterize liquid phase, the conventional parameters for wastewater treatment, such as COD, ammonia, nitrate, nitrite, phosphates, total nitrogen were measured. Additionally, pH, temperature, dissolved oxygen, and alkalinity were measured, to check if the system was working at optimal conditions.

For solid phase characterization, Total and Volatile Suspended Solids (TSS and VSS) were determined following Standard Methods (APHA-AWWA-WPCF, 1999). Biomass was characterized by means of parameters such as sludge volumetric index, granules particle size distribution and techniques of digital image analysis, electronic microscopy and stereomicroscope. On the other hand, identification of different populations present in the biomass samples (granular sludge taken from the UASB stage; suspended and biofilm biomass taken from the MBR stage) was carried out by Fluorescent In Situ Hybridization (FISH). To obtain the distribution

of bacteria and archaea in the combined UASB-MBR system, a wide variety of specific FISH probes was applied and visual comparison of the results was performed. Confocal laser microscopy was used to acquire images of the bacteria with questionable results obtained by conventional microscopy.

Finally, the methodology applied for membrane filtration control and monitoring was described, such as critical flux measurement or chosen foulants concentrations determination procedures.

In **Chapter 3** a new combined UASB-MBR system will be introduced. The system is composed of a first methanogenic UASB stage, and a second MBR stage with two interconnected chambers: aerobic, with biofilm growing on small carrier elements and with biomass growing in suspension, and filtration chamber, with hollow fiber membrane module. The goal of the first methanogenic chamber was to diminish the COD of the raw wastewater, producing biogas rich in methane, and decrease the sludge production. In the second MBR stage, the remaining soluble biodegradable COD was oxidized by the heterotrophic bacteria. In the filtration chamber of the MBR stage, the membrane module could be operated at higher fluxes than those reported for AnMBR systems, and similar to those obtained in aerobic MBRs. In this sense, the concept of combined UASB-MBR system proposed in this Thesis was to join the advantages of the methanogenic and aerobic membrane bioreactor processes, by reducing energy requirements for aeration, producing biogas with high methane percentage and a permeate with very low COD content.

To simulate municipal wastewater flow, a synthetic wastewater was fed to the combined UASB-MBR system. COD in the influent was between 200 and 1200 $mg \cdot L^{-1}$, ammonium concentration around 35 $mg \cdot L^{-1}$ and phosphorous concentration was 8 $mg \cdot L^{-1}$, respectively. OLR in-between 1 and 3 $kgCOD \cdot m^{-3} \cdot d^{-1}$ and a HRT of 13–21 h were applied. Temperature was between 17.5 and 23.2 °C. During the whole operating period the COD removal efficiency was in the range of 90 and 96%, of which in between 40 and 80% was removed in the first methanogenic chamber. The average COD concentration measured in the permeate was around 5 $mg \cdot L^{-1}$. Biogas production with methane content between 75 and 80% was observed. With regard to membrane operation, average permeabilities around 150 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ were achieved, operating with fluxes of 11-15 $L \cdot m^{-2} \cdot h^{-1}$.

Life Cycle Assessment was applied for the evaluation of proposed UASB-MBR system compared to 3 other membrane bioreactor configurations of increasing complexity. It was found that UASB-MBR was the best if acidification impact category was considered, however attention should be paid in global warming and ecotoxicity matters. Moreover, due to the poor elimination of nitrogenous compounds, eutrophication was also pointed out as a bottleneck of the proposed system.

In **Chapter 4** the feasibility of the combined UASB and MBR system for the treatment of dairy wastewater at ambient temperatures was investigated. As in **Chapter 3**, the system consisted of a methanogenic UASB stage and two-compartment post-treatment aerobic MBR stage, with a membrane ultrafiltration module. The objective of the system was to decrease the COD of dairy wastewater, producing a methane rich biogas, diminish overall sludge production, and to obtain high quality effluent due to the implementation of a membrane filtration stage. Since in **Chapter 3** the proposed UASB-MBR system was proved to be feasible for the treatment of low strength wastewater with the average organic loading rate of 1.25 $kgCOD \cdot m^{-3} \cdot d^{-1}$, in **Chapter 4** higher OLRs were applied. The system presented a high tolerance to loading changes (up to 3.9 $kgCOD \cdot m^{-3} \cdot d^{-1}$) and temperature fluctuations (17 – 25 °C). Moreover, the impact of internal recirculation on MBR stage and an overall system performance was studied. The average total and soluble COD removals were above 95%, reaching 99% during the stable operation. The observed overall biomass yield was low, from 0.13 to 0.07 $gVSS \cdot gCOD^{-1}$. Biogas production yield reached 150 $L \cdot kg^{-1}$ of t-COD, with an average methane content of 73%. With respect to membrane performance, permeability values between 140 and 225 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ were obtained, similar to those reported for aerobic MBR systems. The average flux obtained was 13 $L \cdot m^{-2} \cdot h^{-1}$, reaching 19 $L \cdot m^{-2} \cdot h^{-1}$ in stable operation depending on operating conditions. These values were lower than those observed in aerobic MBR systems, but much higher than those referred for methanogenic AnMBRs.

The objective of **Chapter 5** was to evaluate the impact of excess aerobic sludge on the specific methanogenic activity (SMA), in order to establish the maximum allowable aerobic sludge loading that could be applied. Moreover, the potential influence of biopolymers and extracellular polymeric substances, that are generated as a result of excess aerobic sludge hydrolysis, on membrane

performance was determined by assessing the fouling potential of the liquid broth, taking into account parameters such as specific cake resistance (SCR) and sludge filterability. These assays were performed to assess the impact on SMA of different fractions of aerobic sludge, i.e. 0.03, 0.05, 0.10 and 0.15. It means that to 2.5 $gVSS \cdot L^{-1}$ of anaerobic seed sludge 0.075, 0.125, 0.250 and 0.375 $gVSS \cdot L^{-1}$ of aerobic sludge were added, respectively. It was found that a low amount of aerobic sludge leads to an increased SMA and a high membrane fouling potential. Results showed that addition of 0.15 fraction of aerobic sludge caused more than 20% SMA decrease.

The increase in biopolymers, characterized as biopolymeric cluster (BPC), extracellular polymeric substances (EPS), and soluble microbial products (SMP) could be ascribed to aerobic sludge hydrolysis. A clear positive correlation between the concentration of colloidal fraction of BPC (cBPC) and specific resistance to filtration (SRF), and negative correlation between cBPC and supernatant filterability (SF) measured at the end of SMA tests (in relation with aerobic sludge fraction) was observed, indicating that sludge filtration resistance increases when more aerobic sludge is hydrolyzed, and thus more cBPC is released.

During AnMBR operation, proteins significantly contributed to sludge filterability decrease expressed as SRF and filterability, whereas the carbohydrate fraction of SMP was of less importance due to low concentrations. On the contrary, carbohydrates seemed to improve filterability and diminish SRF of the sludge. Albeit, cBPC increase caused an increase in mean TMP during the AnMBR operation, confirming that cBPC is positively correlated to membrane fouling, which is in agreement with results presented in **Chapter 3** and **Chapter 4**.

In this **Chapter 6** biomass present in the combined UASB-MBR system (previously described in **Chapter 3** and **Chapter 4**) was characterized, by using a wide spectrum of analytical techniques. Among others, morphology description of granular, suspended and biofilm biomass, size distribution and composition of granular sludge, and FISH analysis were applied. To obtain more detailed information about the bacterial populations present in the combined UASB-MBR, DNA extraction, PCR, DGGE and sequencing were performed. From the application of these molecular techniques a heterogeneous distribution of microorganisms present in the granular, suspended and biofilm biomass was revealed. Among *Proteobacteria* phylum, a subclass of *Betaproteobacteria* was the

most dominant, followed by the *Gammaproteobacteria*. *Alphaproteobacteria* was scarce and appeared in coccoid form, while bacteria belonging to *Deltaproteobacteria* were not observed at all. The predominance of members of *Betaproteobacteria* was associated with abundance of nitrifying and denitrifying bacteria. Apart from these microorganisms, *Bacteroidetes*, nitrite-oxidizing bacteria (NOB), *Acidobacteria*, *Firmicutes* and filamentous bacteria belonging to *Chloroflexi* were also detected. Finally, the appearance of some Anammox bacteria, belonging to *Planctomycetales*, was observed during the first operation periods.

Apart from molecular techniques, the role of protozoa in the MBR stage was evaluated. It was revealed that the presence of plastic support and thus development of predators is crucial for stable operation and high flux achievement in the MBR stage. Taking into account the impact of metazoan and protozoa on F/M ratio, this finding is in agreement with the results presented in **Chapter 4**.

The combined UASB-MBR system was operated during more than 3 years. Since the system was subjected to many modifications (OLR, temperature, aerobic/anoxic conditions in MBR chambers, recirculation ratio, etc) within this period a development of big variety of microorganisms was observed. In **Chapter 7**, two interesting and intriguing processes will be described: methane oxidation coupled to denitrification and Anammox, which were observed at the end and at the beginning of the operation of the system, respectively.

The presence of dissolved methane, especially at low temperature, represents an important environmental concern in terms of greenhouse gas (GHG) emissions of wastewaters treated using methanogenic bioreactors. Methane has a global warming potential 25 times higher than carbon dioxide. For low strength wastewaters, dissolved methane might account up to 50% of the produced methane. The dissolved methane is easily desorbed from the effluents, especially if these are either released in the environment or post-treated using aerobic bioreactors. Thus the use of anaerobic technology could increase GHG emissions of wastewater treatment. The use of this dissolved methane as a carbon source for biological denitrification has been proposed as an alternative to reduce both GHGs emissions and nitrogen content of the treated wastewater. In this study the effluent of a UASB reactor was post-treated in an MBR with a first anoxic chamber in order to use dissolved methane as carbon source for denitrification. Up to 60%

and 95% nitrogen removal and methane consumption were observed, respectively. The stripping of the dissolved methane present in the UASB effluent led to a worsening of nitrogen removal in the MBR system. Batch experiments confirmed the presence of microorganisms capable of denitrifying using the dissolved methane as carbon source. Recirculation ratio between the anoxic and aerobic chambers of the MBR system, and either the presence or absence of dissolved methane were shown as the main important parameters governing the denitrification process.

On the other hand, the presence and activity of Anammox bacteria in the combined UASB-MBR system shows its potential to develop a wide variety of populations of microorganisms, depending on the effluent requirements.

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Resumen

Durante los últimos 30-40 años, la legislación ambiental relativa a la conservación del agua dulce y a la reducción de su contaminación ha sido cada vez más restrictiva. Esto ha conducido a un mayor desarrollo tecnológico en el sector del agua. Por otro lado, ha aumentado la visión del tratamiento de aguas como una fuente no despreciable de gases de efecto invernadero. Esto, unido a varias iniciativas gubernamentales, institucionales y de otras organizaciones, ha conducido a considerar la aplicación de tecnologías más sofisticadas.

El proceso de tratamiento anaerobio de aguas residuales tiene más de 100 años. Es un proceso natural en el cual una variedad de especies distintas de dos reinos biológicos diferentes, las Bacterias y las Arqueas, trabajan juntas para convertir residuos orgánicos en gas metano, que es una excelente fuente de energía, pasando por una serie de intermedios. Además de la reducción significativa del contenido en materia orgánica, los microorganismos patógenos son también eliminados. Adicionalmente, la cantidad de exceso de lodo que se produce y las necesidades de nutrientes son mucho menores que con el tratamiento aerobio. Sin embargo, a pesar de que el metano es una buena fuente de energía renovable, también es un poderoso gas de efecto invernadero, y debe prestarse una especial atención si el agua residual ha de tratarse a temperatura ambiente.

Se ha observado una creciente confianza en la tecnología MBR que ha llevado a un incremento exponencial en el número de instalaciones alrededor del mundo, aunque el coste continúa siendo el mayor impedimento para la generalización de su aplicación. Sin embargo, durante los últimos 15 años, tanto el coste de instalación (y particularmente el de las membranas) como los costes operacionales del proceso MBR han descendido significativamente, dando lugar a nuevas oportunidades como la instalación de membranas en plantas de tratamiento de aguas residuales ya existentes a fin de incrementar su capacidad o la calidad del agua sin necesitar de un incremento del espacio ocupado.

Una de las posibles combinaciones es la aplicación de la tecnología de membranas como un post-tratamiento de efluentes anaerobios. En este sentido, la combinación de UASB con MBR para el tratamiento de aguas de baja carga a

temperatura ambiente, tal como se propone en esta Tesis, encaja perfectamente en el estado del arte tecnológico y en la demanda del mercado.

Tomando como punto de partida el conocimiento existente (**Capítulo 1**), en esta Tesis se estudió la puesta en marcha y la operación de un sistema combinado UASB-MBR empleando dos tipos diferentes de aguas residuales. El estudio se centró principalmente en la eliminación de materia orgánica y su conversión a biogás, así como el potencial de eliminación de nitrógeno a temperatura ambiente. Adicionalmente, se evaluó la operación de la membrana y los factores que le afectan. Las aguas residuales estudiadas fueron un agua semi-sintética con un contenido de DQO similar al agua urbana (**Capítulo 3**) y otra similar al agua residual de lechería (**Capítulo 4**), ambas preparadas en el laboratorio de la Universidad de Santiago de Compostela (España).

Teniendo en cuenta que la mayor parte de los procesos biológicos de tratamiento de aguas los llevan a cabo bacterias o arqueas, su identificación y cuantificación es crucial. Técnicas moleculares como FISH (Fluorescent in situ hibrydization, hibridación fluorescente in situ) y DGGE (denaturing gradient gel electrophoresis, electroforesis en gel con gradiente de desnaturalización) permitieron describir y seguir la dinámica de poblaciones durante la operación del sistema combinado UASB-MBR (**Capítulo 6**, **Capítulo 7**), y ayudaron a resolver algunas particularidades observadas (**Capítulo 7**).

Finalmente, es un hecho bien conocido que uno de los cuellos de botella de la tecnología de membranas es el ensuciamiento y las propiedades de filtrabilidad del lodo presente en el líquido. Puesto que en esta Tesis el efluente anaerobio fue tratado en una etapa MBR, los principales factores y su relación con la filtrabilidad del lodo y el ensuciamiento fueron evaluados en ensayos discontinuos a corto plazo y en ensayos a largo plazo en un reactor a escala laboratorio (**Capítulo 5**).

El principal contenido de cada capítulo y los principales objetivos que se consiguieron se detallan en las secciones a continuación.

En el **Capítulo 1**, empezando con una breve perspectiva histórica, se presentan los fundamentos del tratamiento de aguas residuales. Los procesos más importantes como eliminación de materia orgánica, nitrificación, desnitrificación y digestión anaerobia serán explicados, para dar una idea de la complejidad y las interacciones que pueden ocurrir durante los procesos de tratamiento de aguas. En este sentido, puesto que la conversión de materia orgánica y nutrientes se lleva

a cabo principalmente por bacterias y arqueas, se describen los diferentes tipos de biomasa, centrándose en biomasa adherida, en suspensión y crecimiento híbrido.

Uno de los elementos más importantes del sistema UASB-MBR propuesto en esta Tesis es la etapa UASB (Upflow Anaerobic Sludge Blanket, reactor anaerobio de flujo ascendente). Por lo tanto, en el **Capítulo 1** se resumirán el origen y las aplicaciones de esta tecnología. Adicionalmente, se evaluará de acuerdo con la literatura la aplicabilidad de la digestión anaerobia al tratamiento de aguas residuales, especialmente aguas residuales de baja carga.

Por otro lado, teniendo en cuenta que la etapa MBR fue implementada en el sistema combinado UASB-MBR estudiado en esta Tesis, el **Capítulo 1** también se centrará en los fundamentos, desventajas y ventajas de la tecnología MBR comparada con los sistemas convencionales de lodos activos (CAS). Se describirán diferentes tipos de reactores MBR, junto con los factores más importantes que afectan su operación, de los cuales el flujo crítico y los mecanismos de ensuciamiento son los más importantes. Además, se discutirá la eficiencia del MBR anaerobio (AnMBR) para el tratamiento de aguas residuales en comparación con el MBR aerobio. Se hará énfasis en los sistemas que operan a temperaturas ambiente, el cual es también el caso del sistema combinado UASB-MBR propuesto en esta Tesis.

Finalmente, se evaluará la tecnología MBR como un post-tratamiento del efluente de UASB. En este sentido, esta Tesis es un paso adelante en el desarrollo de la tecnología combinada UASB-MBR, puesto que es un intento de resolver los problemas relacionados con las principales desventajas de dicho tratamiento, relacionados con la necesidad de aplicar un post-tratamiento a los efluentes anaerobios, la operación de MBR anaerobios (ensuciamiento, bajos flujos transmembrana) y MBR aerobios (elevado consumo energético y producción de lodo).

En el **Capítulo 2**, se describirán los métodos analíticos empleados en esta Tesis. La metodología se dividió en fase líquida, fase sólida, fase gaseosa, caracterización de la biomasa y comportamiento de la membrana. Para caracterizar la fase líquida, se midieron los parámetros convencionales del tratamiento de aguas residuales, como DQO, amonio, nitrato, nitrito, fosfatos y nitrógeno total. Adicionalmente, se midieron pH, temperatura, oxígeno disuelto y

alcalinidad, a fin de comprobar si el sistema estaba operando en condiciones óptimas.

Para la caracterización de la fase sólida, se midieron los Sólidos en Suspensión Totales y Volátiles (SST y SSV) siguiendo el Standard Methods (APHA-AWWA-WPCF, 1999). La biomasa se caracterizó por medio de parámetros como el índice volumétrico de lodos, la distribución de tamaños de partícula de gránulos y técnicas de análisis digital de imagen, microscopía electrónica y estereomicroscopio. Por otro lado, se llevó a cabo la identificación de las diferentes poblaciones presentes en las muestras de biomasa (lodo granular tomado de la etapa UASB; biomasa en suspensión y en biopelícula tomada de la etapa MBR) por medio de hibridación fluorescente in situ (FISH). Para obtener la distribución de bacterias y arqueas en el sistema combinado UASB-MBR, se empleó una gran variedad de sondas de FISH específicas y se realizó una comparación visual de los resultados. Se usó microscopía confocal laser para tomar imágenes de las bacterias con resultados cuestionables obtenidos por medio de microscopía convencional.

Finalmente, se describió la metodología aplicada para el control y monitorización de la filtración por membrana, tal como medida de flujo crítico o determinación de la concentración de determinados productos responsables del ensuciamiento.

En el **Capítulo 3** se introducirá un nuevo sistema combinado UASB-MBR. El sistema está compuesto por una primera etapa metanogénica UASB, y una segunda etapa MBR con dos cámaras interconectadas: aerobia, con crecimiento de biopelícula en pequeños elementos de soporte y con biomasa creciendo en suspensión, y cámara de filtración, con un módulo de membranas de fibra hueca. El objetivo de la primera cámara metanogénica era disminuir la DQO del agua residual bruta, produciendo biogás rico en metano, y reduciendo la producción de lodo. En la segunda etapa MBR, la restante DQO soluble biodegradable fue oxidada por bacterias heterótrofas. En la cámara de filtración de la etapa MBR, el módulo de membrana pudo ser operado a flujos más elevados que los encontrados en la literatura para sistemas AnMBR, y similares a los encontrados en MBRs aerobios. En este sentido, el concepto de sistema combinado UASB-MBR que se propone en esta Tesis une las ventajas de los procesos metanogénico y de reactor de membranas aerobio, reduciendo los requerimientos de energía para la aireación, produciendo biogás con un elevado porcentaje en metano y un permeado con muy bajo contenido en DQO.

Para simular el flujo de agua residual urbana, se alimentó un agua sintética al sistema combinado UASB-MBR. La DQO en el influente estuvo entre 200 y 1200 $mg \cdot L^{-1}$, la concentración de amonio alrededor de 35 $mg \cdot L^{-1}$ y la concentración de fósforo fue 8 $mg \cdot L^{-1}$, respectivamente. La VCO estuvo entre 1 y 3 $kgDQO \cdot m^{-3} \cdot d^{-1}$ y se aplicó un TRH de 13-21 h. La temperatura estuvo entre 17,5 y 23,2 ºC. Durante todo el período de operación la eficacia de eliminación de DQO estuvo en el rango entre 90 y 96%, del cual entre el 40 y 80% fue eliminado en la primera cámara metanogénica. La concentración media de DQO medida en el permeado fue de alrededor de 5 $mg \cdot L^{-1}$. Se observó producción de biogás con un contenido en metano entre el 75 y 80%. Respecto a la operación de la membrana, se alcanzaron permeabilidades medias alrededor de 150 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$, operando con flujos de 11-15 $L \cdot m^{-2} \cdot h^{-1}$.

Se aplicó Análisis de Ciclo de Vida para la evaluación del sistema UASB-MBR propuesto, en comparación con otras 3 configuraciones de biorreactores de membranas con complejidad creciente. Se encontró que el UASB-MBR era el mejor sistema si se consideraba la categoría de impacto de acidificación. Sin embargo se debía prestar atención a los aspectos de calentamiento global y ecotoxicidad. Además, debido a la pobre eliminación de compuestos nitrogenados, la eutrofización apareció como uno de los cuellos de botella del sistema propuesto.

En el **Capítulo 4** se investigó la aplicabilidad del sistema combinado UASB-MBR para el tratamiento de agua residual de lechería a temperatura ambiente. Como en el **Capítulo 3**, el sistema consistía en una etapa metanogénica UASB y una etapa con dos compartimentos de post-tratamiento MBR aerobio, con un módulo de ultrafiltración por membrana. El objetivo del sistema era reducir la DQO del agua residual de lechería, produciendo un biogás rico en metano, reduciendo la producción global de lodo, y obteniendo un efluente de elevada calidad debido a la filtración por membrana. Puesto que en el **Capítulo 3** se demostró que el sistema UASB-MBR propuesto era apropiado para el tratamiento de aguas residuales de baja carga con una velocidad de carga orgánica media de 1,25 $kgDQO \cdot m^{-3} \cdot d^{-1}$, en el **Capítulo 4** se aplicaron VCOs mayores. El sistema presentó una elevada resistencia a cambios de carga (hasta 3,9 $kgDQO \cdot m^{-3} \cdot d^{-1}$) y fluctuaciones de temperatura (17 – 25 ºC). Además, se estudió el impacto de la recirculación interna en la etapa MBR y en la operación global del sistema. Las eliminaciones medias de DQO total y soluble estuvieron

por encima del 95%, alcanzando el 99% durante la operación estable. El rendimiento en biomasa observado fue bajo, de 0,13 a 0,07 $gSSV \cdot gDQO^{-1}$. El rendimiento de producción de biogás alcanzó 150 $L \cdot kg^{-1}$ de DQO_t, con un contenido medio de metano del 73%. Respecto a la operación de la membrana, se alcanzaron valores de permeabilidad entre 140 y 225 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$, que son similares a los que se encuentran en la literatura para sistemas MBR aerobios. El flujo medio obtenido fue de 13 $L \cdot m^{-2} \cdot h^{-1}$, alcanzando 19 $L \cdot m^{-2} \cdot h^{-1}$ en operación estacionaria, dependiendo de las condiciones de operación. Esos valores fueron menores que los observados en sistemas MBR aerobios, pero muy superiores a los obtenidos en AnMBRs metanogénicos.

El objetivo del **Capítulo 5** fue evaluar el impacto del exceso del lodo aerobio en la actividad metanogénica específica (AME), a fin de establecer la máxima carga de lodo aerobio que podría ser aplicada. Además, se estudió la potencial influencia en la operación de la membrana de los biopolímeros y las sustancias poliméricas extracelulares, que se generan como resultado del exceso de hidrólisis del lodo aerobio. Para ello se observó el potencial de ensuciamiento de la mezcla líquida, teniendo en cuenta parámetros como la resistencia específica de la torta y la filtrabilidad del lodo. Esos ensayos se llevaron a cabo para evaluar el impacto en la AME de diferentes fracciones de lodo aerobio, i.e. 0,03, 0,05, 0,10 y 0,15. Esto significa que para 2,5 $gSSV \cdot L^{-1}$ de lodo anaerobio inoculado, se añadieron 0,075, 0,125, 0,250 y 0,375 $gSSV \cdot L^{-1}$ de lodo aerobio, respectivamente. Se encontró que una baja cantidad de lodo aerobio producía un incremento de la AME y un elevado potencial de ensuciamiento de la membrana. Los resultados indicaron que la adición de una fracción de 0,15 de lodo aerobio causó más de un 20% de descenso de la AME.

El incremento de biopolímeros, caracterizados como clústeres biopoliméricos (CBP), sustancias poliméricas extracelulares (SPE), y productos microbianos solubles (PMS) puede ser atribuido a la hidrólisis de lodo aerobio. Se observó una clara correlación positiva entre la concentración de la fracción coloidal de CBP (cCBP) y la resistencia específica a la filtración (REF), y una correlación negativa entre cCBP y la filtrabilidad del sobrenadante (FS) medida al final de los ensayos de AME (y en relación con la fracción de lodo aerobio). Esto indicó que la resistencia a la filtración del lodo se incrementa cuando se hidroliza más lodo aerobio y, por lo tanto, cuando se libera más cCBP.

Durante la operación del AnMBR, las proteínas contribuyeron significativamente a disminuir la filtrabilidad del lodo, expresado como REF y filtrabilidad, mientras que la fracción de carbohidratos de los PMS fue de menor importancia debido a sus bajas concentraciones. Por la contra, los carbohidratos parecían mejorar la filtrabilidad y disminuir la REF del lodo. No obstante, el incremento de cCBP causó un ascenso de la presión transmembrana durante la operación del AnMBR, confirmando que cCBP está positivamente correlacionada con el ensuciamiento de la membrana, lo cual está de acuerdo con los resultados presentados en el **Capítulo 3** y **Capítulo 4**.

En este **Capítulo 6** se caracterizó la biomasa presente en el sistema combinado UASB-MBR (previamente descrito en el **Capítulo 3** y **Capítulo 4**) por medio de un amplio espectro de técnicas analíticas. Se emplearon entre otras, la descripción morfológica de la biomasa granular, suspendida y en biopelícula, la distribución de tamaños y composición del lodo granular, y análisis FISH. Para obtener información más detallada sobre las poblaciones bacterianas presentes en el sistema combinado UASB-MBR, se llevaron a cabo extracción de ADN, PCR, DGGE y secuenciación. Gracias a la aplicación de estas técnicas moleculares, se obtuvo la distribución heterogénea de microrganismos presentes en la biomasa granular, suspendida y en biopelícula. Dentro del filo *Proteobacteria*, la más dominante fue una subclase de *Betaproteobacteria*, seguida por *Gammaproteobacteria*. La presencia de *Alphaproteobacteria* fue escasa y apareció en forma cocoide, mientras no se observó la presencia de bacterias pertenecientes a *Deltaproteobacteria*. La predominancia de miembros de *Betaproteobacteria* se asoció con la abundancia de bacterias nitrificantes y desnitrificantes. Además de esos microrganismos, también se detectaron *Bacteroidetes*, bacterias oxidantes de nitrito, *Acidobacteria*, *Firmicutes* y bacterias filamentosas pertenecientes a *Chloroflexi*. Finalmente, se observó la aparición de bacterias Anammox, pertenecientes a las *Planctomycetales*, durante los primeros períodos de operación.

Además de las técnicas moleculares, se evaluó el papel de los protozoos en la etapa MBR. Se observó que la presencia del soporte plástico y, por tanto, el desarrollo de predadores es crucial para la operación estable y para alcanzar elevados flujos en la etapa MBR. Teniendo en cuenta el impacto de metazoos y protozoos en la relación alimento/microrganismos, esta observación está de acuerdo con los resultados presentados en el **Capítulo 4**.

El sistema combinado UASB-MBR se operó durante más de 3 años. Puesto que el sistema fue sometido a muchas modificaciones (VCO, temperatura, condiciones aeróbicas/anóxicas en las cámaras MBR, relación de recirculación, etc), se observó el desarrollo de una gran variedad de microrganismos. En el **Capítulo 7** se describirán dos procesos muy interesantes e incluso fascinantes: la oxidación de metano acoplada a desnitrificación y el proceso Anammox, que fueron observados al final y al principio de la operación del sistema, respectivamente.

La presencia de metano disuelto, especialmente a baja temperatura, representa una importante preocupación ambiental en términos de emisiones de gases de efecto invernadero (GEI) procedentes de aguas residuales tratadas por medio de biorreactores metanogénicos. El metano tiene un potencial de calentamiento global 25 veces mayor que el dióxido de carbono. En el caso de aguas de baja carga, el metano disuelto puede suponer hasta el 50% de todo el metano producido. El metano disuelto es fácilmente desorbido de los efluentes, especialmente si éstos son vertidos al medio o post-tratados empleando biorreactores aerobios. Por lo tanto el uso de la tecnología anaerobia podría incrementar la emisión de GEI procedentes del tratamiento de aguas residuales. Se ha propuesto el uso de este metano disuelto como una fuente de carbono para la desnitrificación biológica, como una alternativa tanto para reducir las emisiones de GEI como el contenido de nitrógeno del agua residual tratada. En este estudio el efluente de un reactor UASB fue post-tratado en un MBR con una primera cámara anóxica, a fin de usar el metano disuelto como fuente de carbono para la desnitrificación. Se observaron hasta un 60% y 95% de eliminación de nitrógeno y consumo de metano, respectivamente. El stripping del metano disuelto presente en el efluente del UASB condujo a un empeoramiento en la eliminación de nitrógeno en el MBR. Experimentos discontinuos confirmaron la presencia de microrganismos capaces de desnitrificar empleando el metano disuelto como fuente de carbono. La relación de recirculación entre las cámaras anóxica y aerobio del sistema MBR, así como la presencia o ausencia de metano disuelto, fueron los parámetros más importantes que controlaron el proceso de desnitrificación.

Por otro lado, la presencia y actividad de bacterias Anammox en el sistema combinado UASB-MBR muestra su potencial para desarrollar una amplia variedad de poblaciones de microrganismos, dependiendo de los requerimientos del efluente.

Resumo

Durante os últimos 30-40 anos, a lexislación ambiental relativa á conservación da auga doce e á redución da súa contaminación foi cada vez máis restritiva. Isto conduciu a un maior desenvolvemento tecnolóxico no sector da auga. Doutra banda, aumentou a visión do tratamento de augas como unha fonte non desprezable de gases de efecto invernadoiro. Isto, xunto con varias iniciativas gobernamentais, institucionais e doutras organizacións, conduciu a considerar a aplicación de tecnoloxías máis sofisticadas.

O proceso de tratamento anaerobio de augas residuais ten máis de 100 anos. É un proceso natural no cal unha variedade de especies distintas de dous reinos biolóxicos diferentes, as Bacterias e as Arqueas, traballan xuntas para converter residuos orgánicos en gas metano, que é unha excelente fonte de enerxía, pasando por unha serie de intermedios. Ademais da redución significativa do contido en materia orgánica, os microorganismos patóxenos son tamén eliminados. Adicionalmente, o exceso de lodo que se produce e as necesidades de nutrientes son moito menores que co tratamento aerobio. Con todo, malia que o metano é unha boa fonte de enerxía renovable, tamén é un poderoso gas de efecto invernadoiro, e debe prestarse unha especial atención se a auga residual ten que tratarse a temperatura ambiente.

Observouse unha crecente confianza na tecnoloxía MBR que levou a un incremento exponencial no número de instalacións ao redor do mundo, aínda que o custo continúa sendo o maior impedimento para a xeneralización da súa aplicación. Con todo, durante os últimos 15 anos, tanto o custo de instalación (e particularmente o das membranas) como os custos operacionais do proceso MBR descenderon notablemente, dando lugar a novas oportunidades como a instalación de membranas en plantas de tratamento de augas residuais xa existentes a fin de incrementar a súa capacidade ou a calidade da auga sen necesitar dun incremento do espazo ocupado.

Unha das posibles combinacións é a aplicación da tecnoloxía de membranas como un post-tratamento de efluentes anaerobios. Neste sentido, a combinación de UASB con MBR para o tratamento de augas de baixa carga a temperatura

ambiente, tal como se propón nesta Tese, encaixa perfectamente no estado da arte tecnolóxica e na demanda do mercado.

Tomando como punto de partida o coñecemento existente (**Capítulo 1**), nesta Tese estudouse a posta en marcha e a operación dun sistema combinado UASB-MBR empregando dous tipos diferentes de augas residuais. O estudo centrouse principalmente na eliminación de materia orgánica e a súa conversión a biogás, así como o potencial de eliminación de nitróxeno a temperatura ambiente. Adicionalmente, avaliouse a operación da membrana e os factores que lle afectan. As augas residuais estudadas foron unha auga semi-sintética cun contido de DQO similar á auga urbana (**Capítulo 3**) e outra similar á auga residual de leitería (**Capítulo 4**), ámbalas dúas preparadas no laboratorio da Universidade de Santiago de Compostela (España).

Tendo en conta que a maior parte dos procesos biolóxicos de tratamento de augas lévanos a cabo bacterias ou arqueas, a súa identificación e cuantificación é crucial. Técnicas moleculares como FISH (Fluorescent in situ hibrydization, hibridación fluorescente in situ) e DGGE (denaturing gradient gel electrophoresis, electroforese en xel con gradiente de desnaturalización) permitiron describir e seguir a dinámica de poboacións durante a operación do sistema combinado UASB-MBR (**Capítulo 6**, **Capítulo 7**), e axudaron a resolver algunhas particularidades observadas (**Capítulo 7**).

Finalmente, é un feito ben coñecido que un dos pescozos de botella da tecnoloxía de membranas é o ensuciamento e as propiedades de filtrabilidade do lodo presente no líquido. Posto que nesta Tese o efluente anaerobio foi tratado nunha etapa MBR, os principais factores e a súa relación coa filtrabilidade do lodo e o ensuciamento foron avaliados en ensaios descontinuos a curto prazo e en ensaios a longo prazo nun reactor a escala laboratorio (**Capítulo 5**).

O principal contido de cada capítulo e os principais obxectivos que se conseguiron detállanse nas seccións a continuación.

No **Capítulo 1**, empezando cunha breve perspectiva histórica, preséntanse os fundamentos do tratamento de augas residuais. Os procesos máis importantes como eliminación de materia orgánica, nitrificación, desnitrificación e dixestión anaerobia serán explicados, para dar unha idea da complexidade e das interaccións que poden ocorrer durante os procesos de tratamento de augas. Neste sentido, posto que a conversión de materia orgánica e nutrientes se leva a

cabo principalmente por bacterias e arqueas, descríbense os diferentes tipos de biomasa, centrándose en biomasa adherida, en suspensión e crecemento híbrido.

Un dos elementos máis importantes do sistema UASB-MBR proposto nesta Tese é a etapa UASB (Upflow Anaerobic Sludge Blanket, reactor anaerobio de fluxo ascendente). Polo tanto, no **Capítulo 1** resumiranse a orixe e as aplicacións desta tecnoloxía. Adicionalmente, avaliarase de acordo coa literatura a aplicabilidade da dixestión anaerobia ao tratamento de augas residuais, especialmente augas residuais de baixa carga.

Doutra banda, tendo en conta que a etapa MBR foi introducida no sistema combinado UASB-MBR estudado nesta Tese, o **Capítulo 1** tamén se centrará nos fundamentos, desvantaxes e vantaxes da tecnoloxía MBR comparada cos sistemas convencionais de lodos activos (CAS). Describiranse diferentes tipos de reactores MBR, xunto cos factores máis importantes que afectan á súa operación, dos cales o fluxo crítico e os mecanismos de ensuciamento son os máis importantes. Ademais, discutirase a eficiencia do MBR anaerobio (AnMBR) para o tratamento de augas residuais en comparación co MBR aerobio. Farase énfase nos sistemas que operan a temperatura ambiente, o cal é tamén o caso do sistema combinado UASB-MBR proposto nesta Tese.

Finalmente, avaliarase a tecnoloxía MBR como un post-tratamento do efluente de UASB. Neste sentido, esta Tese é un paso adiante no desenvolvemento da tecnoloxía combinada UASB-MBR, posto que é un intento de resolver os problemas relacionados coas principais desvantaxes de devandito tratamento, relacionados coa necesidade de aplicar un post-tratamento aos efluentes anaerobios, a operación de MBRs anaerobios (ensuciamento, baixos fluxos transmembrana) e MBRs aerobios (elevado consumo enerxético e produción de lodo).

No **Capítulo 2**, describiranse os métodos analíticos empregados nesta Tese. A metodoloxía dividiuse en fase líquida, fase sólida, fase gasosa, caracterización da biomasa e comportamento da membrana. Para caracterizar a fase líquida, medíronse os parámetros convencionais do tratamento de augas residuais, como DQO, amonio, nitrato, nitrito, fosfatos e nitróxeno total. Adicionalmente, medíronse pH, temperatura, osíxeno disolto e alcalinidade, a fin de comprobar se o sistema estaba operando en condicións óptimas.

Para a caracterización da fase sólida, medíronse os Sólidos en Suspensión Totais e Volátiles (SST e SSV) seguindo o Standard Methods (APHA-AWWA-WPCF, 1999). A biomasa caracterizouse por medio de parámetros como o índice volumétrico de lodos, a distribución de tamaños de partícula de gránulos e técnicas de análise dixital de imaxe, microscopía electrónica e estereomicroscopio. Doutra banda, levouse a cabo a identificación das diferentes poboacións presentes nas mostras de biomasa (lodo granular tomado da etapa UASB; biomasa en suspensión e en biopelícula tomada da etapa MBR) por medio de hibridación fluorescente in situ (FISH). Para obter a distribución de bacterias e arqueas no sistema combinado UASB-MBR, empregouse unha gran variedade de sondas de FISH específicas e realizouse unha comparación visual dos resultados. Usouse microscopía confocal láser para tomar imaxes das bacterias con resultados cuestionables obtidos por medio de microscopía convencional.

Finalmente, describiuse a metodoloxía aplicada para o control e monitorización da filtración por membrana, tal como medida de fluxo crítico ou determinación da concentración de determinados produtos responsables do ensuciamento.

No **Capítulo 3** introducirase un novo sistema combinado UASB-MBR. O sistema está composto por unha primeira etapa metanoxénica UASB, e unha segunda etapa MBR con dúas cámaras conectadas: aerobia, con crecemento de biopelícula sobre pequenos elementos de soporte e con biomasa crecendo en suspensión, e cámara de filtración, cun módulo de membranas de fibra oca. O obxectivo da primeira cámara metanoxénica era diminuír a DQO da auga residual bruta, producindo biogás rico en metano, e reducindo a produción de lodo. Na segunda etapa MBR, a restante DQO soluble biodegradable foi oxidada por bacterias heterótrofas. Na cámara de filtración da etapa MBR, o módulo de membrana puido ser operado a fluxos máis elevados que os atopados na literatura para sistemas AnMBR, e similares aos atopados en MBRs aerobios. Neste sentido, o concepto de sistema combinado UASB-MBR que se propón nesta Tese une as vantaxes dos procesos metanoxénico e de reactor de membranas aerobio, reducindo os requirimentos de enerxía para a aireación, producindo biogás cunha elevada porcentaxe en metano e un permeado con moi baixo contido en DQO.

Para simular o fluxo de auga residual urbana, alimentouse unha auga sintética ao sistema combinado UASB-MBR. A DQO no influente estivo entre 200 e 1200 $mg \cdot L^{-1}$, a concentración de amonio ao redor de 35 $mg \cdot L^{-1}$ e a concentración de fósforo

foi 8 $mg \cdot L^{-1}$, respectivamente. A VCO estivo entre 1 e 3 $kgDQO \cdot m^{-3} \cdot d^{-1}$ e aplicouse un TRH de 13-21 h. A temperatura estivo entre 17,5 e 23,2 ºC. Durante todo o período de operación a eficacia de eliminación de DQO estivo no rango entre 90 e 96%, do cal entre o 40 e 80% foi eliminado na primeira cámara metanoxénica. A concentración media de DQO medida no permeado foi de ao redor de 5 $mg \cdot L^{-1}$. Observouse produción de biogás cun contido en metano entre o 75 e 80%. Respecto á operación da membrana, alcanzáronse permeabilidades medias ao redor de 150 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$, operando con fluxos de 11-15 $L \cdot m^{-2} \cdot h^{-1}$.

Aplicouse Análise de Ciclo de Vida para a avaliación do sistema UASB-MBR proposto, en comparación con outras 3 configuracións de biorreactores de membranas con complexidade crecente. Atopouse que o UASB-MBR era o mellor sistema se se consideraba a categoría de impacto de acidificación. Con todo debíase prestar atención aos aspectos de quentamento global e ecotoxicidade. Ademais, debido á pobre eliminación de compostos nitroxenados, a eutrofización apareceu como un dos pescozos de botella do sistema proposto.

No **Capítulo 4** investigouse a aplicabilidade do sistema combinado UASB-MBR para o tratamento de auga residual de leitería a temperatura ambiente. Como no **Capítulo 3**, o sistema consistía nunha etapa metanoxénica UASB e unha etapa con dous compartimentos de post-tratamento MBR aerobio, cun módulo de ultrafiltración por membrana. O obxectivo do sistema era reducir a DQO da auga residual de leitería, producindo un biogás rico en metano, reducindo a produción global de lodo, e obtendo un efluente de elevada calidade debido á filtración por membrana. Posto que no **Capítulo 3** se demostrou que o sistema UASB-MBR proposto era apropiado para o tratamento de augas residuais de baixa carga cunha velocidade de carga orgánica media de 1,25 $kgDQO \cdot m^{-3} \cdot d^{-1}$, no **Capítulo 4** aplicáronse VCOs maiores. O sistema presentou unha elevada resistencia a cambios de carga (ata 3,9 $kgDQO \cdot m^{-3} \cdot d^{-1}$) e flutuacións de temperatura (17 – 25 °C). Ademais, estudouse o impacto da recirculación interna na etapa MBR e na operación global do sistema. As eliminacións medias de DQO total e soluble estiveron por encima do 95%, alcanzando o 99% durante a operación estable. O rendemento en biomasa observado foi baixo, de 0,13 a 0,07 $gSSV \cdot gDQO^{-1}$. O rendemento de produción de biogás alcanzou 150 $L \cdot kg^{-1}$ de DQO_t, cun contido medio de metano do 73%. Respecto da operación da membrana, alcanzáronse valores de permeabilidade entre 140 e 225 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$, que son similares aos que se atopan na literatura para sistemas MBR aerobios.

O fluxo medio obtido foi de 13 $L \cdot m^{-2} \cdot h^{-1}$, alcanzando 19 $L \cdot m^{-2} \cdot h^{-1}$ en operación estacionaria, dependendo das condicións de operación. Eses valores foron menores que os observados en sistemas MBR aerobios, pero moi superiores aos obtidos en AnMBRs metanoxénicos.

O obxectivo do **Capítulo 5** foi avaliar o impacto do exceso do lodo aerobio na actividade metanoxénica específica (AME), a fin de establecer a máxima carga de lodo aerobio que podería ser aplicada. Ademais, estudouse a potencial influencia sobre a operación da membrana dos biopolímeros e as sustancias poliméricas extracelulares, que se xeran como resultado do exceso de hidrólise do lodo aerobio. Para iso observouse o potencial de ensuciamento da mestura líquida, tendo en conta parámetros como a resistencia específica da torta e a filtrabilidade do lodo. Eses ensaios leváronse a cabo para avaliar o impacto na AME de diferentes fraccións de lodo aerobio, i.e. 0,03, 0,05, 0,10 e 0,15. Isto significa que para 2,5 $gSSV \cdot L^{-1}$ de lodo anaerobio inoculado, engadíronse 0,075, 0,125, 0,250 e 0,375 $gSSV \cdot L^{-1}$ de lodo aerobio, respectivamente. Atopouse que unha baixa cantidade de lodo aerobio producía un incremento da AME e un elevado potencial de ensuciamento da membrana. Os resultados indicaron que a adición dunha fracción de 0,15 de lodo aerobio causou máis dun 20% de descenso da AME.

O incremento de biopolímeros, caracterizados como clústeres biopoliméricos (CBP), substancias poliméricas extracelulares (SPE), e produtos microbianos solubles (PMS) pode ser atribuído á hidrólise do lodo aerobio. Observouse unha clara correlación positiva entre a concentración da fracción coloidal de CBP (cCBP) e a resistencia específica á filtración (REF), e unha correlación negativa entre cCBP e a filtrabilidade do sobrenadante (FS) medida ao final dos ensaios de AME (e en relación coa fracción de lodo aerobio). Isto indicou que a resistencia á filtración do lodo se incrementa cando se hidroliza máis lodo aerobio e, polo tanto, cando se libera máis cCBP.

Durante a operación do AnMBR, as proteínas contribuíron de xeito significativo a diminuír a filtrabilidade do lodo, expresado como REF e filtrabilidade, mentres que a fracción de carbohidratos dos PMS foi de menor importancia debido ás súas baixas concentracións. Pola contra, os carbohidratos parecían mellorar a filtrabilidade e diminuír a REF do lodo. No entanto, o incremento de cCBP causou un ascenso da presión transmembrana durante a operación do AnMBR, confirmando que a cCBP está positivamente correlacionada co ensuciamento da

membrana, o cal está de acordo cos resultados presentados no **Capítulo 3** e **Capítulo 4**.

Neste **Capítulo 6** caracterizouse a biomasa presente no sistema combinado UASB-MBR (previamente descrito no **Capítulo 3** e **Capítulo 4**) por medio dun amplo espectro de técnicas analíticas. Empregáronse entre outras, a descrición morfolóxica da biomasa granular, suspendida e en biopelícula, a distribución de tamaños e composición do lodo granular, e análise FISH. Para obter información máis detallada sobre as poboacións bacterianas presentes no sistema combinado UASB-MBR, leváronse a cabo extracción de ADN, PCR, DGGE e secuenciación. Grazas á aplicación destas técnicas moleculares, obtívose a distribución heteroxénea de microorganismos presentes na biomasa granular, suspendida e en biopelícula. Dentro do filo *Proteobacteria*, a máis dominante foi unha subclase de *Betaproteobacteria*, seguida por *Gammaproteobacteria*. A presenza de *Alphaproteobacteria* foi escasa e apareceu en forma cocoide, mentres non se observou a presenza de bacterias pertencentes a *Deltaproteobacteria*. A predominancia de membros de *Betaproteobacteria* asociouse coa abundancia de bacterias nitrificantes e desnitrificantes. Ademais deses microorganismos, tamén se detectaron *Bacteroidetes*, bacterias oxidantes de nitrito, *Acidobacteria*, *Firmicutes* e bacterias filamentosas pertencentes a *Chloroflexi*. Finalmente, observouse a aparición de bacterias Anammox, pertencentes ás *Planctomycetales*, durante os primeiros períodos de operación.

Ademais das técnicas moleculares, avaliouse o papel dos protozoos na etapa MBR. Observouse que a presenza do soporte plástico e, xa que logo, o desenvolvemento de predadores é crucial para a operación estable e para alcanzar elevados fluxos na etapa MBR. Tendo en conta o impacto de metazoos e protozoos na relación alimento/microorganismo, esta observación está de acordo cos resultados presentados no **Capítulo 4**.

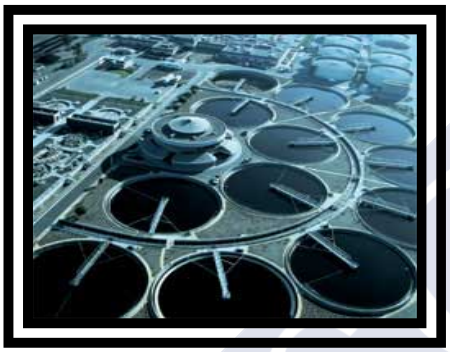
O sistema combinado UASB-MBR operouse durante máis de 3 anos. Posto que o sistema foi sometido a moitas modificacións (VCO, temperatura, condicións aerobias/anóxicas nas cámaras MBR, relación de recirculación, etc), observouse o desenvolvemento dunha gran variedade de microorganismos. No **Capítulo 7** describiranse dous procesos moi interesantes e mesmo fascinantes: a oxidación de metano acoplada á desnitrificación e o proceso Anammox, que

foron observados ao final e ao principio da operación do sistema, respectivamente.

A presenza de metano disolto, especialmente a baixa temperatura, representa unha importante preocupación ambiental en termos de emisións de gases de efecto invernadoiro (GEI) procedentes de augas residuais tratadas por medio de biorreactores metanoxénicos. O metano ten un potencial de quecemento global 25 veces maior que o dióxido de carbono. No caso de augas de baixa carga, o metano disolto pode supoñer ata o 50% de todo o metano producido. O metano disolto é facilmente desorbido dos efluentes, especialmente se estes son vertidos ao medio ou post-tratados empregando biorreactores aerobios. Polo tanto o uso da tecnoloxía anaerobia podería incrementar a emisión de GEI procedentes do tratamento de augas residuais. Propúxose o uso deste metano disolto como unha fonte de carbono para a desnitrificación biolóxica, como unha alternativa tanto para reducir as emisións de GEI como o contido de nitróxeno da auga residual tratada. Neste estudo o efluente dun reactor UASB foi post-tratado nun MBR cunha primeira cámara anóxica, a fin de usar o metano disolto como fonte de carbono para a desnitrificación. Observáronse ata un 60% e 95% de eliminación de nitróxeno e consumo de metano, respectivamente. O stripping do metano disolto presente no efluente do UASB conduciu a un empeoramento na eliminación de nitróxeno no MBR. Experimentos descontinuos confirmaron a presenza de microorganismos capaces de desnitrificar empregando o metano disolto como fonte de carbono. A relación de recirculación entre as cámaras anóxica e aerobia do sistema MBR, así como a presenza ou ausencia de metano disolto, foron os parámetros máis importantes que controlaron o proceso de desnitrificación.

Doutra banda, a presenza e actividade de bacterias Anammox no sistema combinado UASB-MBR mostra o seu potencial para desenvolver unha ampla variedade de poboacións de microorganismos, dependendo dos requirimentos do efluente.

Chapter 1



Introduction

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SUMMARY

In this Chapter, starting with a brief historical background, the fundamentals of wastewater treatment will be presented. The most crucial processes such as organic matter removal, nitrification, denitrification and anaerobic digestion will be explained, to give the Reader an overview on the complexity and interactions that might occur during wastewater treatment processes. In these sense, since organic matter and nutrient conversion are driven mostly by bacteria and archaea, different types of biomass forms will be described, focusing on attached, suspended and hybrid growth.

One of the crucial elements of combined UASB-MBR studied in this work is the Upflow Anaerobic Sludge Blanket (UASB) stage. Therefore, in this Chapter the origin and worldwide application of this technology will be resumed. Additionally, the applicability of anaerobic digestion in wastewater treatment, especially concerning low-strength wastewater, will be evaluated.

On the other hand, since MBR stage was implemented in the combined UASB-MBR system studied in this Thesis, this Chapter will also focus on MBR technology fundamentals, drawbacks and advantages over conventional activated sludge (CAS) systems. Different types of MBR reactors will be described, together with the most important factors influencing their operation, with critical flux and fouling mechanisms being of most importance. Moreover, anaerobic MBR (AnMBR) effectiveness in wastewater treatment in comparison with aerobic MBR will be discussed, will the emphasis on those systems working at ambient temperatures, which is also the case for the combined UASB-MBR proposed in this Thesis.

Finally, MBR technology as a UASB effluent post-treatment option will be evaluated. In this sense, the present study is a step forward into the development of combined UASB-MBR technology, since it is an attempt to

resolve problems related to the main drawbacks of such a treatment, related with the need of post-treatment of anaerobic effluents, the operation of anaerobic MBR (fouling, low membrane fluxes) and aerobic MBR (high energy consumption and sludge production).

1.1 FUNDAMENTALS OF WASTEWATER TREATMENT

Victor Hugo in *Les Miserables* (1892) said "The history of men is reflected in the history of sewers". Wastewater treatment dates back to 3500 – 2500 BC to Mesopotamian Empire, which was the first civilization to formally address sanitation problems. In the ruins of Ur and Babylonia, there are remains of homes which were connected to a drainage system to carry away wastes (Lofrano & Brown, 2010) as well as latrines leading to cesspits.

The ancient Egyptians, well known for their many achievements, had bathrooms and toilets seats made of limestone, connected to drainage channels (Herakopolis, B.C.E. 2100).

The Indus Valley (1500 BC) was far advanced in wastewater management and there is where the world´s first urban sanitation system was discovered. in Harrapa and Mohenjo-Daro (Pakistan). Wastewater was channelled to covered drains that lined the major streets (figure 1-1, left). Some houses, presumably those of wealthier inhabitants, include rooms that appear to have been set aside for bathing. This civilization, which disappeared when the river moved its course, was rediscovered in 1930s.

Although sewer and water pipes were not inventions of the Romans, since they were already present in other Eastern civilizations, they were certainly perfected by them. The most famous ancient wastewater collector, the Cloaca Maxima (figure 1-1, right) was constructed 600 – 500 BC in Ancient Rome, in order to drain local marshes and remove the waste of one of the world's most populous cities. The effluent was carried to the River Tiber, which ran beside the city. But soon it was insufficient to handle the flow of wastewater. Therefore, it was enlarged in the following centuries, extended and roofed over (Wiesmann *et al.*, 2007). In any case The Romans were brilliant managers and engineers and their systems rivalled modern technology.

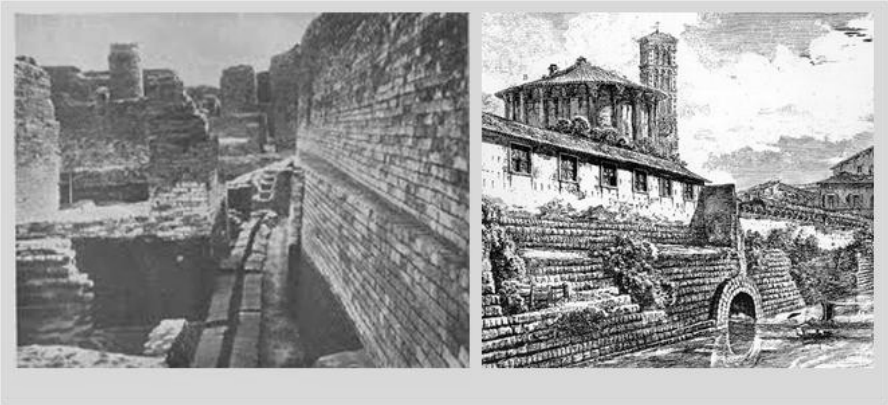

Figure 1-1 Mohenjo-Daro drainage system (source: Govt. of India)

The Greeks were forerunners of modern sanitation systems. Archaeological studies have established unequivocally that, the origin of modern technologies of water management dates back to ancient Greece (300 BC to 500 AD). At the Palace of Minos in Knossos, toilets similar to Egyptian ones were found connected to a closed sewer which still exists and is working after 4000 years. The Ancient Greeks had public latrines which drained into pipes which conveyed the wastewater and stormwater to a collection basin outside the city. From there, brick-lined conduits conveyed the wastewater to agricultural fields where it was used for irrigation and to fertilise crops and orchards (Lofrano & Brown, 2010).

When the Roman Empire collapsed, the sanitary dark ages began and lasted for over a thousand years (476–1800). The culture of water as a source of health and wellness which had not only marked the Roman civilization but many more civilizations before then, was abandoned (Lofrano & Brown, 2010).

While the 18th century brought about the Industrial Revolution, it was not until the 19th century that any changes were made in the way water was managed, mostly hindered by economic, social and institutional constraints.

Figure 1-2 summarizes the evolution of wastewater treatment technologies beginning form the design of cesspit, in which inlet and outlet pipes dipped below the water surface thus forming a water seal (the "fosses Mouras") and septic tanks, going through the process of activated sludge, and finishing with modern technologies such as membrane filtration or Anaerobic Ammonia Oxidation (Anammox) process. Since 1900s, the

purposes of biological wastewater treatment could be summarized as: (1) removal of organic matter in order to prevent excessive DO depletion in receiving waters, (2) removal of suspended and/or colloidal solid to avoid their accumulation in receiving waters, and (3) reduction of the concentration of pathogenic organisms released to the receiving waters (Tchobanoglous *et al.*, 2004). In this sense, the combination of aerobic, anoxic and anaerobic biological process allows to achieve desired quality of the effluent, optimizing the cost and energy requirements.

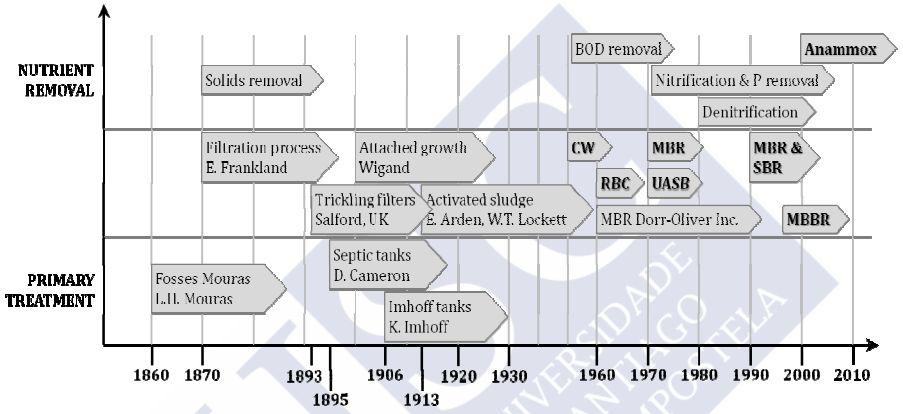

Figure 1-2 Evolution of wastewater treatment. CW – constructed wetlands; RBC – rotating biological contactor; UASB – Up-flow Anaerobic Sludge Blanket; MBR – Membrane Bioreactor; SBR – Sequencing Batch Reactor; MBBR – Moving Bed Biofilm Reactor (adapted and modified from Lofrano & Brown, 2010)

The main differences between aerobic/anoxic and anaerobic processes are collected in table 1-1.

Table 1-1 Comparison of Aerobic/anoxic and Anaerobic wastewater treatment systems (adapted from Grady et al., 1999)

	Wastewater treatment system	
Feature	**Aerobic/anoxic**	**Anaerobic**
Organic removal efficiency	High	High

Effluent quality	Excellent	Moderate to poor
Sludge production	High	Low
Nutrient requirements	High	Low
Energy requirements	High	Low to moderate
Temperature sensitivity	Low	High
Methane production	No	Yes
Nutrient removal	Possible	Negligible

1.1.1 Aerobic and anoxic processes

In aerobic/anoxic systems, heterotrophic bacteria use oxygen or nitrate as their terminal electron acceptor while using biodegradable organic matter as an energy and carbon source for growth (table 1-1). The presence of dissolved oxygen allows for the growth of autotrophic nitrifiers, which use ammonia as an electron donor, producing nitrate. In contrast, when both dissolved oxygen and nitrate are absent, alternative electron acceptors must be used. Examples of typical aerobic/anoxic reactions for wastewater treatment and corresponding microorganisms are collected in table 1-2.

Table 1-2 Classification of aerobic microorganisms by electron donor, electron acceptor, sources of cell carbon and end products (adapted from Tchobanoglous et al., 2004).

Type of bacteria	Common reaction name	Carbon source	Electron donor (substrate oxidized)	Electron acceptor	Products
Aerobic heterotrophic	Aerobic oxidation	Organic compounds	Organic compounds	O_2	CO_2, H_2O
Aerobic autotrophic	Nitrification	CO_2	NH_3^-, NO_2^-	O_2	NO_2^-, NO_3^-
Type of bacteria	**Common reaction name**	**Carbon source**	**Electron donor (substrate oxidized)**	**Electron acceptor**	**Products**
	Iron oxidation	CO_2	Fe(III)	O_2	Ferric Iron Fe(III)

	Sulphur oxidation	CO_2	H_2S, S^0, $S_2O_3^{2-}$	O_2	SO_4^{2-}
	Aerobic methane oxidation (AMO)	CH_4	CH_4	O_2	CO_2, H_2O
Facultative heterotrophic	Denitrification anoxic reaction	Organic compounds	Organic compounds	NO_2^-, NO_3^-	N_2, CO_2, H_2O
Anaerobic autotrophic	Anammox (Anaerobic	CO_2	NH_3^-,	NO_2^-	N_2, H_2O
	Anoxic methane oxidation	CH_4	CH_4	NO_2^-, NO_3^-	N_2, CO_2, H_2O

1.1.1.1 Organic matter oxidation

The Urban Waste Water Treatment Directive (Council Directive 91/271/EEC) is focused mainly on COD (chemical oxygen demand), BOD (biological oxygen demand) and TSS (total suspended solids), and require between 70 and 90% removal of each. Typical organic materials that are found in municipal wastewater include carbohydrates, fats, proteins, urea, soaps and detergents. All of these compounds contain carbon, hydrogen, and oxygen, and, in most cases, organically bound nitrogen, sulphur and phosphorus. During biochemical degradation, these elements are biologically transformed from organic forms to mineralized forms (i.e., NH_3, NH_4, NO_3, SO_4, and PO_4).

During the process of aerobic oxidation the conversion of organic matter is carried out by mixed bacterial cultures, which is represented by the following equation:

$$C_{10}H_{19}O_3N + \text{nutrients} \rightarrow CO_2 + NH_3 + C_5H_7O_2N + \text{other end products} \quad \text{eq. 1.1}$$

The formula $C_{10}H_{19}O_3N$ is used to represent the organic matter in wastewater which serves as electron donor (see also table 1-1), while the oxygen serves as electron acceptor. The term $C_5H_7O_2N$ represents new bacteria. Their endogenous respiration in shown as resulting in relatively simple end products and energy, although stable organic end products can be also formed (equation 1.2):

$$C_5H_7O_2N + 5O_2 \rightarrow 5CO_2 + 2H_2O + NH_3 + \text{energy} \qquad \text{eq. 2.2}$$

For organic matter removal pH in the range of 6.9 to 9.0 is tolerable, while optimal performance occurs near a neutral value. A dissolved oxygen concentration of 2 mg $O_2 \cdot L^{-1}$ is commonly used in activated sludge systems. Depending on the treated wastewater, care must be taken to assure that sufficient nutrients (N and P) are available for the amount of organic matter to be treated (Tchobanoglous *et al.*, 2004).

1.1.1.2 Nitrification

The nitrification process is carried out into two steps: (1) nitritation process, in which the ammonia (NH_4-N) is oxidized to nitrite (NO_2-N), and (2) nitratation process, in which nitrite is oxidized to nitrate (NO_3-N). The first stage of nitrification includes two enzymes: ammonia monooxygenase (amoA), that catalyzes the oxidation of ammonia to hydroxylamine, and hydroxylamine oxidoreductase (HAO), that catalyzes the oxidation of hydroxylamine to nitrite. Nitrification is the initial step of the biological nitrogen removal and is carried out by two phylogenetically independent groups of autotrophic aerobic bacteria, namely, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). All known chemolithoautotrophic AOB belong to the phylum *Proteobacteria*, divided in two monophyletic groups: *Gamma-* and *Betaproteobacteria*. Among AOB, *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosorobrio* are the most commonly mentioned bacteria genera, while *Nitrobacter*, *Nitrococcus*, *Nitrospira*, *Nitrospina* and *Nitrocystis* are autotrophic bacteria usually responsible for nitrite oxidation (Mtecalf & Eddy, 2004). Recently, it was discovered that ammonium oxidation can also be performed by archaea (AOA) (Konneke et al., 2005) and methanotrophic bacteria (Hanson & Hanson, 1996).

The complete process of nitrification may be represented by these simplified equations:

Ammonia oxidation reaction

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O \qquad \text{eq. 1.3}$$

Nitrite oxidation reaction

$$2NO_2^- + O_2 \rightarrow 2NO_3^-$$ eq. 1.4

The total oxidation reaction

$$NH_4^+ + 2\,O_2 \rightarrow NO_3^- + 2\,H^+ + H_2O$$ eq. 1.5

The whole metabolism of the bacteria including their growth (combination of anabolism and catabolism) is described by means of the following stoichiometric equation (eq. 1.6), where the fixation of inorganic carbon and its equilibrium are present.

$$NH_4^+ + 1.83\,O_2 + 1.98\,HCO_3^- \rightarrow 0.021\,C_5H_7O_2N + 0.98\,NO_3^- + 1.041\,H_2O + 1.88\,H_2CO_3$$ eq.1.6

1.1.1.3 Denitrification

Biological denitrification is an integral part of biological nitrogen removal and is used especially where there are concerns for eutrophication and where groundwater must be protected against elevated concentrations of NO_3-N. In the denitrification process the nitrate and/or nitrite present in the wastewater is reduced to molecular nitrogen gas by means of heterotrophic bacteria (table 1-2). The process requires the presence of organic carbon source as electron donor, e.g. acetic acid or methanol and a nitrogen oxide (nitrate or nitrite) which acts as the last electron acceptor in the respiratory chain substituting the O_2 molecule. The reduction process is carried out by subsequent steps through nitrogen compounds in different oxidation states (eq. 1.7).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$ eq. 1.7

In the biological nitrogen removal process, the electron donor can be the soluble organic matter present in the treated wastewater (eq. 1.8), the organic matter produced during endogenous decay (eq. 1.9) and/or exogenous source such as methanol or acetate (eq. 1.10).

$$C_{10}H_{19}O_3N + 10\,NO_3^- \rightarrow 5\,N_2 + 10\,CO_2 + 3\,H_2O + NH_3 + 10\,OH^-$$ eq. 1.8

$$5\,CH_3OH + 6\,NO_3^- \rightarrow 3\,N_2 + 5\,CO_2 + 7\,H_2O + 6\,OH^-$$ eq. 1.9

$$5\,CH_3COOH + 8\,NO_3^- \rightarrow 4\,N_2 + 10\,CO_2 + 6\,H_2O + 8\,OH^- \qquad \text{eq. 1.10}$$

From the stoichiometry it can be seen that the denitrification process causes an increase of the alkalinity of the medium, being 3.57 g of alkalinity (expressed as $CaCO_3$) per g of NO_3-N reduced. The oxygen equivalent of using nitrite as electron acceptor is 2.86 g O_2 per g NO_3-N.

Denitrifying ability is distributed into wide variety of bacterial groups, covering more than 50 genera (Figueroa-Leiro, 2011). However, denitrifying activity has been found also in some archaea and in fungi (Oishi and Kusuda, 2003; Cabello *et al.*, 2004). Most of denitrifiers are facultative anaerobic heterotrophs (less frequently autotrophs), which means that in oxic conditions they carry out full aerobic respiration.

1.1.2 Anaerobic processes

Anaerobic processes are those biological treatment processes that occur in the absence of oxygen and other oxidizers.

Table 1-3 Classification of anaerobic microorganisms by electron donor, electron acceptor, sources of cell carbon and end products (adapted from Tchobanoglous et al., 2004).

Type of bacteria	Common reaction name	Carbon source	Electron donor (substrate oxidized)	Electron acceptor	Products
Anaerobic heterotrophic	Acid fermentation	Organic compound	Organic compounds	Organic compounds	Volatile fatty acids
	Iron reduction	Organic compound	Organic compounds	Fe(III)	Fe(II), CO_2, H_2O
	Sulfate reduction	Organic compound	Organic compounds	SO_4	H_2S, CO_2, H_2O
	Methanogenesis	Organic compound	Volatile fatty acids	CO_2	CH_4
Methanotrophic archaea and SRB*	Anaerobic methane oxidation (AnMO)	CH_4	CH_4	SO_4^{2-}	H_2S, CO_2, H_2O

* SRB - Sulfate-reducing bacteria

1.1.2.1 *Anaerobic digestion*

Anaerobic digestion is considered an attractive method for energy-efficient treatment of a variety of wastes other than sewage sludge, including animal manure, crop waste, food processing waste, distillery waste and municipal waste. Most of the carbon in these wastes is converted to methane, while many of the nutrients are retained, making treated sludge an excellent fertilizer. Anaerobic digestion has also been considered as a method for turning biomass into energy.

Anaerobic processes have been used for the treatment of domestic and industrial wastewater for well over a century (McCarty & Smith, 1986). These processes differ from conventional aerobic treatment in that no aeration is applied. The absence of oxygen leads to controlled anaerobic conversions of organic pollutants to carbon dioxide and methane, the latter of which can be utilized as energy source.

The digestion process can be distinguished into four different phases (figure 1-2): hydrolysis, acidogenesis, acetogenesis and methanogenesis.

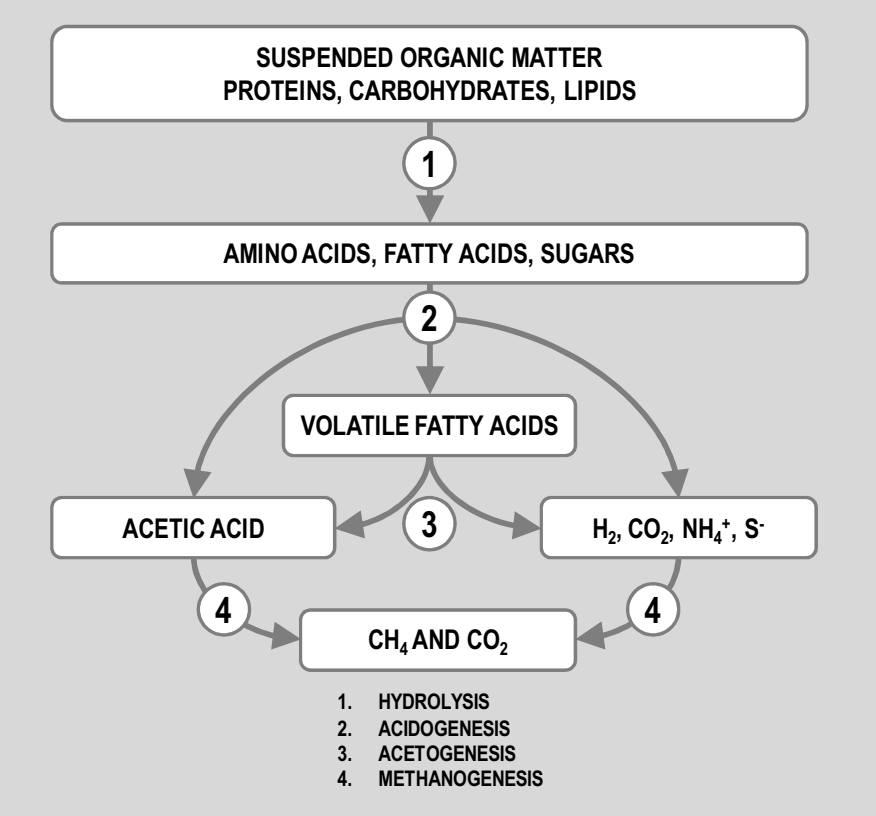

Figure 1-3 Scheme of anaerobic digestion steps

Hydrolisis

This process consist of bacterial hydrolysis of the complex organic materials (figure 1-3) in order to break down complex insoluble organic polymers and convert them into dissolved compounds with a lower molecular weight; in other words, to make them available for other bacteria (Lettinga, 1995). Proteins are converted via (poly)peptides to amino acids, carbohydrates are transformed into soluble sugars (mono- and disaccharides) and lipids are converted into long chain fatty acids and glycerine. In practice, the hydrolysis rate can be limiting for the overall rate of anaerobic digestion, especially taking into account the conversion of lipids in lower temperatures.

Acidogenesis

Facultative microorganisms (those that live either in the presence or absence of oxygen) and obligate anaerobic bacteria then convert dissolved compounds into simple organic compounds (volatile fatty acids, alcohols, lactic acid) and mineral compounds such as carbon dioxide, hydrogen, ammonia, and hydrogen sulphide gas (Lettinga, 1995). Diversity of acidogenic bacteria is important, especially taking into account accidental presence of oxygen, which otherwise might become toxic to obligate anaerobs such as methanogenic bacteria.

Acetogenesis

Acetogenic bacteria convert these resulting products of acidogenesis into the final products for methane production: acetate, hydrogen, and carbon dioxide (Lettinga, 1995). As can be seen in figure 1-3 a fraction of approximately 70% of initial COD is converted into acetic acid and the reminder of electron donor capacity is concentrated in the form of hydrogen.

Methanogenesis

Methanogenesis is often the rate limiting step of the overall anaerobic digestion process, although at lower temperatures it might be hydrolysis (van Haandel & Lettinga, 1994; Lettinga, 1995). The biochemistry of biogas production dictates that CO_2 and CH_4 must be produced simultaneously to achieve stable operation. The product CO_2/CH_4 ratio is governed by the type of substrates used. There are two main pathways (also some others that are currently regarded as exceptions and less important) of biochemical pathways, that result in biogas: (1) acetoclastic pathway, where methane is produced from acetate, and (2) hydrogenotrophic pathway, where methane is produced from the reduction of carbon dioxide by hydrogen. The stoichiometrical representation of these processes is as follows:

acetotrophic methanogenesis:

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \text{eq. 1.11}$$

hydrogenotrophic methanogenesis:

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \qquad \text{eq. 1.12}$$

Moreover, hydrogenotrophic bacteria grow faster than those utilizing acetic acid, therefore acetotrophic methanogens (acetoclastic) are usually rate limiting with respect to the conversion of complex macromolecules in wastewater into biogas.

Methane forms the main part of the biogas obtained (60-70%), together with carbon dioxide (30-40%), nitrogen gas and negligible content of hydrogen sulphide gas. In this sense the energy of 1 m^3 of biogas is equal to 1 m^3 of natural gas. The composition and enrichment of biogas depends on the degraded material and the process of anaerobic digestion.

1.1.3 Types of biomass in wastewater treatment

Biological treatment is one of the most popular types of wastewater treatment, which more or less mimics some of the natural processes found in a self-purifying receiving body. Mainly this processes consist of organic degradation and nitrogen conversion through bacterial action. These can be done by attached or suspended microorganisms, giving rise to two main families of wastewater treatment processes: the fixed film (or attached growth) processes, and the suspended growth processes, such as the activated sludge. Among these two groups there is a variety of biomass forms, which are described in figure 1-4.

1.1.3.1 Attached and/or granular growth

Fixed film processes are based on the capacity of different microorganisms to grow on surfaces. They tend to attach to solid surfaces due to various reasons:

- substrate availability,
- protection from a harmful environment, particularly at high-velocity water currents,
- interaction of physical forces like attraction, adsorption and adhesion (Senthilnathan & Ganczarczyk, 1990).

Fixed-film or attached growth systems include trickling filters, bio-towers, and rotating biological contactors, where the biomass grows on media and the wastewater passes over its surface (figure 1-4). Attached microorganisms possess some advantageous properties compared to suspended microorganisms. Often they exhibit:

- increased persistence in the system,
- faster growth rate,
- increased metabolic activity,
- greater resistance to toxicity (Senthilnathan and Ganczarczyk, 1990).

1.1.3.2 Suspended growth

Suspended-growth systems include activated sludge, where the biomass is mixed with the wastewater and can be operated in a smaller space than trickling filters that treat the same amount of water. However, fixed-film systems are more able to cope with drastic changes in the amount of biological material and can provide higher removal rates for organic material and suspended solids than suspended growth systems (EPA, 2004).

1.1.3.3 Hybrid growth

Biomass support systems consist of immersing various types of support media in an activated sludge reactor to favour the growth of fixed bacteria. The support can be fixed in the reactor or can consist of mobile media such as foam pads, small carriers, etc. (figure 1-4). These hybrid systems should allow a reduction in the aeration tank volume following the introduction of biomass support to meet a certain objective, and thus an increase in the treatment system stability and performance (Gebara, 1999). The main advantages of these systems are improved nitrification and an increase in sludge settleability (Wanner *et al.*, 1988; Muller, 1998).

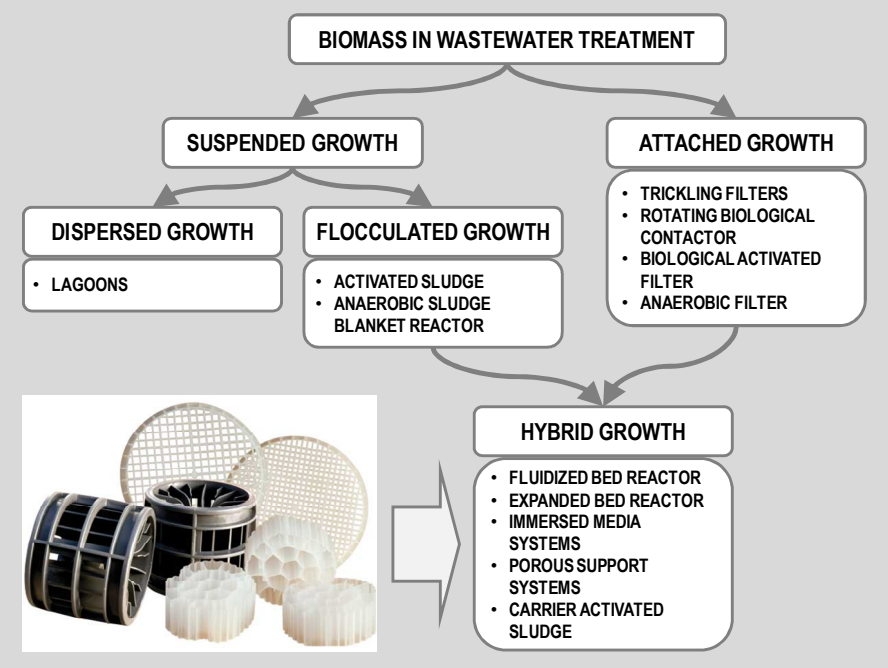

Figure 1-4 Systematics of biomass forms in wastewater treatment systems (adapted from Senthilnathan & Ganczarczyk, 1990).

1.2 UP-FLOW ANAEROBIC SLUDGE BED (UASB) REACTOR

1.2.1 Anaerobic digestion in wastewater treatment

The first application of anaerobic digestion for wastewater treatment can be dated at the end of the XIX century. M. Mouras (France) developed the system where settleable solids from sewage were "liquefied" (McCarty, 1981). Later on, a variety of anaerobic treatment systems was proposed, such as the septic tank or Imhoff tank. In both systems the wastewater flows through the upper part while the anaerobic sludge remains at the bottom of the tank, allowing the biodegradation of settleable solids. Within the years some modifications were done, such as combining the Imhoff tank with a heated digester. However, the overall efficiency of early anaerobic systems was around 30-50%, due to the low content (one-third to one-half) of settleable fraction in the influent wastewater. On the other

hand, to achieve higher removal rate of organic matter, longer contact between the substrate and the anaerobic biomass should be provided. This problem was solved by the development of so-called high-rate systems, where the biomass is retained either by immobilization of biomass or simply by applying solid-liquid separation, with the return of the separated solids to the reactor.

Different anaerobic high-rate reactors could be used for treating either industrial or municipal sewage, e.g.: Anaerobic Filter (AF), Upflow Anaerobic Sludge Blanket (UASB), Expanded Granular Sludge Blanket (EGSB) and Fluidized Bed (FB) reactor.

From all these systems the UASB and its modifications are the most popular anaerobic reactors for treating both municipal and industrial wastewaters - at present close to 80% of all full-scale anaerobic installations are sludge bed reactors. The UASB reactor (figure 1-5) was developed in the 1970s by Prof. Lettinga and his group at University of Wageningen (Netherlands). It is by far the most widely used high rate anaerobic sewage treatment system (van Haandel & Lettinga, 1994). The success of UASB reactor relies on the establishment of a dense sludge bed in the bottom of the reactor formed by accumulation of incoming suspended solids and bacterial growth (usually forming flocs and granules). These dense aggregates tend to have very good settling properties and are nor susceptible to washout from the system under proper reactor conditions (L. Seghezzo, 2004). Therefore, retention of the sludge enables good treatment performance. Natural turbulence, caused by the influent flow, and the biogas production provides good mixing, which enables good wastewater-biomass contact. What is more, in the same time high grade energy is produced as biogas.

UASB reactor consists of the following sections: sludge bed or ¨blanket¨, in which all biological processes take place and Gas-Liquid-Solid (GLS) separator, which is the most characteristic part of UASB systems (figure 1-5). It is located at the top of the reactor, which enables to recover elevated solids back to the reaction zone, while the produced bubbles of biogas are collected. Therefore UASB reactor acts as a primary clarifier, a bioreactor and a sludge digester combined.

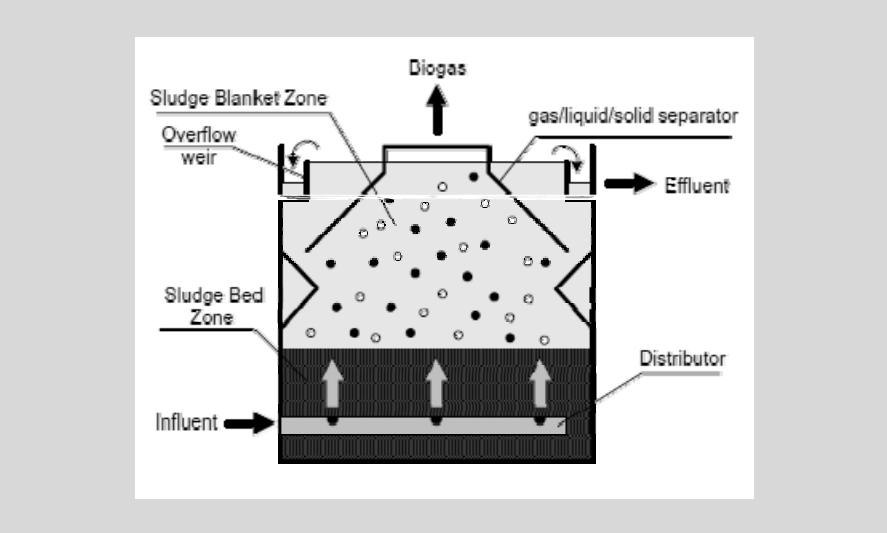

Figure 1-5 The schematic representation of UASB reactor (Copyright © 2008 - 2009 Trident Innovations Sdn. Bhd.)

1.2.2 Worldwide applications of UASB reactors

In many tropical countries, UASB reactor technology offers a simple and effective way of reducing organic pollutant emissions. Today, UASB technology for domestic wastewater treatment has been implemented in many regions, e.g. India, Pakistan, China, Columbia, Brazil, Mexico, Indonesia and Egypt (Hulshoff Pol et al., 1998). Some of these plants use the biogas that is generated from the conversion of the pollutants. The energy generated is more than sufficient for their energy demand (van Haandel & Lettinga, 1994).

Anaerobic treatment of domestic wastewater can be very interesting and cost-effective in countries were the priority in discharge control is in removal of organic pollutants. Anaerobic biomass has very low biomass yield. However, at low temperatures the growing rate of these microorganisms, and thus the capacity for degrading organic compounds diminish. For this reason, it is important to avoid any loss of anaerobic biomass with the treated water that could diminish the capacity of the anaerobic reactor for treating wastewater. Anaerobic bacteria can adapt quite easily to low temperatures, and high-rate anaerobic treatment has been achieved at psychrophilic conditions (Kato, 1994; Kato *et al.*, 1994;

Rebac *et al.*, 1995; Elmitwalli *et al.*, 1999), including some experiences with domestic sewage (Lettinga *et al.*, 1983; Grin *et al.*, 1983, 1985; de Man *et al.*, 1986, 1988; Sanz & Fdz-Polanco, 1990; van der Last & Lettinga, 1992; Wang, 1994). On the other hand, anaerobic bacteria can tolerate a wide variety of toxicants (Speece, 1983).

1.2.3 UASB reactors in low-strength wastewater treatment

The application of anaerobic technology has been mostly directed towards the treatment of medium and high strength wastewater. However, Kato *et al.* (1994) proved that high treatment performance could be obtained by UASB reactors treating low strength wastewater (above 90% of COD removal). It was also demonstrated that the temperature decrease may influence the efficiency of the system. Therefore, in this work the application of hybrid reactor, including UASB system, was studied, to ensure maximum COD elimination. Moreover, for the efficient application of UASB reactors in the treatment of municipal sewage, several factors still need to be clarified. For example, the application of granular sludge bed reactors for the treatment of wastewaters with a high content of suspended solids can affect the sludge bed development in different ways, like blocking liquid distribution systems, diluting the granular sludge bed with inactive material and favouring growth on the particle surface rather than in granular biomass (Lettinga & Hulshoff Pol, 1991). Additionally, the following question should be answered: what is the capacity of this type of reactor for removing soluble and suspended solids COD separately, what are the operating parameters with which to control the reactor, and what are the critical values of the sludge retention time, granulation, optimum height of the sludge bed and blanket, etc (Agraval *et al.*, 1997).

1.3 MEMBRANE BIOREACTORS

1.3.1 Fundamentals of membrane technology

Membrane filtration is a rapidly expanding field in water treatment. There are many different types of filters available in a wide range of pore sizes

and configurations. In addition, there are numerous possible applications for membrane filtration ranging from the removal of relatively large particulate material to the removal of dissolved compounds.

A membrane is defined as a semi-permeable thin layer of material capable of separating contaminants as a function of their physical/chemical characteristics. The degree of selectivity (which component will pass through the membrane) is determined by the size and the chemical characteristics of the membrane and the material being filtered. The main separation mechanisms are (figure 1-6):

- Filtration: microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF);
- Diffusion and solubility of some compounds into the membrane: reversed osmosis (RO), gas permeation (GP) and NF;
- Electric potential

The coarsest membrane, associated with microfiltration (MF), can reject particulate matter. The most selective membranes, associated with reverse osmosis (RO), can reject singly charged (i.e. monovalent) ions, such as sodium (Na^+) and chloride (Cl^-). Given that the hydraulic diameter of these ions is less than 1 nm, it stands to reason that the pores in an RO membrane are very small. Indeed, they are only visible using the most powerful of microscopes (Judd, 2011).

The key definitions in membrane filtration are listed below:

- Feed water is the influent water for the membrane system; if the raw water is of poor quality, some membrane systems may utilize pre-treatment steps prior to adding it to the membrane unit of the treatment plant.
- Transmembrane Pressure (TMP) is the change in the pressure of the water as it passes through the membrane.
- Specific flux is the flux of the membrane divided by the TMP of the membrane itself. The lower the specific flux, the more pressure loss through the system and the more expensive it is to operate the system. Temperature corrected specific flux for a membrane system is calculated by dividing a system´s temperature corrected flux by the membranes´ TMP.

- Permeate is the filtrate from a membrane filter. It is called permeate due to the way that the feed water permeates through the membrane.

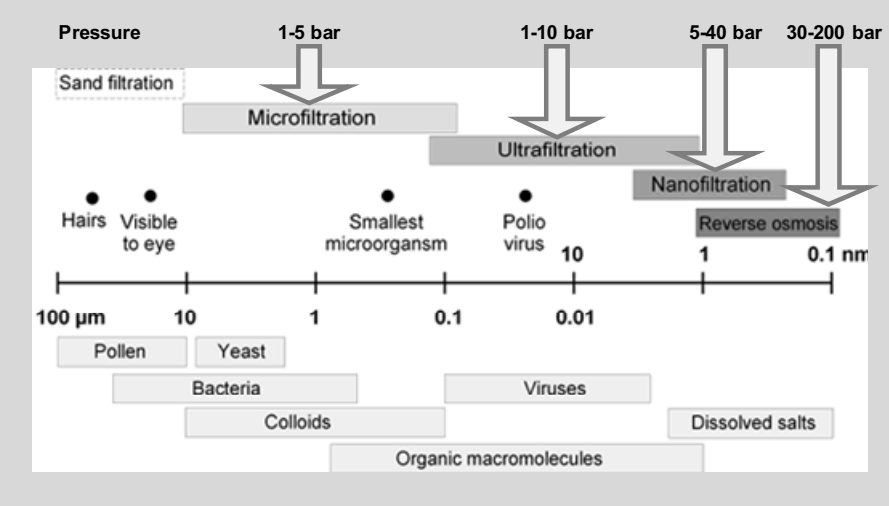

Figure 1-6 Membrane separation processes overview

1.3.2 Membrane Bioreactors

The first reference of the use of membrane systems dates back to 1969 (section 1.1). An ultrafiltration membrane was used for separating the treated waste water from the biomass in an active sludge system. The combination of the two technologies has lead to the development of the Membrane bioreactors (MBR) (Brindle & Stephenson, 1996). The majority of current biological treatment systems using membranes are modifications of the activated sludge process, where the secondary settler, used in traditional systems, has been substituted with membrane filtration units for separating the microorganisms in suspension from the water treated.

According to how the membrane is integrated with the bioreactor, two MBR process configurations can be identified (Judd, 2011): side-stream and submerged (figure 1-7). In side-stream MBRs membrane modules are placed outside the reactor, and the reactor mixed liquor circulates over a recirculation loop that contains the membrane. In submerged MBRs, the membranes are placed inside the reactor, submerged in the mixed liquor.

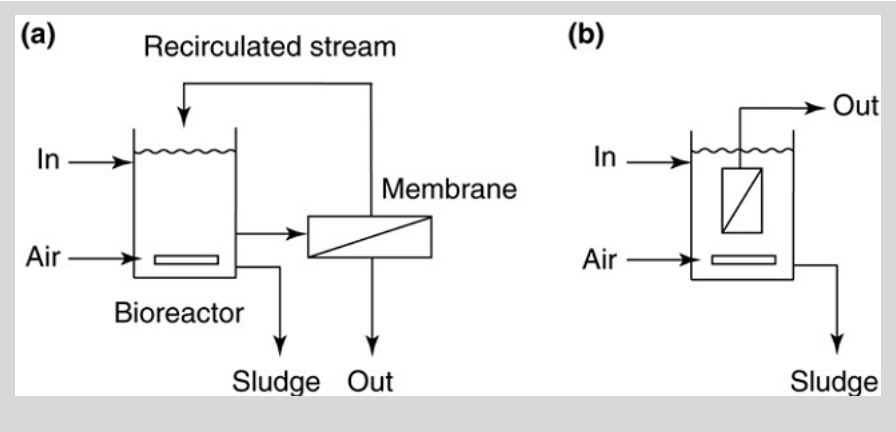

Figure 1-7 MBR process configurations: a) external (side-stream), and b) submerged (immersed). Adapted from: Judd & Jefferson, 2003.

For the side-stream configuration, a high cross-flow fluid velocity provided by a recirculation pump is designed to reduce deposition of suspended solids at the membrane surface. Although this configuration is simple and provides more direct hydrodynamic control of fouling, the energy demand is relatively high. The submerged configuration, on the other hand, relies on coarse bubble aeration to produce in-tank recirculation and suppress fouling. Although the energy demand of the submerged system can be up to two orders of magnitude lower than that of side-stream systems (van Dijk & Roncken 1997; Gander *et al.* 2000), submerged systems operate at a lower flux and so demand more membrane area.

The membranes used for this purpose are porous microfiltration or ultrafiltration membranes manufactured with organic or inorganic materials arranged on hollow fibre, plate or tubular (Figure 1-8), modules which can be placed inside or outside of the biological reactor (Günder & Krauth, 1998; Buisson *et al.*, 1998; Günder & Krauth, 1999; Ghyoot & Verstraete 2000). There are also different patents based on the use of different membrane filtration modules which can or could be used in the separation of the waste water treated in biomass in suspension bioreactors (US patents 5,558,774 and 6,303,035).

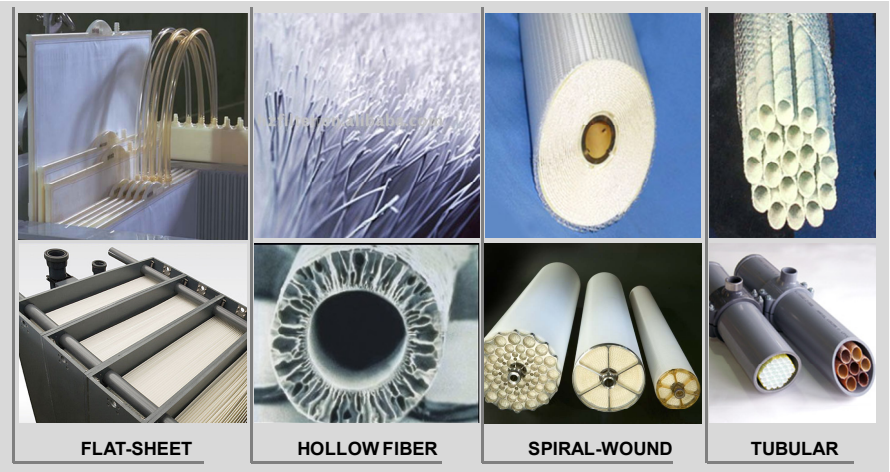

Figure 1-8 Different modules of membranes used in MBRs

Membrane bioreactors (MBR) ensure biomass retention by the use of micro or ultrafiltration modules. Since biomass is physically retained inside the reactor, there is no risk of cells washout and the conversion capacity is apparently non-dependent on the formation of biofilms or granules. Moreover, since the permeate is free of solids or cells, less post-treatment steps are required if reuse or recycle is of interest, in comparison with sludge bed technologies (Jeison & van Lier, 2008). So far, the main drawback of MBR systems is related with membrane costs, energy requirements and membrane fouling (van Dijk & Roncken, 1997; Choo *et al.*, 2000; Stowa, 2002). However, important advances have been made in the development of new types of membranes, of which the costs have been significantly reduced (Judd, 2011). In addition, research is being conducted in order to find reactor configurations and operational procedures that reduce fouling and energy consumption.

1.3.3 Critical flux

The amount of water that could be filtered by surface unit is limited, among others, by the increasing fouling tendency observed, especially when high fluxes are applied. This causes a drop of capacity of the filtration material, resulting in decrease of permeated water.

The critical flux concept was introduced over 10 year ago, and has proven useful to characterize membrane fouling in membrane applications,

especially in MBRs (Bacchin *et al.*, 2006). The critical flux was originally defined as the flux below which no fouling occurs (Field *et al.*, 1995; Howell, 1995). So, the critical flux is the value at which TMP starts to deviate from the pure water behaviour. The latter is the now called strong definition of the critical flux.

1.3.4 Fouling mechanisms and factors affecting it

Membrane fouling is definitively the main drawback of the application of MBRs for wastewater treatment (Flemming *et al.*, 1997). It is a complex phenomena resulting from interactions between the membrane material and the components in the activated sludge liquor, essentially being the exopolymers (EPS). Microbial EPS are high molecular-weight mucous secretions from microbial cells. They can play an important role for floc formation in activated sludge liquors (Sanin & Vesilind 2000; Liao *et al.* 2001). The EPS matrix is very heterogeneous, with polymeric materials arising including polysaccharides, proteins, lipids, and nucleic acids (Bura *et al.* 1998; Nielson & Jahn 1999). Since EPS provide a highly hydrated gel matrix in which microorganisms are embedded, they provide a significant barrier to permeate flow in the MBR. As soon as the membrane surface comes into contact with the biological suspension, deposition of biosolids onto it takes place leading to flux decline. Since this cake layer is largely readily removable from the membrane if an appropriate physical washing protocol is employed, it is often classified as reversible fouling. On the other hand, internal fouling caused by the adsorption of dissolved matter into the membrane pores and pore blocking is considered irreversible and is generally only removed by chemical cleaning. Also mineral substances present in the sludge play a non-negligible role in membrane fouling (Judd & Jefferson, 2003). Recent studies have quantified the fouling caused by each fraction of the mixed liquor (suspended solids, colloids and solutes), membrane itself and operation conditions (figure 1-9). Recently, many MBR studies have identified EPS as the most significant biological factor responsible for membrane fouling.

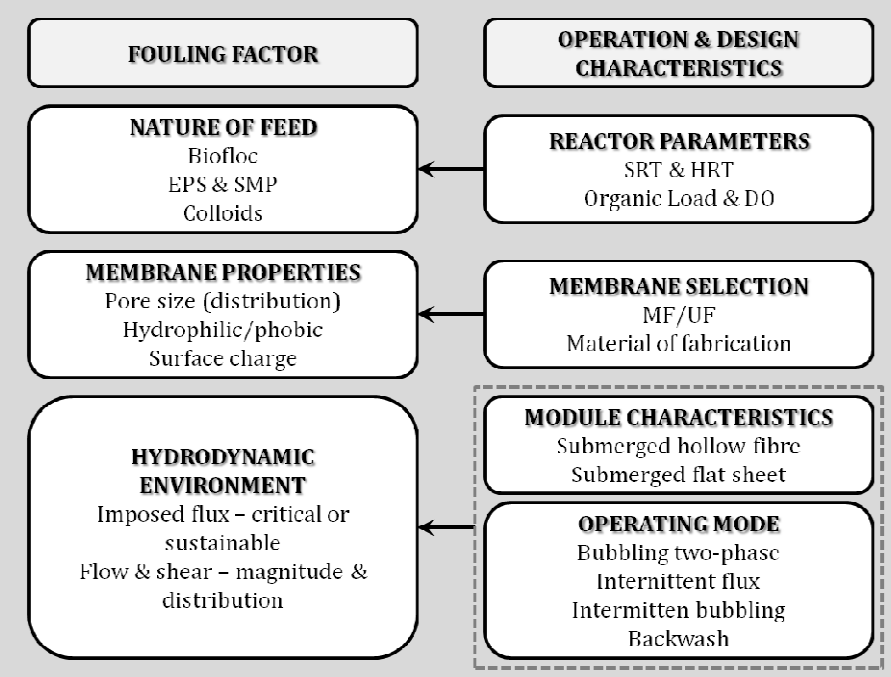

Figure 1-9 Factors fouling factors and operation and design characteristic. Adapted from Zhang et al., 2006.

In MBRs for wastewater treatment, fouling is not avoidable, and indeed the absolute absence of fouling is not at all a necessary condition for the long term operation of MBRs (Jeison, 2006; Judd, 2011). However, various techniques can be used to reduce membrane fouling:

- Feeding pre-treatment (e.g. precipitation of sparingly soluble ions or other problematic compounds that may foul the membrane).
- Reduction of applied flux (in order to operate under sub-critical conditions).
- Physical cleaning, among which the following strategies are usually applied:
    - Relaxation periods;
    - Periodic backwashing (with permeate or air in submerged membrane modules);
    - Air scouring;
    - Water rinsing/flushing.

- Chemical cleaning, among which the following strategies are the most commonly applied:
    - *In situ* maintainance chemical cleaning;
    - *Ex situ*/On site (without removing of membrane module, but emptying the filtration chamber).
- Application of other strategies, such as flux enhancers (e.g. polymers such as PermaCare® MPE™ of Nalco, MPH or MPL of Kurita; powdered activated carbon (PAC); metal salts; chitosan or starch);

1.4 ANAEROBIC MEMBRANE BIOREACTORS (AnMBR)

The anaerobic MBR (AnMBR) is a combination of an anaerobic reactor coupled with the membrane unit. Over the last decade, the adaptation of AnMBRs has made membrane reactors a promising alternative to conventional wastewater treatment. The AnMBR has the advantages of aeration-energy savings, possible biogas recovery, and lower sludge production, resulting in competitive capital and operating costs. However, negligible or no ammonia, total nitrogen, or phosphorus removal can be expected from an anaerobic MBR process (Baek & Pagilla, 2006). Up to now, several types of anaerobic bioreactor process coupled with membranes have been studied for treating different types of wastewaters.

In the case of municipal wastewater, AnMBRs could have potential application in the removal of organic carbon or biochemical oxygen demand (BOD) from the wastewater. Use of an anaerobic process was previously not feasible for BOD removal in municipal wastewater because of the poor settleability of anaerobic sludge in gravity settlers and the potential for odours (Baek & Pagilla, 2006). In the case of the anaerobic MBR, the bioreactor is a closed unit, and the solid–liquid separation is performed by a membrane filtration unit. Thus, the two drawbacks, which precluded the use of the anaerobic-sludge process for BOD removal from municipal wastewater, could be circumvented by using the anaerobic MBR.

1.4.1 AnMBR in wastewater treatment

Anaerobic process has been used successfully during the past decades. In that sense, considering the low growth rates of anaerobic bacteria, Anaerobic Membrane bioreactors technology is likely to be feasible if the system has to be operated at high biomass concentrations. Liao *et al.* (2006) reviewed the application of AnMBRs, which have been tested with synthetic, food processing, industrial, high solids content, and municipal wastewaters at laboratory, pilot, and full scale. According to these authors, the opportunity for AnMBR application to dilute wastewaters also appears strong, while application to highly concentrated soluble wastewaters is likely limited, since the efficiency obtained with the MBR is comparable with that of anaerobic system alone. On the other hand, large-scale use of AnMBRs in wastewater treatment will require a significant decrease in price of the membranes, and the type of membranes used can also significantly affect fouling in an AnMBR. Several researchers have tried to develop cost-effective membranes using low cost materials such as non-woven filters (Ho *et al.*, 2007; An *et al.*, 2009).

Anaerobic processes are often operated at mesophilic (35 °C) and thermophilic (55 °C) temperatures. However, for wastewaters with a low organic content (e.g., municipal wastewater), the methane production cannot cover the heating requirement and operation would be better under ambient temperatures (An *et al.*, 2009). Although operation at ambient temperatures appears technically feasible, SRTs need to be lengthened, e.g., two times as long as mesophilic operation may be required, and the hydrolysis of solids is also restrained due to the lower temperature compared to mesophilic or thermophilic operation (Liao *et al.*, 2006). Membrane may alleviate some of those challenges because of its high solids retention capability.

Examples of the application of various configurations of AnMBRs in municipal wastewater treatment (real and synthetic) at ambient temperatures are presented in tables 1-4 and 1-5.

Table 1-4 Examples of AnMBRs in municipal wastewater treatment

Plant	Volume	Temp	Type of wastewater	COD removal	Membrane properties				Reference
					Type/configuration	Material	Pore size	Flux	
	(L)	(°C)		(%)	(-)	(-)	(µm)	(L/m2·h)	
AnMBR	4	20	synthetic wastewater	95	-	PTFE	1	5	Ho and Sung (2010)
AnMBR	4	15	synthetic wastewater	85	-	PTFE	1	5	Ho and Sung (2010)
AMBR	-	25	synthetic municipal wastewater	96	laminated non-woven filter	PTFE	12	4 - 12	Ho et al. (2007)
AnMBR	180	25	domestic wastewater	88	external side-stream dead-end	-	0.2	3.75, 7.50 and 11.25	Lew et al. (2009)
AnMBR	18	14 - 25	domestic wastewater	60 - 95	submerged	polyethylene	0.03	5 - 10	Wen et al. (1999)
AnMBR	10	25 - 32	presettled primary effluent	25 - 70	tubular	poly vinylidene fluoride	0.1	0.1 - 10 TMP	Baek and Pagilla (2006)
AnMBR	50	37	municipal wastewater	88	cross-flow	PVDF on PE	25 nm	-	Saddoud et al. (2007)
UASB+AnMBR	9	30	synthetic wastewater	98 - 99	side-stream	-			Bailey et al. (1994)
AD*+AnMBR	12.9	15 - 20	real municipal wastewater	52 - 87	non-woven fabric	PET	0.64	5	An et al. (2009)
SAM**	20	ambient	municipal wastewater	95	flat-sheet	-	0.25	12 - 15.4	Cho et al. (2005)
SAMBR***	21.6	35	synthetic wastewater and sucrose		flat sheet (Kubota)	polyethylene	0.4	2 - 5	Spagni et al. (2010)
SAnA-MBR****	5	30	synthetic wastewater	99	capillary membrane	polyethersulphone	0.02	5 - 14	Zhang et al. (2005)

*Anaerobic Digester, **Sequencing Anoxic/anaerobic Membrane bioreactor, ***Submerged Anaerobic MBR, ****Staged Anaerobic and Aerobic MBR

Table 1-5 Examples of hybrid AnMBR in municipal wastewater treatment

Plant	Volume	Temp	Type of wastewater	COD removal	Membrane properties				Reference
					Type/configuration	Material	Pore size	Flux	
	(L)	(°C)		(%)	(-)	(-)	(μm)	(L/m2·h)	
HyMER* (Anox+An+Aer)	190	ambient	domestic wastewater	-	submerged flat panel	PES, modified polyethersulfone	0.3	21.9	Liu et al. (2008)
HyVAB** (An+Aer)+MBR	-	-	pretreated municipal wastewater	56 - 90	double-face flat sheet	PVDF	0.14	23 and 30	Phattaranawik and Leiknes (2010)
HMBR*	45	-	diluted terephthalic acid wastewater	-	dead-end flat-shaped hollow-fiber	polyethylene	0.1 - 0.2	4.5	Yang et al. (2006)
HMBR*	45	-	synthetic and diluted terephthalic acid wastewater	-	dead-end flat-shaped hollow-fiber	polyethylene	0.1 - 0.2	3.0, 4.5 and 6.0	Yang et al. (2009)

*Hybrid MBR, **Hybrid Vertical Anaerobic sludge–aerobic Biofilm reactor

Hu and Stuckey (2006) achieved 90% soluble COD removal efficiency at a 3 h HRT with an inlet organic concentration of 460 mg/L, using two Anaerobic Membrane Bioreactors (AnMBR) with both, flat sheet and hollow fibre modules. Ho and Sung (2010) investigated the performance of a cross-flow AnMBR treating synthetic municipal wastewater. They achieved more than 95% Chemical Oxygen Demand (COD) removal, with permeate concentration lower than 40 mg/L. Hu *et al.* (2009) proposed a hybrid reactor, based on the installation of aerating membrane into an anaerobic baffled reactor (HMABR). The results demonstrated that after the installation of membrane module, total Volatile Fatty Acids (VFA) and COD concentration in the HMABR effluent were decreased by 68.1 and 59.5% respectively, with increased nitrogen removal efficiency by 83.5%, at influent COD concentration of 1600 mg/L and NH^4–N concentration of 80 mg/L. This demonstrates that the AnMBR can treat low-strength wastewater with similar treatment performance as aerobic MBRs.

One of the main drawbacks of using AnMBR is related with membrane fouling and the maximum operating flux that can be achieved. Feasible flux has a strong influence on both the capital and operation costs of the process. Most of the authors working with AnMBRs reported fluxes in the range of 5–15 $L \cdot m^{-2} \cdot h^{-1}$ at temperatures above 30 °C (Zhang *et al.*, 2005; Saddoud *et al.*, 2007; Trzcinski & Stuckey, 2009). Jeison and van Lier (2006) obtained critical flux values in the range 16–23 $L \cdot m^{-2} \cdot h^{-1}$ under thermophilic (30 °C), and 5–21 $L \cdot m^{-2} \cdot h^{-1}$ under mesophilic (55 °C) conditions. In the case of domestic wastewater treated at ambient temperatures, operating fluxes are significantly lower. Lew *et al.* (2009) reported 11.25 $L \cdot m^{-2} \cdot h^{-1}$ at 25 °C, while Wen *et al.* (1999), operating a laboratory scale anaerobic bioreactor coupled with a membrane filtration worked with flux of 5 $L \cdot m^{-2} \cdot h^{-1}$. Similar results were obtained by Ho and Sung (2010), who operated with flux set on 5 $L \cdot m^{-2} \cdot h^{-1}$ and the temperature of 15 and 20 °C. Moreover, Spagni *et al.* (2010) demonstrated that the applicable fluxes obtained in AnMBR ranged between 2 and 5 $L \cdot m^{-2} \cdot h^{-1}$ depending strongly on operational conditions and rapid membrane fouling was usually observed. Therefore, the fluxes obtained in AnMBR are lower than those observed in aerobic MBR, that are in the range between 20 and 30 $L \cdot m^{-2} \cdot h^{-1}$ (Judd, 2002; Wen *et al.*, 2004).

Limited number of studies was devoted to the AnMBRs performance (Jeison & van Lier, 2006, 2007, 2008) focusing, for example, on the factors affecting operational flux (e.g. cake layer formation). It was proved that cake

formation is the limiting factor for the application of AnMBRs. Moreover, biomass concentration and temperature showed to be an important factor determining the critical flux (e.g. under mesophilic conditions biomass concentration affects critical flux linearly, while termophilic conditions reduced drastically that effect). Even though the cake formation showed to be mainly reversible in short-term experiments, particle deposition proceeded fast once critical flux was reached. However, side-stream membrane filtration would improve the performance of such reactors, achieving 3 times higher fluxes (Jeison & van Lier, 2008).

1.4.2 Parameters governing permeate flux

Fouling in anaerobic membrane bioreactors (AnMBRs) represents one of the most significant barriers to their more widespread implementation for both municipal and industrial wastewater treatment. According to Berube *et al.* (2006) the optimal membrane system for an AnMBR would consist of an organic, hydrophilic, and negatively charged membrane with pore size of approximately 0.1 μm. Membranes with a larger nominal pore size may foul more readily as a result of clogging by macro-colloids, which can completely block the entrance of the pores, while those with a smaller nominal pore size are expected to foul more readily as a result of clogging by micro-colloids, which can adsorb to the surface of the pores. The size of the biosolid particles and concentration of soluble microbial products in the mixed liquor affect permeate flux. Higher concentration of microbial products may be present in the mixed liquor when an AnMBR is operated with relatively low operating temperatures. Consequently, higher temperatures can have beneficial effects on permeate flux by reducing the concentration of microbial products and viscosity of the permeate. On the other hand, there is always a possibility of additional treatment, such as application of Powdered Activated Carbon (PAC).

1.5 MBR AS A POST-TREATMENT OF UASB EFFLUENTS

1.5.1 Main post-treatment options for UASB effluent currently in use

In spite of their great advantages, anaerobic reactors hardly produce effluents that comply with usual discharge standards established by environmental agencies. Therefore, the effluents from anaerobic reactors usually require a post-treatment step as a means to adapt the treated effluent to the requirements of the environmental legislation and protect the receiving water bodies (Chernicharo, 2006). The main role of the post-treatment is to complete the removal of organic matter, as well as to remove constituents little affected by the anaerobic treatment, such as nutrients (N and P) and pathogenic organisms (viruses, bacteria, protozoans and helminths).

In comparison with a conventional treatment plant composed of primary sedimentation tank followed by the aerobic biological treatment, UASB reactor followed by aerobic biological treatment presents some key advantages (Chernicharo, 2006).

- the primary sedimentation tanks, sludge thickeners and anaerobic digesters, can be replaced with UASB reactors. In this configuration the UASB reactors acts as the aerobic sludge thickener and digester;
- power consumption for aeration in AS systems preceded by UASB reactors will be substantially lower compared to CAS;
- thanks to the lower sludge production in anaerobic systems and to their better dewaterability, excessive sludge generated in anaerobic/aerobic systems will be much lower than that from aerobic systems alone;
- the construction and operational costs of a treatment plant with UASB reactor followed by aerobic biological treatment are lower than in the case of CAS, reaching 20–50% of investment savings and 40–50% savings on operation and maintenance costs.

The main post-treatment options for UASB wastewater treatment currently in use worldwide, with their most important characteristics, are collected in figure 1-10.

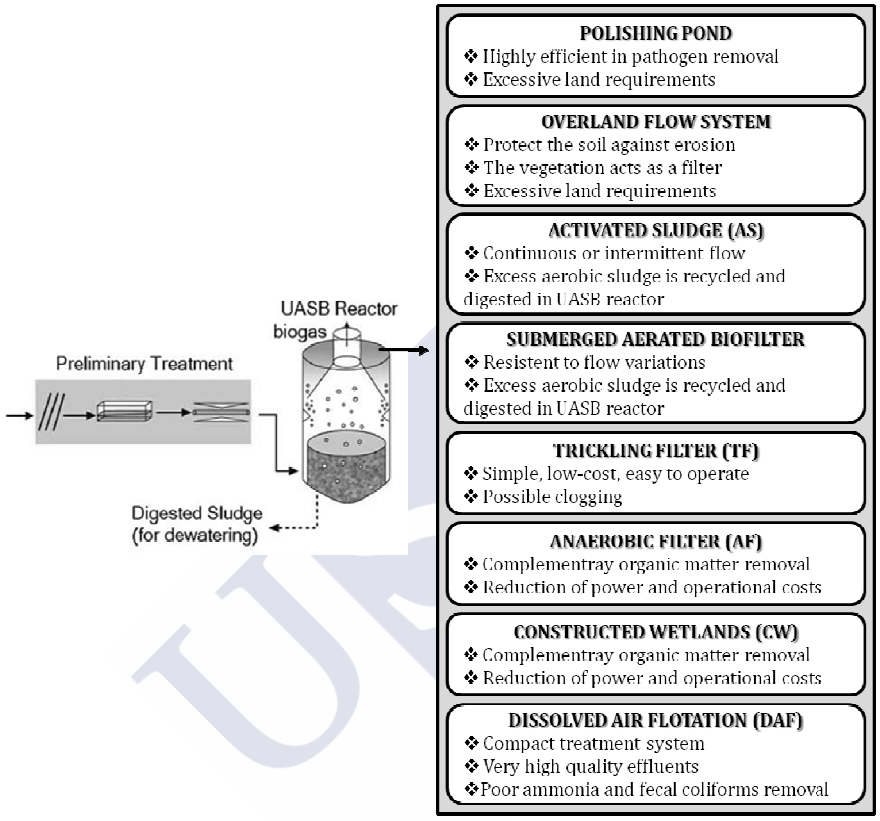

Figure 1-10 The main post-treatment options for UASB wastewater treatment with their most important characteristics. Adapted from: Chernicharo, 2006.

Table 1-6 presents a quantitative comparison of the main systems applied as a UASB post-treatment, focusing on average effluent characteristics such as BOD, COD and nutrients concentration.

Table 1-6 Average effluent concentrations and typical removal efficiencies of organic matter and nutrients in domestic wastewater. Adapted from: Chernicharo, 2006.

System	Average quality of the effluent and average removal efficiency					
	BOD_5 (mg/L) [%]	COD (mg/L) [%]	TSS (mg/L) [%]	NH_4-N (mg/L) [%]	Total N (mg/L) [%]	Total P (mg/L) [%]
UASB reactor	70-100 [60-75]	180-270 [55-70]	60-100 [65-80]	>15 [<50]	>20 [<60]	>4 [<35]
UASB + polishing ponds	40-70 [77-87]	100-180 [70-83]	50-80 [73-83]	10-15 [50-65]	15-20 [50-65]	<4 [>50]
UASB + overland flow	30-70 [77-90]	90-180 [70-85]	20-60 [80-93]	10-20 [35-65]	>15 [<65]	>4 [<35]
UASB + activated sludge	20-50 [83-93]	60-150 [75-88]	20-40 [87-93]	5-15 [50-85]	>20 [<60]	>4 [<35]
UASB + submerged aerated biofilter	20-50 [83-93]	60-150 [75-88]	20-40 [87-93]	50-15 [50-85]	>20 [<60]	>4 [<35]
UASB + high rate trickling filter	20-60 [80-93]	70-180 [73-88]	20-40 [87-93]	>15 [<50]	>20 [<60]	>4 [<35]
UASB + anaerobic filter	40-80 [75-87]	100-200 [70-80]	30-60 [80-90]	>15 [<50]	>20 [<60]	>4 [<35]
UASB + dissolved air flotation	20-50 [83-93]	60-100 [83-90]	10-30 [90-97]	>20 [<30]	>30 [<30]	1-2 [75-88]

1.5.2 Membrane bioreactors as a post-treatment of UASB effluents

As discussed in previous sections, membrane bioreactors (MBRs) have been receiving increasing attention due to their capability of producing high-quality effluents that comply with most water reclamation standards. Chong *et al.* (2012) reviewing performance enhancements of UASB reactors for domestic sludge treatment, highlighted the potential of MBR technology as such application. According to Buntner *et al.* (2011; 2013, submitted) and Sánchez *et al.* (2013) an MBR, used as a UASB post-treatment in pilot plant studies, have been shown to achieve excellent COD removal efficiencies, high membrane fluxes and biogas production rich in methane. Herrera-Robledo *et al.* (2011) also showed the effectiveness of a UASB-MBR in producing an effluent with COD, SS and pathogen contents that met Mexican municipal wastewater reclamation criteria. However, in this work, the MBR was used as a tertiary ultrfiltration unit, and membrane module

was operated only 8/24 h, with cleaning procedure applied after every working cycle. Finally, An *et al*. (2008, 2009) presented a study of a UASB-MBR system with simultaneous nitrogen removal and methanogenesis when treating low-strength synthetic wastewater enriched with organic carbon and ammonium chloride. The authors also investigated the influence of the sludge recirculation ratio on the TN removal efficiency. It was observed, that this removal increased from 48.1% to 82.3% when the sludge recirculation ratio was increased from 50% to 800%, via shortcut biological nitrogen removal process during with the ammonium nitrogen was oxidized to the form of nitrite instead of nitrate, consuming less TOC (or COD) (An *et al*., 2008, 2009). A recycling ratio of 400% was recommended to obtain high carbon and nitrogen removal efficiencies over 56.3% of methane in the biogas produced (An *et al*., 2009). However, these authors operated at mezophilic conditions. Eventually, more studies are on-going to develop cost-effective membranes, membrane-fouling control and optimisation, including thorough investigations of the fouling mechanisms, as well as operational optimisation and design.

The present study is a step forward into the development of combined UASB-MBR technology, since it is an attempt to resolve problems related to the main drawbacks of such a treatment, related with the need of post-treatment of anaerobic effluents, the operation of anaerobic MBR (fouling, low membrane fluxes) and aerobic MBR (high energy consumption and sludge production).

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Chapter 2

Materials & Methods

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

SUMMARY

In this chapter, the analytical methods used in this Thesis are described. In order to characterize liquid phase, the conventional parameters such as COD, ammonia, nitrate, nitrate, phosphates, total nitrogen were measured. Additionally, pH, temperature, dissolved oxygen, and alkalinity were measured, to check if the system was working at optimal conditions.

On the other hand, biomass characterisation present in the different stages of experimental setup was performed. Total and Volatile Suspended Solids (TSS and VSS) were determined following Standard Methods (APHA-AWWA-WPCF, 1999). Biomass was characterized also by means of parameters such as sludge volumetric index, granules particle size distribution and techniques of digital image analysis, electronic microscopy and stereomicroscope. Identification of different populations present in the biomass samples was carried out by Fluorescent In Situ Hybridization (FISH). To obtain the distribution of bacteria in the combined UASB-MBR system, the FISH technique was applied to granular, biofilm and suspended biomass. Confocal laser microscopy was used to obtain images of the bacteria with questionable results obtained by conventional microscopy.

Finally, the methodology applied for membrane filtration control and monitoring is described.

The specific analytical methods used in a single part of the work are described in the corresponding chapter, as well as the corresponding experimental set-ups.

2.1 COMBINED UASB-MBR SYSTEM

For the purpose of this Thesis (Chapter 3, Chapter 4, Chapter 6 and Chapter 7) a combined UASB-MBR system was designed and constructed (figure 2-1). The system consisted of three chambers, connected in series: 1) methanogenic UASB reactor, 2) aerobic/anoxic chamber with biofilm growing on plastic support and in suspension, and 3) membrane filtration chamber.

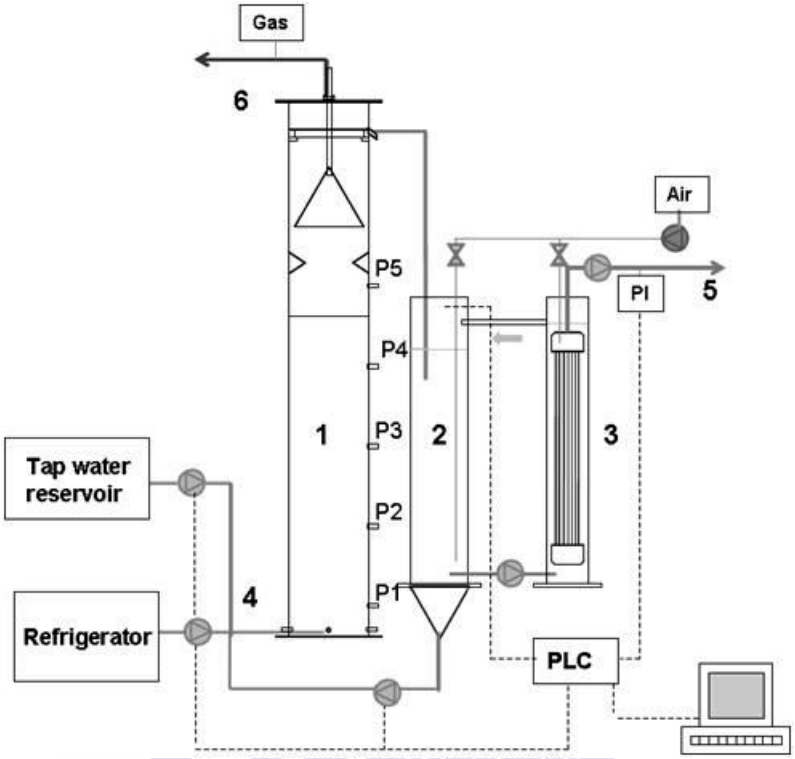

Figure 2-1 Schematic diagram of the combined UASB-MBR system. (1) UASB stage, (2) Aerobica/anoxic chamber with biofilm and suspended biomass, (3) Membrane filtration chamber, (4) Feeding and recirculation, (5) Permeate (backwashing), (6) Biogas outlet. P1, P2, P3, P4 and P5 corresponds to the sampling ports of UASB stage.

The volume of each chamber, both total and effective, is given in table 2-1.

Table 2-1 Total and effective volumes of the bioreactor.

Volume (L)	UASB stage	Aerobic/anoxic chamber	Membrane filtration chamber
Total	141	42	22
Effective	133	37	20

The UASB reactor was seeded with 50 L of anaerobic biomass (figure 2-2a) with concentration of around 27 g VSS·L^{-1}, originating from the Internal Circulation (IC) anaerobic reactor of a brewery industry located in Galicia (Spain), whereas 5 L of biomass from a MBR pilot plant treating urban wastewater was employed as an aerobic biomass inoculum.

Reactor was fed using synthetic wastewater composed of diluted skimmed milk, $NaHCO_3$ and trace elements. During the first operation days, some other chemicals were added to the feeding (NH_4Cl, Na_2HPO_4, KH_2PO_4). The concentration of each compound is given in table 2.2. The feeding was primarily stored at room temperature, however, due to the rapid decomposition of the milk added, from day 141 a refrigerator was employed to ensure the homogenous feeding of the system.

Table 2-2 Feeding composition.

Compound	Concentration (mg/L)	Compound	Concentration (g/d)
NH_4Cl	150*	$FeCl_3 \cdot 6H2O$	0.72
Na_2HPO_4	25*	H_3BO_3	0.072
KH_2PO_4	12*	$CuSO_4 \cdot 5H_2O$	0.0144
$NaHCO_3$	200	KI	0.0144
		$ZnSO_4 \cdot 7H_2O$	0.0576
* Eliminated from the feeding after 40 days of operation (Chapter 3)		$CoCl_2 \cdot 6H_2O$	0.072
		$MnCl_2 \cdot 4H_2O$	0.0576

The effluent of the UASB reactor was led to the aerobic biofilm stage which consists of a 36 L aerobic bioreactor with 18.5 L (50 % of the effective volume) of Kaldnes K3 support (figure 2-2b). Finally, the filtration stage was carried out in a 20 L aerobic chamber, where a membrane module Zenon ZW10 (figure 2-2c) with a surface area of 0.9 m^2 was employed. This module consists of PVDF hollow-fibre membrane, with a pore size of 0.04 µm. The membrane was operated in cycles of 7.5 min with a permeation period of 7 min and a backwashing period of 0.5 min. The filtration chamber was aerated in order to minimize membrane fouling. The operation of the system was controlled by a PLC (Siemens S7-200) connected to a computer. Trans-membrane Pressure (TMP) data was measured with an analogue pressure sensor (Efector500 PN-2009) and collected in the PC via an analogue PLC module Siemens EM 235.

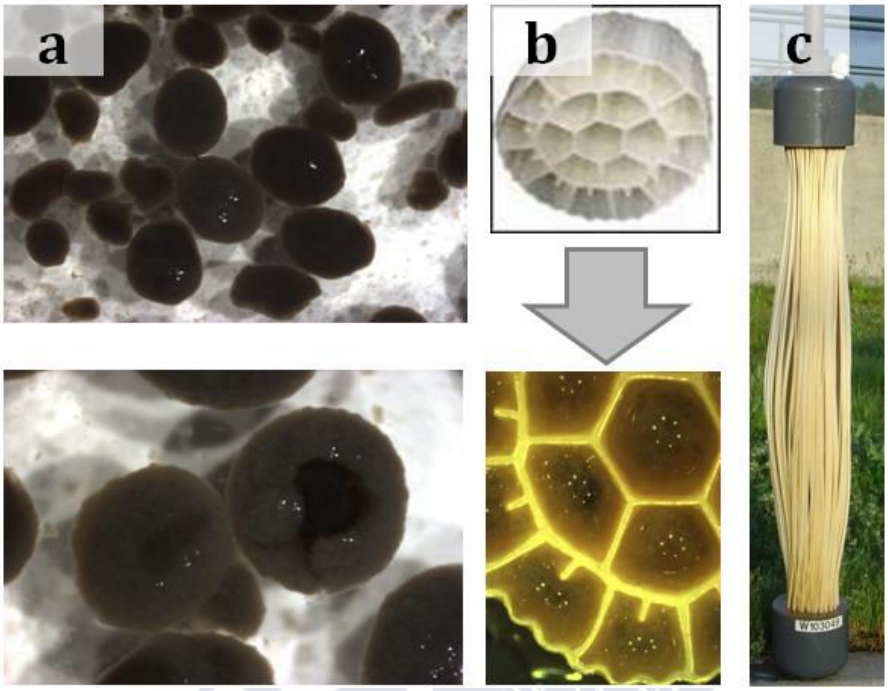

Figure 2-2 (a) Anaerobic granules inoculum taken from the IC reactor; (b) Kaldnes K3 support without (up) and with biomass (down); (c) membrane module ZW-10, Zenon.

The study was performed during 3 operating campaigns: (1) start-up and low-strength wastewater treatment, (2) dairy wastewater treatment, (3) denitrification with dissolved methane. The details of the operating campaigns are detailed in table 2-3.

Table 2-3 Details of three operating campaigns

Periods	Campaign 1 Days	Campaign 2 Days	Campaign 3 Days
I	0 – 77	0 – 32	0 – 84
II	78 – 114	33 – 194	85 – 120
III	115 – 175	195 – 292	121 – 150
IV	176 – 220	-	151 – 169
V	-	-	170 – 198
Vi	-	-	199 – 233

2.2 LIQUID PHASE ANALYTICAL METHODS

In this section, the methods used for the determination of the conventional parameters of wastewater and sludge are described. For soluble fraction analysis, the samples were previously filtered with a pore size of 0.45 µm in order to remove suspended solids.

2.2.1 Carbon compounds

2.2.1.1 Chemical Oxygen Demand (COD)

Chemical Oxygen Demand (COD) is defined as the amount a specified oxidant (potassium dichromate) required to oxidise the organic matter present in a liquid sample (wastewater) in an acid medium. A catalyst (silver sulphate) is used to improve the oxidation of some organic compounds. After digestion, the remaining unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed, being the amount of oxidable matter calculated in terms of oxygen equivalent.

The total and soluble Chemical Oxygen Demand (t-COD and s-COD) were determined following the method described by Soto *et al.* (1989), which is a modification from the method 5220C of the Standard Methods for the *Examination of Water and Wastewater* (APHA-AWWA-WPCF, 1999). The difference between total and soluble COD is that t-COD is determined using the raw sample, while for s-COD determination, the sample is previously filtered through nitrocellulose fibre filters (Whatman, GFC) with a pore size of 0.45 µm.

Reagents preparation

a). Standard potassium dichromate digestion solution:

- "concentrated" : 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ are dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 are added. The solution is cooled to room temperature and, finally, diluted to 1000 mL.
- "diluted" : 2.44 g of $K_2Cr_2O_7$ and 17 g of $HgSO_4$ are dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 are

added. The solution is cooled to room temperature and, finally, diluted to 1000 mL.

b). Sulphuric acid reagent: 10.7 g of Ag_2SO_4 are added to 1 L of concentrated H_2SO_4. The solution is used after 2 days of preparation.

c). Ferroin indicator solution: 1.485 g of $C_{18}H_8N_2{\cdot}H_2O$ (phenanthroline monohydrate) and 0.695 g of $SO_4Fe{\cdot}7H_2O$ are dissolved in 100 mL of distilled water.

d). Standard potassium dichromate solution 0.05 N. 1.226 g of $K_2Cr_2O_7$, previously dried at 105 ºC for 2 hours, are dissolved in 500 mL of distilled water.

e). Standard ferrous ammonium sulphate titrant (FAS):

- "concentrated" : 0.035*N*: 13.72 g of $Fe(NH)_4(SO)_2{\cdot}6H_2O$ are dissolved in distilled water. Then, 40 ml of concentrated H_2SO_4 are added and, finally, the solution is cooled and diluted to 1000 mL.
- "diluted" : 0.016*N*: 6.28 g of $Fe(NH)_4(SO)_2{\cdot}6H_2O$ are dissolved in distilled water. Then, 20 ml of concentrated H_2SO_4 are added and, finally, the solution is cooled and diluted to 1000 mL.

Determination procedure

This procedure is applicable to samples with COD concentrations between 90-900 $mg{\cdot}L^{-1}$ (referred as "concentrated") or COD<90 $mg{\cdot}L^{-1}$ (referred as "diluted"). Place 2.5 mL of sample in 10-mL Pyrex tubes. Add 1.5 mL of digestion solution (concentrated or diluted, respectively) and 3.5 mL of sulphuric acid reagent slowly on the wall of the tube slightly inclined (to avoid mixing). A blank sample using distilled water is prepared in the same way. This blank acts as "reference", representing the COD of the distilled water. After being sealed with Teflon and tightly capped, the tubes are finally mixed completely and placed in the block digester (HACH 16500-100) preheated to 150ºC. The duration of the digestion period is 2 h.

After digestion, the tubes are cooled to room temperature. Then, the content of the tubes is transferred to a beaker and, once added 1-2 drops of ferroin indicator, the solution is titrated under rapid stirring with standard FAS (concentrated or diluted, respectively). The FAS solution is standardised daily as follows: Put 5 mL of distilled water into a small beaker.

Add 3.5 mL of sulphuric acid reagent. Cool to room temperature and add 5 mL of standard potassium dichromate solution (0.05 N). Add 1-2 drops of ferroin indicator and titrate with FAS titrant. The end-point is a sharp colour change from blue-green to reddish brown. Molarity of FAS solution is calculated with the following equation (2-1):

$$M_{fas} = \frac{5 \cdot 0.05}{V_{fas}} \qquad \text{eq. 2-1}$$

where:

M_{fas}: molarity of FAS (mol·L^{-1}), and

V_{fas}: volume of FAS consumed in the titration (mL).

The COD is calculated with the following equation (2-2):

$$COD = \frac{(A - B) \cdot M_{fas} \cdot 8000}{V} \qquad \text{eq. 2-2}$$

where:

COD: Chemical Oxygen Demand (mg O_2·L^{-1}),

A: mL of FAS consumed by the blank,

B: mL of FAS consumed by the sample,

M_{fas}: molarity of FAS (mol·L^{-1}), and

8000: milliequivalent weight of oxygen x 1000 mL·L^{-1}.

Interferences

Oxidation of most organic compounds is 95 to 100% of the theoretical value. Chlorine, bromide, iodine, and any other reagent that reacts with silver ion and inhibits the catalytic activity of silver can interfere. For more detailed information see method 5220A of Standard Methods.

2.2.1.2 Total Dissolved Organic Carbon (TOC) and Inorganic Carbon (IC)

Organic carbon in liquid samples may include a variety of organic compounds in different oxidation states. Total Organic Carbon (TOC) is a more convenient and direct expression of total organic content than COD,

but does not provide the same information. Unlike COD, TOC is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen and hydrogen, and inorganics that can contribute to the oxygen demand measured by COD (APHA-AWWA-WPCF, 1999). To determine the quantity of organically bound carbon, the organic molecules must be broken down and converted to a single carbon molecular form that can be measured quantitatively. The TOC concentration was determined by a Shimadzu analyzer (TOC-5000) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. The instrument is connected to an automated sampler (Shimadzu, ASI-5000-S). The TC concentrations are determined from the amount of CO_2 produced during the combustion of the sample at 680 °C, using platinum immobilised over alumina spheres as catalyst. The IC concentrations are obtained from the CO_2 produced in the chemical decomposition of the sample with H_3PO_4 (25%) at room temperature. The CO_2 produced is optically measured with a nondispersive infrared analyzer (NDIR) after being cooled and dried. High purity air is used as carrier gas with a flow of 150 mL min^{-1}. A curve comprising 4 calibration points in the range of 0 to 1 g C L^{-1}, using potassium phthalate as standard for TC and a mixture of sodium carbonate and bicarbonate (Na_2CO_3/$NaHCO_3$, 3:4 w/w) for IC, is used for the quantification (Fig. 2-3).

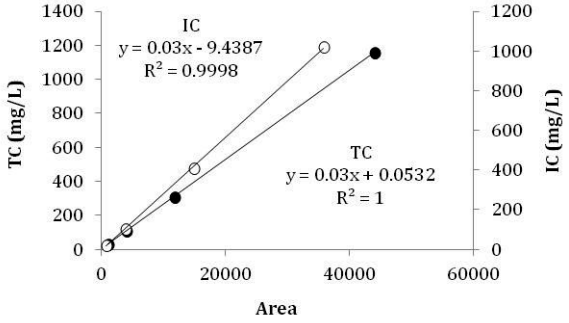

Figure 2-3 Example of calibration curve to determine TC and IC concentrations.

2.2.1.3 Volatile Fatty Acids (VFA)

Volatile Fatty Acids (VFA), acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric, are intermediate products of the anaerobic digestion. A VFA

accumulation reflects a kinetic disequilibrium between the acids producers and the acids consumers (Switzembaum *et al.*, 1990) and it is an indicator of process destabilization.

VFA are determined by gas chromatography (HP, 5890A) equipped with a Flame Ionization Detector (FID) and an automatic injector (HP, 7673A). The determination is performed in a glass column (3 m long and 2 mm of internal diameter) filled with Chromosorb WAW (mesh 100/120) impregnated with NPGA (25%) and H_3PO_4 (2%). The column, injector and detector temperatures are 105, 260 and 280°C, respectively. Gas N_2, previously saturated with formic acid before entering into the injector, is used as carrier gas with a flow of 24 mL/min. Air and H_2 are used as auxiliary gases with flows of 400 and 30 mL/min, respectively. VFA, after being separated in the column according to their molecular weights, are burnt in a H_2-air flame and finally measured in the FID at 280°C. The quantification of the sample is made with a 6-8 point calibration curve for each acid in the range of 0-1 g/L, using pivalic acid as internal standard (Figure 2-4).

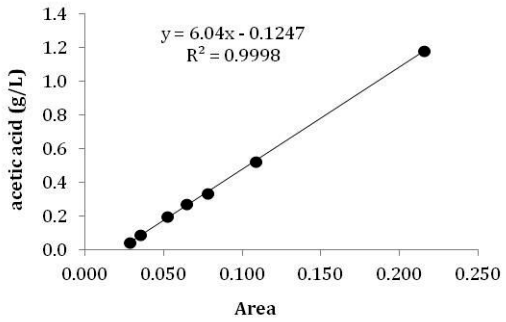

Figure 2-4 Example of calibration curve for the acetic acid.

2.2.2 Nitrogen compounds

2.2.2.1 Ammonium nitrogen

Ammonium nitrogen is measured by a colorimetric method (Wheatherburn, 1967), based on the reaction of NH_3 with HClO and phenol, forming a

strong-blue compound (indophenol) which can be colorimetrically determined using a spectrophotometer (Cecil CE 7200) at 635 nm.

Reagents preparation

a). Solution 1: Phenol-nitroprusside: 15 g of phenol and 0.05 g of sodium nitroprusside are added to 250 mL of buffer solution. The buffer solution was prepared adding 30 g of $Na_3PO_4 \cdot 12\ H_2O$, 30 g $Na_3C_6H_5O_7 \cdot 2H_2O$ and 3 g EDTA per litre, adjusted to pH 12.

b). Solution 2: Hipochloride: 15 mL of commercial bleach are mixed with 200 mL of NaOH 1 N and filled up to 500 mL with distilled water.

Determination procedure

Place 2.5 mL of sample (diluted if necessary to get a maximum concentration of 1 mg NH_4^+-N·L^{-1}), previously centrifuged of filtered through 0.45 μm nitrocellulose filter, and add, 1.0 and 1.5 mL of solution 1 and 2, respectively. After waiting 45 min (the time necessary for the reaction described before to complete) at room temperature, the concentration of NH_4^+-N is measured in a spectrophotometer at 635 nm. The quantification is done with a 5-7 points calibration curve in the range of 0-1 mg NH_4^+-N·L^{-1}, using NH_4Cl as standard (Fig. 2-5).

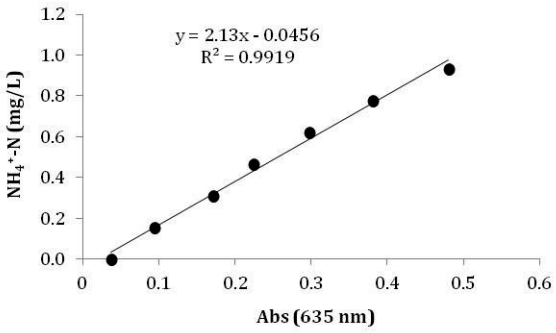

Figure 2-5 Example of calibration curve for ammonium concentration determination.

Free ammonia was calculated according to the method by Anthonisen *et al.* (1976).

2.2.2.2 *Nitrite*

Nitrite concentration in wastewater is determined following the method 4500-NO_2^--B described in *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 1999). This method is suitable for for concentrations of 5 to 1000 μg NO_2^--N·L^{-1}.

Nitrite is determined through the formation of a reddish purple azo dye produced at pH 2.0-2.5 by coupling diazotized sulphanilamide with N-(1-napththyl)-ethylenediamine dihydrochloride (NED dihydrochloride).

Reagents preparation

a). Sulphanilamide: 10 g of sulphanilamide are dissolved in 100 mL of concentrated HCl and 600 mL of distilled water. After cooling, the volume is filled up to 1 L with distilled water.

b). NED: 0.5 g of NED are dissolved in 500 mL of distilled water.

Determination procedure

To 5 mL of sample (diluted if necessary to fit the concentration range of the method), previously filtrated through 0.45 μm nitrocellulose filter, 0.1 mL of each solution (sulphanilamide and NED) are added. After waiting 20 min for colour stabilisation, the sample is measured in a spectrophotometer (Cecil CE 7200) at 543 nm, providing a light path of 1 cm or longer. The quantification is done with 6-8 points calibration curve in the range of 0-0.30 mg NO_2^--N·L^{-1}, using $NaNO_2$ as standard (Figure 2-6).

Interferences

NCl_3 impart a false red color when color reagent is added. The following ions interfere because of precipitation and should be avoided: Sb^{3+}, Au^{3+}, Bi^{3+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^{2+}, chloroplatinate ($PtCl_6^{2-}$), and metavanadate (VO_3^{2-}). For further details see method 4500-NO_2^- B of Standard Methods.

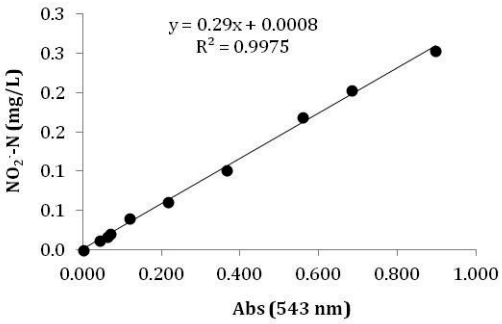

Figure 2-6 Example of calibration curve used for nitrite determination.

2.2.2.3 Nitrate

Nitrate concentration in wastewater is determined following the method 4500-NO_3^--B described in Standard Methods for the *Examination of Water and Wastewater* (APHA-AWWA-WPCF, 1999).

Measurement of UV absorption at 220 nm enables rapid determination of NO_3^- ions. Because dissolved organic matter also may absorb at 220 nm and NO_3^- does not absorb at 275 nm, a second measurement at 275 nm is used to correct the NO_3^- value. Acidification with 1*N* HCl is designed to prevent interference from hydroxide or carbonate concentrations up to 1000 mg $CaCO_3 \cdot L^{-1}$. Chloride has no effect on determination.

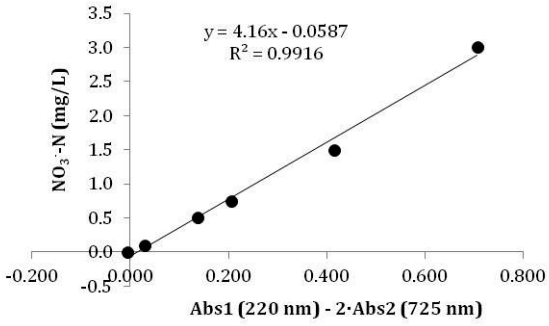

Figure 2-7 Example of calibration curve used for nitrate determination.

Determination procedure

Place 5 mL of sample (diluted if necessary to get a maximum concentration of NO_3^--N of 2.5 $mg \cdot L^{-1}$) and add 0.1 mL of HCl 1N. Afterwards, the absorbance at 220 and 275 nm is measured in a spectrophotometer (Cecil CE 7200). The absorbance related to nitrate is obtained by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm. The quantification is done with a 6-8 points calibration curve in the range of 0-2.50 mg NO_3^--$N \cdot L^{-1}$, using KNO_3 as standard (Figure 2-7).

Interferences

Dissolved organic matter, surfactants, NO_2^-, and Cr_6^+ interfere. For further details see method 4500-NO_3^- B of Standard Methods.

2.2.2.4 *Dissolved Total (TN) and Inorganic (IN) Nitrogen*

TN was determined in a total organic nitrogen analyzer (Rosemount-Dohrmann DN-1900) equipped with a quimioluminiscence detector with two channels. One channel determines the Total Nitrogen (TN), by oxidation at high temperature, and the other determines the Inorganic Nitrogen (IN), by a chemical reduction.

All the nitrogen present in the water is catalytically oxidised to nitrous oxide (NO). The process for TN determination occurs in two steps. The first step is a catalytic (Cu as catalyst) oxidation in the combustion tube at 850 °C and with pure oxygen (1 atm) as carrier gas. The second one is the chemical reduction of residual NO_2 with H_2SO_4 at 80 °C and catalyzed by $VaCl_3$. For the IN determination, only the second step (chemical reduction) is used. The NO obtained in the two steps is dried and forced to react with O_3 producing an unstable excited state NO_2*. The change back of this oxide to its fundamental state releases a proton, from which the determination of TN and IN is carried out by quimioluminiscence, using a multiplicator tube. The instrument is calibrated with a certified standard solution (KNO_3, 20 mg $N \cdot L^{-1}$) using a response factor method.

2.2.3 Phosphorus compounds

2.2.3.1 Phosphates

The method is based on the absorbance measurement at the radiation of 880 nm (Method 4500-PE of Standard Methods APHA-AWWA-WPCF, 1999). Minimum concentration that can be detected with this method is 10 $\mu gP \cdot L^{-1}$.

Ammonium molybdate and antimony potassium tartrate react with orthophosphate in acid medium to form phosphomolybdic heteropolyacid. This compound is reduced by ascorbic acid into molybdate blue.

Reagents preparation

Reagent A: Sulphuric acid 5N: Dissolve 70 mL of concentrated H_2SO_4 in 500 mL of distilled water.

Reagent B: Solution of antimony potassium tartrate. 1.3715 g of $K(SbO)C_4H_4O_6 \cdot 0.5H_2O$ are dissolved in 500 mL of distilled water. This solution must be kept in a bottle with glass top in order to be preserved.

Reagent C: Solution of ammonium molybdate. 20 g of $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ are dissolved in 500 mL of distilled water. This solution must be kept in a bottle with glass top in order to be preserved.

Reagent D: Ascorbic acid 0.01M. Dissolve 1.76 g of ascorbic acid in 100 mL of distilled water. This solution is stable for one week and should be kept at 4 °C.

Combined reagent: To prepare 100 mL of the combined reagent, the reagents A to D are mixed according to the following volumes:

- 50 mL of reagent A,
- 5 mL of reagent B,
- 15 mL of reagent C,
- 30 mL of reagent D.

The mixture must be stirred after the addition of each reagent, following the mentioned order. This combined reagent is stable for 4 hours.

Determination procedure

A sample of 5 mL is taken and one drop of phenolphthalein indicator solution (0.5-1 g phenolphthalein in 1 L of ethanol at 80% concentration) is added. If red color appears, reagent A (H_2SO_4 5N) is added (drop by drop) until the red color disappears. Then, 0.8 mL of the combined reagent is added and the mixture is stirred with a vortex stirrer. After 10 minutes but before 30 minutes, the absorbance at 880 nm is measured with a spectrophotometer Cecil CE 7200 and the results are given by comparison with a calibration curve (figure 2-8), done with commercial solution of phosphate (1000 $mg \cdot L^{-1}$). A blank with reagents must be also measured as a reference.

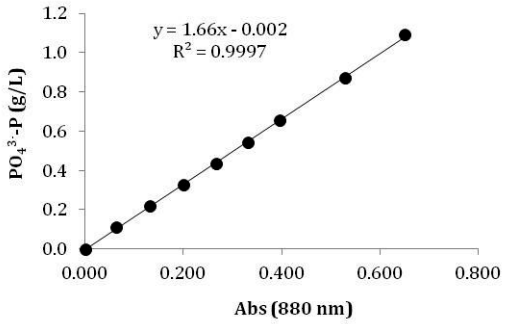

Figure 2-8 Example of a calibration curve used for phosphates determination.

Interferences

Arsenates react with solution of ammonium molybdate and produce blue color similar to that formed with phosphates. Hexavalent Cr and NO2- also interfere and give lower results: 3% if its concentration is of 1 $mg \cdot L^{-1}$ and 10-15% with 10 $mg \cdot L^{-1}$.

2.2.3.2 Total phosphorus

In order to analyze the soluble total phosphorus, the sample is digested to hydrolyze the polyphosphates to orthophosphate and then this latter compound can be measured with the previously described colorimetric method.

A sample of 50 mL is taken and one drop of phenolphthalein indicator solution is added. If red color appears, some drops of reagent A (H_2SO_4 5N)

are slowly added until the red color disappears. Then, 1 mL of H_2SO_4 solution (300 mL of concentrated H_2SO_4 diluted to 1 L with distilled water) and 0.4 g of solid $(NH_4)2S_2O_8$ are added. The mixture is gently boiled by a heater during 30-40 min in order to have a final volume about 10 mL. Organo-phosphorous compounds like AMP may need up to 1.5-2 h to be completely digested. The mixture is cooled and diluted to 30 mL with distilled water. A drop of phenolphthalein indicator solution is added and the mixture is neutralized with NaOH 1N till pale pink color is obtained. Then the phosphorus concentration is determined with the colorimetric method previously described.

2.2.4 Other control parameters

2.2.4.1 pH

The pH is one of the key parameters measured in wastewater treatment systems, since its control is important to maintain the biological activity of the microorganisms involved in the treatment process. The pH measurements were performed with different electrodes, such as the one of Crison Instruments S.A., 52-03, equipped with an automatic compensatory temperature device (Crison Instruments, S.A., 21-910-01) and connected to a measure instrument (pH mV^{-1}). The sensibility of the instrument is ±1 mV, corresponding to 0.01 pH units. The electrode is calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

2.2.4.2 Alkalinity

Alkalinity is a measure of the ability of a solution to neutralize acids to the equivalence point of carbonate or bicarbonate and therefore is responsible for the value of pH. The alkalinity is equal to the stoichiometric sum of the bases in solution. In the natural environment carbonate alkalinity tends to make up most of the total alkalinity due to the common occurrence and dissolution of carbonate rocks and presence of carbon dioxide in the atmosphere. Other common natural components that can contribute to alkalinity include borates, hydroxide, phosphates, silicates, nitrate, dissolved ammonia, the conjugate bases of some organic acids and sulphide.

Alkalinity measurement may be useful as a stability indicator of the anaerobic degradation process. A typical symptom of the abnormal operation of an anaerobic reactor is the increase of the organic acids concentration, which occurs when their production exceeds their consumption.

Total alkalinity (A_T) can be considered, approximately, as a sum of the alkalinity due to the presence of bicarbonate and volatile fatty acids (VFA), expressed as $CaCO_3$. Partial alkalinity (A_P), measured by the titration till pH 5.75, corresponds to the alkalinity of bicarbonate (Jenkins *et al.*, 1983), while the intermediate alkalinity (A_I), which is the difference between A_T (titration till pH 4.3) and A_P, represents – in an approximate form – the alkalinity due to the VFA concentration (Ripley *et al.*, 1986).

Various authors established that the relation between A_I and A_T is an adequate parameter of the anaerobic digestion process, and should not exceed the value of 0.3 (Ripley *et al.*, 1986; Switzembaum *et al.*, 1990; Soto *et al.*, 1993; Wentzel *et al.*, 1994) to avoid the accumulation of the VFA in the system.

Determination of the alkalinity is realised based on the modified method 2320A of APHA-AWWA-WPCF (1999) and consists of the titration of the centrifuged or filtrated sample with H_2SO_4 (with titrated normality) at two points of pH: 5.75 (which corresponds to the partial alkalinity) and 4.30 (which corresponds to the total alkalinity).

Values of the alkalinity are expressed as mg $CaCO_3 \cdot L^{-1}$ and are calculated as follows:

$$A_P = A \cdot N \cdot 50000/V \qquad \text{eq. 2-3}$$

$$A_T = B \cdot N \cdot 50000/V \qquad \text{eq. 2-4}$$

being:

V: volume of the sample (25 mL)

N: normality of H_2SO_4

A: volume of H_2SO_4 (mL) necessary to reach pH 5.75

B: volume de H_2SO_4 (mL) necessary to reach pH 4.3

Interferences

Soaps, oily matter, suspended solids, or precipitates may coat the glass electrode and cause a sluggish response, therefore additional time between titrant addition should be allowed. Samples should not be filtered, diluted, concentrated or altered.

2.2.4.3 Dissolved oxygen (DO)

A dissolved oxygen probe (AQUALITYC, model OXI-921) connected to a meter (M-Design Instruments TM-3659) was used to control DO concentration in the reactor.

2.2.4.4 Temperature

Temperature was measured using the probe for dissolved oxygen measurement (AQUALITYC, model OXI-921) connected to a meter (M-Design Instruments TM-3659)

2.3 SOLID PHASE ANALYTICAL METHODS

2.3.1 Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

Solids refer to matter suspended or dissolved in water or wastewater. Determination of solids concentration is important in the control if biological and physical wastewater treatment processes and for assessing compliance with regulatory agency wastewater effluent limitations. Solids present in water can be organic or inorganic. Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) are determined following the methods 2540D and 2540E, respectively, described in Standard Methods for the Examination of Water and Wastewater (APHA-AWWA-WPCF, 1999).

Determination procedure

For the determination of total suspended solids (TSS), a selected (in order to yield a residue between 2.5 and 200 mg) well-mixed sample volume is

filtered through a weighed glass-fibre filter (Whatman, GF/C, 4.7 cm of diameter, 1.2 µm of pore size) and the residue retained on the filter is dried to a constant weight (2h) at 103 to 105ºC using aluminium weighing dishes. The increase in weight of the filter represents the total suspended solids and is calculated as follows:

$$TSS = (A - B) * 1000/V \quad \text{eq. 2-5}$$

where:

TSS – total suspended solids ($mg \cdot L^{-1}$)

A – weight of the filter + dried residue (mg)

B – weight of the filter (mg)

V – sample volume (mL)

To determine the volatile solids (VSS), the residue from method 2540D is ignited to constant weight at 550ºC during half an hour. The remaining solids represent the fixed total, dissolved, or suspended solids while the weight lost on ignition is the volatile solids. This determination offers a rough approximation of the amount of organic matter present in the solid fraction of wastewater, activated sludge and industrial wastes. VSS concentration is calculated as follows:

$$VSS = (A - B) * 1000/V \quad \text{eq. 2-6}$$

$$FS = (B - C) * 1000/V \quad \text{eq. 2-7}$$

where:

VSS – volatile suspended solids ($mg \cdot L^{-1}$)

FS – fixed solids ($mg \cdot L^{-1}$)

A – weight of residue + filter before ignition (mg)

B – weight of residue + filter after ignition (mg)

C – weight of filter (mg)

Interferences

In order to avoid interferences it is necessary to exclude large floating particles or submerged agglomerates of nonhomogenous material from the sample. Highly mineralized water containing significant concentrations of

calcium, magnesium, chloride, and/or sulphate may be hygroscopic and require prolonged drying, proper desiccation and rapid weighing. Residues dried at 103 to 105ºC may retain not only water of crystallization but also some mechanically occluded water. If oil and grease is present, the sample should be treated with blender. In the case of VSS, negative error may be produced by loss of volatile matter during drying. Also, if the sample has high fixed solids concentration, VSS determination may be subjected to considerable error. For further details see method 2540 "SOLIDS" of Standard Methods.

2.4 GASEOUS PHASE ANALYTICAL METHODS

2.4.1 Biogas production

Biogas production was measured by Ritter MILLIGASCOUNTER® Type MGC-10 (figure 2-9).

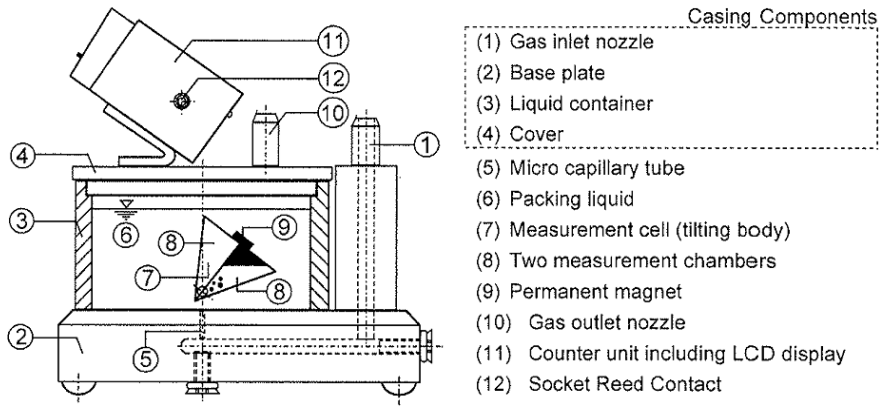

Figure 2-9 Ritter MilliGascounter® and its components.

As indicated in figure 2-9, the gas to be measured flows in via the gas inlet nozzle (1), through the micro capillary tube (5) located in the base of MilliGascounter and up into the liquid casing which is filled with a packing liquid (6). The gas rises as small gas bubbles through the packing liquid, up

and into the measurement cell (7),. The measurement cell consists of two measuring chambers (8), which are filled alternatively by the rising gas bubbles. When a measuring chamber is full, the buoyancy of the filled chamber causes the measurement cell to abruptly tip over into such a position, that the second measuring chamber begins to fill and the first empties. The measurement of gas volume therefore occurs in discrete steps by counting the tilts of the measurement cell (7) with a resolution of approximately 10 mL (=contents of a measuring chamber). This "residual error" (=max. 10 mL) caused by the resolution should be taken into account when estimating/calculating the total measurement error. Through the combination of a permanent magnet (9) and a magnetic sensor (reed contact), this tilting procedure creates a pulse which is registered by the counter unit (11). The measured gas escapes through the gas outlet nozzle (10). For further information please refer to the manufacturer´s manual.

2.4.2 Biogas composition

To measure biogas composition a gas chromatograph HP 5890 Series II with the column of Porapack Q 80/100 2m x 1/8" (SUPELCO) is used. 1 mL of well-mixed sample should be injected through the septum at the following conditions: oven temperature (column) set on 35 °C; injector and the detector temperature set on 110 °C. The obtained peaks corresponded to the percentage of the N_2, CH_4, CO_2 and H_2S content in the sample.

2.5 BIOMASS CHARACTERIZATION

2.5.1 Sludge Volumetric Index

The Sludge Volumetric Index (SVI) determination is defined in the Standard Methods for the Treatment of Water and Wastewater (APHA-AWWA-WPCF, 1999) as the volume in millilitres occupied by 1 g of a suspension after 30 min settling. However, as suggested at the "1st IWA-Workshop Aerobic Granular Sludge" (de Kreuk *et al.*, 2005) and by Schwarzenbeck *et al.* (2004) another parameter, the SVI_5 (SVI after 5 minutes of settling) was used instead of SVI_{30} (SVI after 30 minutes of settling) since it is more

representative for granular biomass. A low SVI_{30} does not necessarily imply sludge granulation and vice versa. Nevertheless a granular sludge bed does consolidate much faster, i.e., the terminal SVI_{30} is already reached after 5 minutes of settling.

2.5.2 Average diameter of the granules

Changes in morphology of the granules were followed by image analysis (Tijhuis *et al.*, 1994). Images of the granular sludge were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (Stemi 2000-C, Zeiss). For digital image analysis the programme Image ProPlus® was used. The procedure followed is represented in figure 2-10 and is as follows:

I) convert the original image of granules to black and white mode since it simplifies the image processing

II) define the range of colours corresponding to the area of interest in the image, i.e. the granules

III) export the data of interest selected with the software (e.g., area, perimeter, roundness, sphericity, average diameter, etc.) to a worksheet

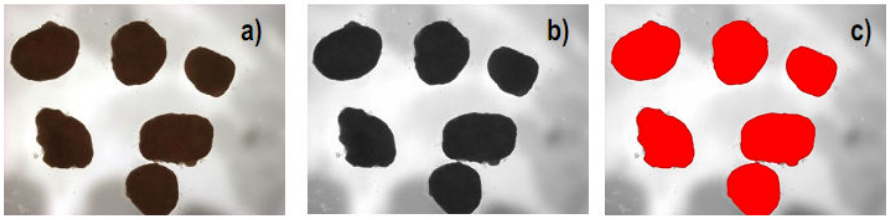

Figure 2-10 (a) Original image of a sample of granules, (b) Image of the granules converted to black and white, (c) Area recognized by the software in red once the threshold levels are defined by the user.

The average diameter obtained from the programme corresponded to the mean feret diameter of the granules. The feret diameter is calculated as an average value from the shortest and the longest measured segment in the granule (Fig. 2-11).

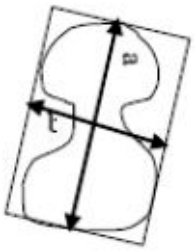

Figure 2-11 Longest and shortest segments in a granule to estimate the feret diameter of a granule.

2.5.3 Specific Methanogenic Activity

The method used in this work was adopted from Soto *et al.* (1993).

Methanogenic activity is a velocity at which anaerobic bacteria produce CH_4 by degrading organic substrates under anaerobic conditions. Methanogenic activity is developed in three phases: acclimatisation (lag phase), exponential (growth) and stationary. Specific methanogenic activity refers to the methanogenic activity per gram of biomass.

Determination procedure

Cultivation medium (1 L): 0.3 g of the yeast extract, 1 mL of the resazurine solution (0.1%), 0.5 g of cisteine and 2 g of sodium bicarbonate. pH should be adjusted at 7. The cultivation medium volume should be calculated as follows:

$$V_{medium} = V_{total} - V_{substrate} - V_{Na_2S \cdot H_2O} - V_{NaHCO_3} - V_{sludge} \quad \text{eq. 2-8}$$

Being:

V_{total}: total volume of the liquid phase, mL

$V_{substrate}$: volume of the VFA solution, mL

$V_{Na2S·H2O}$: volume of the reducer solution, mL

V_{sludge}: volume of the sludge added to achieve the concentration of 1.5 – 2.5 $gVSS \cdot L^{-1}$.

The step by step procedure is as follows:

a). introduction into the bottles (total volume 122 mL) of the previously calculated amount of cultivation medium;

b). addition of the reducer solution and $NaHCO_3$;

c). addition of the sludge;

d). pH adjustment between 7.0 and 7.1;

e). bubbling of N_2/CO_2 85/15% (or N_2) gas mixture;

f). sealing of the bottles with a septum and placing a valve with a needle in the middle of the septum;

g). introducing the bottles into the thermostatic bath;

h). after 30 min, when the thermal equilibrium is attained, addition of the substrates into the flasks and homogenization by shaking gently.

The biogas production was determined as the increment of pressure in the headspace of the vials, measured by means of a pressure transducer device.

Calculations

The calculations are carried out as follows:

To transfer the pressure measured in mV to mmHg, the calibration curve of the pressure transducer device is used:

$$P(mmHg) = 2.758 \cdot P(mV) + 760 \qquad \text{eq. 2-9}$$

Being:

P(mmHg): gas pressure expressed in mmHg;

P (mV): gas pressured measured with the pressure transducer device.

To calculate the partial pressure of methane, the following equation is used:

$$PpCH_4(mmHg) = \frac{P(mmHg) \cdot \%CH_4}{100} \qquad \text{eq. 2-10}$$

Being:

$PpCH_4$(mmHg): partial pressure of CH_4 expressed in mmHg;

$\%CH_4$: percentage of methane measured with gas chromatography.

Next, to calculate the amount of moles of CH_4, the equation of ideal gases is used:

$$nCH_4 = \frac{PpCH_4 \cdot V_G}{R \cdot T} \qquad \text{q. 2-11}$$

Being:

nCH_4: moles of methane;

V_G: volume of the gas phase in a bottle (mL);

R: the ideal gas coefficient, 0.082 atm·L·mol·K^{-1};

T: temperature (K).

Finally, the following relation is used to calculate the COD value of methane produced:

$$1mMCH_4 = 0.064gCOD \qquad \text{eq. 2-12}$$

Specific methanogenic activity is expressed as the amount of CH_4 (as g COD) produced per day and per g VSS taking into account the maximum slope.

2.5.4 Identification of bacteria populations by FISH

The abundance of the different populations of microorganisms present in the sludge samples of the reactors was researched by Fluorescent In Situ Hybridization (FISH). With this technique specific regions in 23S or 16S rRNA are detected with fluorescently labelled probes. If the corresponding domain, phylum, genus or species is present, the probe hybridizes to the targeted sequence and can later be detected microscopically. According to Amann *et al.* (1995) a typical FISH protocol includes four steps (Fig. 2-12): the fixation and permeabilization of the sample; hybridization of the targeted sequence to the probe; washing steps to remove unbound probe; and the detection of labelled cells by microscopy or flow cytometry. This protocol must be applied to disrupted biomass; therefore, the granules must be disintegrated before starting the procedure. To achieve the granular biomass breakage, biomass is sonicated for 1 min at 65% of amplitude using a probe sonicator (UP200s, Dr. Hielscher).

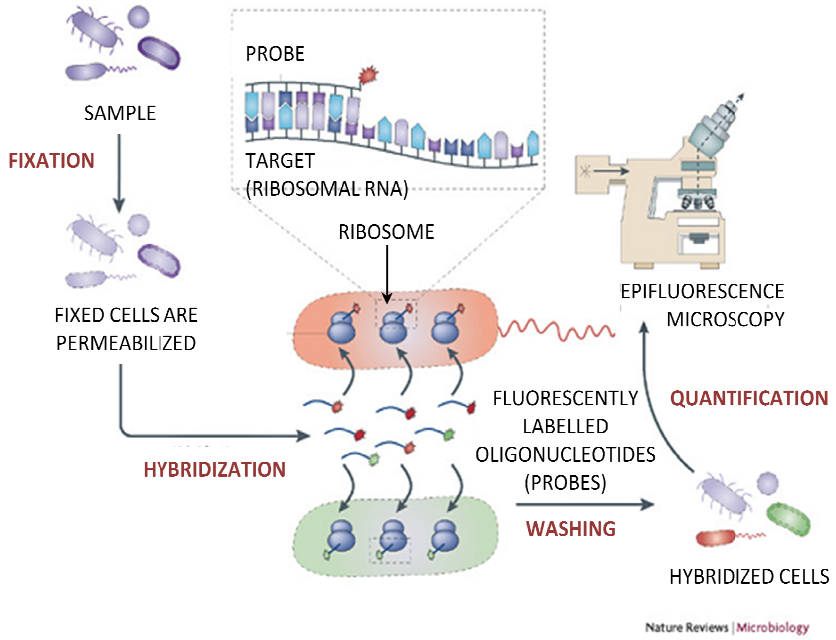

Figure 2-12 Basic steps of FISH technique. (Adapted from Amann & Fuchs, 2008).

During hybridization the cells are exposed to high temperatures, detergents and osmotic gradients. Thus fixation of the cells is essential in order to maintain the morphological integrity of the cells. Fixation of cells with glutaraldehyde results in considerable autofluorescence of the specimen. Autofluorescence is minimized by fixation in freshly prepared (not older than 24 h) 4% paraformaldehyde solution in PBS.

After fixation, the cells are immobilized on a microscopic slide and used for hybridization with 16S rDNA probes. In order to avoid non-specific binding of the rDNA probes, the hybridization is done at stringent conditions (46 °C, 0-65% formamide) and specimens are washed with wash buffer (48 °C). The targeted organisms can be detected by the characteristic fluorescence.

The fluorochromes used to detect the hybridized rRNA were fluos (5(6)-carboxyfluorescein-Nhydroxysuccinimide ester) and Cy3 (indocarbocyanine). To visualize all cells in a sample the stain 4,6-diamidino-2-phenylindole (DAPI) was used. Its application can provide insight into the existence of archaeobacteria and eukaryotes, like e.g. protozoa. For analysis of the slides an epifluorescence microscope

(Axioskop 2 plus, Zeiss) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics) was used. The probes applied in this study are listed and detailed in Chapter 6 and Chapter 7.

For further discussion it has to be kept in mind that samples can never be 100% representative. Thus the fact that no bacteria of a certain kind were present in the sample can always be attributed to unrepresentative sampling or error during procedure (e.g. hybridization process) as well.

2.6 MEMBRANE PERFORMANCE

2.6.1 Analytical methods

2.6.1.1 Extracellular Polymeric Substances (EPSs) and Soluble Microbial Products (SMP)

Extracellular polymeric substances (EPSs) mainly consist of polysaccharides and proteins. To determine their concentration, the sample must be analysed according to the protocol proposed by the members of AMEDEUS & EUROMBRA during the meeting which took place in Berlin, 1 of June 2006. The method of extraction consists of a modification of the method used by Zhang *et al.* (1999). The procedure is as follows:

a). The sample of 200 mL of biomass is centrifuged at 5000 rpm during 20 minutes.

b). The supernatant is removed.

c). Carbohydrate and protein fraction is analyzed in order to obtain SMP concentration.

d). 200 mL of deionised water is added to the remaining biomass and carefully shaken (manually) and the sample is placed in the oven at 80 °C, during 10 minutes.

e). The tubes, still warm, are centrifuged at 5000 rpm during 20 minutes.

f). The supernatant is filtered with the fiberglass filter. Carbohydrate and protein fraction is analyzed in order to obtain EPS concentration.

2.6.1.2 Carbohydrates

Polysaccharides (PS) concentrations were analysed using a modified phenol–sulphuric acid method proposed by Dubois *et al.* (1956).

Reagents preparation

The following reagents are necessary in order to carry out the procedure:

Reagent A: Phenol solution 5 % (v/v)

Reagent B: Sulphuric acid (97 %)

Determination procedure

a). A sample of 1.0 mL is thoroughly mixed with 1.0 mL of reagent A and left for 10 minutes at room temperature.

b). 5.0 mL of reagent B are added rapidly (in stream) and left for 5 minutes at room temperature for cooling.

c). The test tube is then mixed again.

d). After 25 minutes, the absorbance at 490 nm is measured with a spectrophotometer Cecil CE 7200.

A blank with reagents must be also measured as a reference. The quantification is done with 6-8 points calibration curve in the range of 0-100 $mg \cdot L^{-1}$, using D-glucose monohydrate.

Nitrate and nitrite interferences over carbohydrate concentration have been reported by Drews *et al.* (2008). The quantification of this interference is given by the equation:

$$C_{PS} = C_{PS,measured} - 0.099 \cdot C_{N\text{-}NO3\text{-}} - 1.9 \cdot C_{N\text{-}NO2\text{-}} \quad \text{eq. 2-13}$$

2.6.1.3 Proteins

Determination of proteins was done according a modified method based on Lowry *et al.* (1951) and Frølund *et al.* (1996). First the proteins are pretreated with copper ion in alkali solution, and then the aromatic

aminoacids in the treated sample reduce the phosphomolybdatephosphotungnstic acid present in the Folin reagent.

Reagents preparation

The following reagents are necessary in order to carry out the procedure:

Reagent A: Solution of sodium hydroxide (NaOH) 143 mM and sodium bicarbonate (Na_2CO_3) 270 mM.

Reagent B: Solution of cupric sulfate ($CuSO_4$) 57 mM

Reagent C: Solution of sodium Tartrate ($Na_2C_4H_4O_6$) 124 mM

Reagent D: Mixture of reagents A, B, C in ratio of 100:1:1. Reagent D has to be done freshly.

Reagent E: Solution of Folin-Ciocalteu-reagent (1:2 in deionised water)

Determination procedure

a). A sample of 1.5 mL is rapidly mixed with 2.1 mL of reagent D and left for 10 minutes at room temperature.

b). 0.3 mL of reagent E are added rapidly and mixed.

c). After 45 minutes, the absorbance at 750 nm is measured with a spectrophotometer Cecil CE 7200.

A blank with reagents must be also measured as a reference. The quantification is done with 6-8 points calibration curve in the range of 0-250 $mg \cdot L^{-1}$, using protein standard bovine serum albumin (BSA).

2.6.1.4 Colloidal fraction of biopolymer clusters (cBPC)

Another category of organic compounds that has been identified in the liquid phase of MBR sludge and in the cake sludge on membrane surfaces consists of biopolymer clusters (BPC) ranging from 2.5 to 60 µm in size. Based on confocal laser scanning microscopy (CLSM) examination, BPC are free and independent organic solutes that are different from biomass flocs and EPS and much larger than SMP (Wang & Li, 2008; Sun *et al.*, 2008). In this work only colloidal fraction of BPC was considered and the procedure to its determination was as follows: concentration of total Dissolved Organic Carbon (t-DOC) was measured with a Shimadzu analyser (TOC-

5000). The difference in t-DOC concentration between the sludge mixture after filtration through a 0.45 µm nitrocellulose filter and the permeate was assigned to the colloidal fraction of BPC (cBPC) in the liquid phase of the sludge mixture suspension.

2.6.2 Filtration characteristics

2.6.2.1 Filterability

Filterability assays are realized with the filtration Amicon 8200 chamber (Millipore). This chamber has the volume of 180 mL and 63 mm diameter. Membrane durapore filters (Millipore) of PVDF (model HVLP09050) with pore size of 0.45 µm are used. The evolution of the filtrate volume is measured with respect of time. To achieve better precision of the measurement the analytic scale Sartorius BP 1200 is used. The mass of filtrated liquid versus time gives the filterability value.

2.6.2.2 Flux and Permeability

The membrane used in this work has an area of 0.9 m^2. Therefore flux can be calculated as:

$$J = \frac{Q}{S} \qquad \text{eq. 2-14}$$

Where:

J: Flux expressed in $L \cdot m^{-2} \cdot h^{-1}$,

Q: flux expressed in $L \cdot h^{-1}$,

S: membrane area expressed in m^2.

Therefore, permeability can be calculated as:

$$P = \frac{J}{TMP} \qquad \text{eq. 2-15}$$

Where:

P: permeability expressed in $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$,

TMP: Transmembrane Pressure in bar.

2.6.2.3 Critical Flux

The critical flux was determined according to the modified flux-step method proposed by van der Marel *et al.* (2009). The criterion employed was that the increment of TMP with respect to time was higher than 10 Pa/min (Le-Clech *et al.* 2003).

2.6.3 Maintenance of the membrane module

The membrane washing performed were either a mechanical washing with tap water, or a chemical maintenance cleaning (when necessary).

2.6.3.1 Phisical Cleaning

The maintenance cleaning could be performed inside the reactor and the procedure was the following:

1). mechanical rinsing with tap water;

2). backwashing with chlorinated water (250 ppm Cl_2:1) for 1 h.

2.6.3.2 Chemical Cleaning

Chemical cleaning was performed outside the membrane chamber only when permeability value was below 50 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$, approximately. The cleaning procedure was:

1). mechanical cleaning,

2). submerging the membrane in chlorinated water (2000 ppm Cl_2:1) for 2 h,

3). backwashing with chlorinated water (2000 ppm Cl_2:1) for 1 h.

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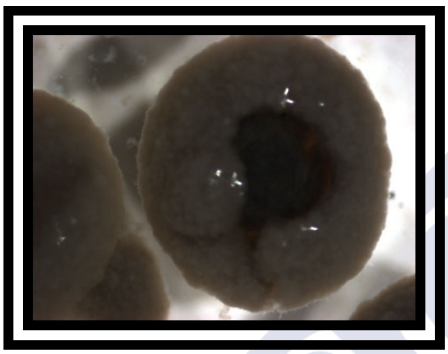
Chapter 3



Start-up and operation of the combined UASB-MBR system for the treatment of low-strength wastewaters at ambient temperatures[1]

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[1] Parts of this chapter were published in the following documents:

D. Buntner, A. Sánchez Sánchez and J.M. Garrido (2011). *Three stages MBR (methanogenic, aerobic biofilm and membrane filtration) for the treatment of low-strength wastewaters*. Water Science & Technology, 64 (2), pp 397–402.

A. Hospido, I. Sánchez, G. Rodriguez-Garcia, A. Iglesias, **D. Buntner**, R. Reif, M.T. Moreira, G. Feijoo (2012). Are all membrane reactors equal from an environmental point of view? Desalination, 285, pp 263–270.

D. Buntner, J.M. Garrido and J.M. Lema (2011). *Reactor biológico de membranas de tres etapas, metanogénica, aerobia y de filtración, para la depuración de aguas residuales*. Patent application ES 2385002 A1, Priority claimed: 27/7/2009.

SUMMARY

The use of a new combined UASB-MBR process with a first methanogenic UASB stage, and a second MBR stage with aerobic biofilm growing on small carrier elements maintained in suspension and with membrane filtration module is proposed. The objective of the first methanogenic chamber is to diminish COD of the raw wastewater, producing a biogas rich in methane, and decrease the sludge production. In the second stage, the remaining soluble biodegradable COD is oxidized by the heterotrophs. In the filtration chamber of the MBR stage, the membrane modules could be operated at higher fluxes than those reported for AnMBR systems, and similar to those obtained in aerobic MBRs. In this sense, the concept of these combined UASB-MBR system is to join the advantages of the methanogenic and aerobic membrane bioreactor processes, by reducing energy requirements for aeration, producing biogas with high methane percentage and a permeate with very low COD content.

A synthetic wastewater was fed to the combined UASB-MBR system. COD in the influent was between 200 and 1200 $mg \cdot L^{-1}$, ammonium ranged from 10 to 35 mg/L and phosphorous concentration was 8 $mg \cdot L^{-1}$, respectively. OLR in-between 1 and 3 $kgCOD \cdot m^{-3} \cdot d^{-1}$ and a HRT of 13–21 h were applied. Temperature was between 17.5 and 23.2 °C. During the whole operating period the COD removal efficiency was in the range of 90 and 96%, of which in between 40 and 80% was removed in the first methanogenic chamber. The average COD concentration measured in the permeate was around 5 $mg \cdot L^{-1}$.Biogas production with methane content between 75 and 80% was observed. With regard to membrane operation, average permeabilities around 150 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ were achieved, operating with fluxes of 11-15 $L \cdot m^{-2} \cdot h^{-1}$.

Life Cycle Assessment was applied for the evaluation of proposed UASB-MBR system compared to 3 other membrane bioreactor configurations of increasing complexity. It was found that UASB-MBR was the best if acidification impact category was considered, however attention should be paid in global warming and ecotoxicity matters. Moreover, due to the poor elimination of nitrogenous compounds, eutrophication was also pointed out as a bottleneck of the proposed system.

3.1 INTRODUCTION

3.1.1 Anaerobic treatment of urban wastewater

The application of anaerobic processes for treating diluted waste streams has received high attention in recent years. One of the reasons is that it may guarantee the process sustainability with regard to the use of the aerobic processes, due to the lower energy consumption, generation of a biogas with a high methane content and diminution of biomass production. One of the most popular anaerobic systems for treating wastewaters is the Upflow Anaerobic Sludge Blanket (UASB) reactor. Due to its simplicity and compactness this technology has been proposed and applied to the treatment of various industrial wastewaters and even domestic wastewater in warm regions (Seghezzo *et al.*, 1998). However, due to the presence of residual biodegradable organic matter and nutrients, anaerobic effluents require an adequate post treatment to reach the local standards for discharge and/or agricultural reuse (Elmitwalli *et al.*, 2002; Tawfik *et al.*, 2005; Chernicharo, 2006). Moreover, anaerobic (UASB) systems are not recommended for treating domestic wastewater in mild or cold regions due to the diminution of activity with temperature and wash out of a fraction of the anaerobic biomass with the effluent. Both effects, diminution of activity and biomass wash out, may decrease the methanogenic bioreactor capacity, especially at low temperatures. In this sense, the use of filtration membranes allows avoiding the observed loss of biomass, and could make wastewater treatment feasible even at lower temperatures (Judd, 2006).

3.1.2 Anaerobic Membrane Bioreactors (AnMBRs) in urban wastewater treatment

Over the last decade, the adaptation of membranes coupled with anaerobic biological processes has made membrane reactors a promising alternative to conventional wastewater treatment. Hu & Stuckey (2006) achieved 90% soluble Chemical Oxygen Demand (COD) removal efficiency at a 3 h HRT with an inlet concentration of 460 $mg \cdot L^{-1}$, using two Anaerobic Membrane Bioreactors (AnMBR) with both, flat sheet and hollow fibre modules. Ho & Sung (2010) investigated the performance of a cross-flow AnMBR treating synthetic municipal wastewater. They achieved more than 95% COD

removal, with permeate concentration lower than 40 $mg \cdot L^{-1}$. Hu *et al.* (2009) proposed a hybrid reactor, based on the installation of aerating membrane into an anaerobic baffled reactor (HMABR). The results demonstrated that after the installation of membrane module, total Volatile Fatty Acids (VFA) and COD concentration in the HMABR effluent were decreased by 68.1 and 59.5% respectively, with increased nitrogen removal efficiency by 83.5%, at influent COD concentration of 1600 $mg \cdot L^{-1}$ and NH_4^+–N concentration of 80 $mg \cdot L^{-1}$. Finally, Giménez *et al.* (2011) and Robles *et al.* (2013) studied a hollow-fibre submerged anaerobic MBR (HF-SAnMBR) at pilot scale, treating municipal wastewater at temperature range from 15 to 33 °C. The plant reached up to 87 % COD removal with membrane fluxes between 9 and 13.3 $L \cdot m^{-2} \cdot h^{-1}$. Moreover, critical flux obtained (normalized to 20 °C) was lower than 14 $L \cdot m^{-2} \cdot h^{-1}$. This demonstrates that the AnMBR can treat low-strength wastewater with similar treatment performance as aerobic MBRs. However, the membrane fouling propensity of the bioreactor liquor is significantly higher for anaerobic treatment, such that fluxes and permeabilities are generally much lower than for the aerobic MBRs (Judd, 2006). Moreover, nutrients (such as nitrogen and phosphorous) elimination is highly limited.

3.1.3 MBR as a post-treatment of UASB effluents in urban wastewater treatment

Anaerobic pre-treatment of domestic wastewater combined with the MBR technology can serve a viable and cost-effective alternative due to its low production of excess sludge, production of energy in form of biogas, high quality of the effluent and applicability in small and large scales. Moreover, the sludge treatment is largely simplified, since it can be treated directly in the UASB unit, therefore there is no need for separate thickener and digester (Chernicharo, 2007).

3.1.4 Life Cycle Assessment

Life Cycle Assessment (LCA) evaluates the environmental load linked to a process, product or service by collecting all the related inputs and outputs through the whole life cycle and the quantification of the environmental impacts associated (Baumann H. & Tillman, 2004; ISO 14040). LCA has been

extensively applied for wastewater treatment. Among the impact categories, the following ones are usually taken into account:

- Eutrophication – this category addresses the impacts from nitrogen and phosphorus in bioavailable forms on aquatic and terrestrial ecosystems. This impact category has been identified as one of the most important criteria to assess the efficiency of a wastewater treatment plant (European Commission, 2010).
- Acidification - this category addresses the impacts associated to the emission of airborne acidifying chemicals. The principal acidifying pollutants are SO_2, NO_X, HCl and NH_3, which are mainly generated in processes of electricity production (European Commission, 2010).
- Global warming – is caused by the emission of greenhouse gases and activities which have an important influence in their atmospheric concentration (European Commission, 2010).
- Terrestrial ecotoxicity and freshwater aquatic toxicity – Toxicity effects in LCA are based on the relative risk and associated consequences of pollutants released into the environment by means of models that account for the fate of toxic substance, exposure and differences in toxicological response (European Commission, 2010).

This selection is in agreement with the set of categories usually evaluated when applied LCA to wastewater treatment.

Recently, Hospido et al. (2011) presented a life cycle assessment of a number of MBR configurations, including the combined UASB-MBR system described in this Thesis. Since the LCA has only been applied to MBRs in a few cases, the work of Hospido et al. (2011) aimed to increase the knowledge related to the environmental performance of MBRs by evaluating different configurations and discussing the possible correlation between operational conditions and environmental profiles. The MBRs under study were:

- An aerobic MBR with two stages: aerobic, and hollow fibre membrane filtration (volume=0.18m^3), for the removal of organic

matter and partial elimination of nitrogen: only nitrification takes place. For a more detailed description see (Reif *et al.*, 2008).

- An pre-denitrification MBR based on three connected tanks: one anoxic (0.20 m^3), one aerobic (0.20 m^3) and the hollow fibre membrane unit (0.10 m^3) for the removal of organic matter and nitrogen. For further information see (Expósito, 2010).
- A modified UCT1 configuration, composed by one anaerobic tank (0.47 m^3), two anoxic tanks (0.71 and 0.46 m^3), one aerobic tank (0.94 m^3) and the hollow fibre membrane compartment (1.40 m^3). The system removes organic matter, nitrogen and phosphorus. A more detailed description of this pilot plant can be found in (Iglesias et al., 2010).
- A combined UASB-MBR system discussed in this Thesis.

3.2 OBJECTIVES

In this work an alternative to overcome problems related with the operation of AnMBR (fouling) and aerobic MBR (high energy consumption and sludge production) is proposed: the use of a new combined UASB-MBR process with a first methanogenic UASB stage, and a second MBR stage divided into two chambers: one with aerobic biomass growing both onto plastic support carrier and in suspension, and a second one with membrane filtration module. The objective of the first methanogenic stage is to reduce the COD of the raw wastewater, producing a biogas rich in methane, and diminish the sludge production. In the second stage, the remaining soluble biodegradable COD is oxidised by the heterotrophs. In the filtration chamber, the membrane module might be operated at higher fluxes that those reported for AnMBR systems, and similar to those referred for aerobic MBRs. In this sense, the concept of the combined UASB-MBR system is to join the advantages of the methanogenic technology and aerobic membrane bioreactor processes and make the anaerobic treatment feasible even for diluted wastewaters at low temperatures.

3.3 MATERIALS AND METHODS

3.3.1 Bioreactor and the strategy of operation

A 170 L combined UASB-MBR system was operated, with a methanogenic UASB stage, and an MBR with an aerobic (with biomass growing as biofilm and in suspension) and a membrane filtration chambers connected in series (figure 3-1). The effluent of the 120 L UASB stage was led to the aerobic stage which consists of a 36 L aerobic bioreactor with 18.5 L (50 % of the apparent volume) of Kaldnes K3 support. Recirculation from the aerobic chamber of MBR stage to UASB stage was implemented, with the ratio R=1. Finally, the membrane filtration was carried out in a 20 L aerobic chamber, where a membrane module Zenon ZW10 with a surface area of 0.9 m^2 was employed. This module consists of PVDF hollow-fibre membrane, with a pore size of 0.04 μm. The membrane was operated in cycles of 7.5 min with a permeation period of 7 min and a backwashing period of 0.5 min. The filtration chamber was aerated in order to minimize membrane fouling. The operation of the system was controlled by a PLC (Siemens S7-200) connected to a computer. Trans-membrane Pressure (TMP) data was measured with an analogue pressure sensor (Efector500 PN-2009) and collected in the PC via an analogue PLC module Siemens EM 235.

The UASB reactor was seeded with 50 L of anaerobic biomass (27 g $VSS \cdot L^{-1}$) from the Internal Circulation (IC) anaerobic reactor of a brewery industry located in Galicia (Spain), whereas 5 L of biomass from a MBR pilot plant treating urban wastewater was employed as an aerobic biomass inoculum.

The study was performed during 220 days and the operation could be divided in four different periods:

Period I (From day 0 until day 77)

During the start-up permeate flux was fixed at 11 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$, and increased to 15 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ after day 30. Feeding COD concentration was fixed around 600 $mg \cdot L^{-1}$. The reactor was fed using synthetic wastewater composed of diluted skimmed milk, $NaHCO_3$ and trace elements. During the first operation days, some other chemicals were added to the feeding (NH_4Cl, Na_2HPO_4, KH_2PO_4) but were eliminated on day 40, since ammonia and phosphorous were present in the effluent of the system. From day 58

to 84, sludge purges from the sampling port P4 (figure 3-1) in the UASB reactor were performed.

Period II (From day 78 until day 114)

In this period the COD concentration was increased from 600 to 900 $mg \cdot L^{-1}$ because of the addition of methanol to the synthetic wastewater used during the Period I. The additional COD concentrations were 300 $mg \cdot L^{-1}$ from day 78 till day 98, and 30 $mg \cdot L^{-1}$ from day 98 on. Methanol was added in order to carry out micropollutants removal experiments (data not shown). During this period permeate flux varied between 12 and 15 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ due to the increase of membrane fouling.

Period III (From day 115 until day 175)

On this period, the COD concentration in the feeding was increased from 900 to 1200 $mg \cdot L^{-1}$. The system was purged from day 141 on, because of the high suspended solids concentration in the reactor. These purges took place from the sampling port P5 (figure 3-1) in the UASB reactor and from membrane chamber due to the accumulation of biomass. Permeate flux varied between 12 and 15 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$.

Period IV (From day 176 until day 220)

On this period the recirculation from aerobic chamber to UASB stage was turned off. The COD concentration was maintained at 1200 $mg \cdot L^{-1}$. The system was purged and the purges took place from the sampling port P5 (figure 3-1) in the UASB reactor and from membrane chamber, as in period III. Permeate flux varied between 12 and 15 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$.

Figure 3-1 Schematic diagram of the three stage pilot-scale MBR. (1) UASB stage, (2) Biofilm aerobic chamber of MBR, (3) Membrane filtration chamber, (4) Feeding and recirculation, (5) Permeate (backwashing), (6) Biogas outlet. P1, P2, P3, P4 and P5 are the sampling ports.

3.3.2 Analytical methods

Temperature, pH, alkalinity and the concentrations of Dissolved Oxygen (DO), Volatile Suspended Solids (VSS), total and soluble Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), ammonium, nitrite, nitrate, phosphate and total phosphorus were determined according to the Standard Methods (APHA 1998). The soluble COD concentration (s-COD) was measured by filtering the samples using 0.45 µm nitrocellulose membrane filters (HA, Millipore) and the permeate was assigned to Biopolymer Clusters (BPC) in the liquid phase of the sludge mixture suspension (Sun *et al.*, 2008). Concentrations of total Dissolved Organic Carbon (t-DOC) and total dissolved Inorganic Carbon (IC) were measured with a Shimadzu analyser (TOC-5000). Soluble Microbial Products (SMP) as

carbohydrates were determined by centrifuging the biomass for 5 min at 5000 rpm (Heraeus, Labofuge 200) and filtering the supernatant through 0.22 µm glass fibre filters (GF 52, Schleicher and Schuell). The carbohydrate concentration was determined following the method of Dubois *et al.* (1956). Biogas composition was measured in a gas chromatograph HP 5890 Series II with the column of Porapack Q 80/100 2m x 1/8" (SUPELCO).

With respect to the membrane operation, trans-membrane pressure (TMP) and permeability were measured continuously. The difference in tDOC concentration between the sludge mixture after filtration through a 0.45 µm filter and the permeate was assigned to the BPC in the liquid phase of the sludge mixture suspension. The critical flux was determined according to the modified flux-step method proposed by Van der Marel *et al.* (2009).

3.3.3 LCA analysis

Detailed information on the procedure of LCA analysis can be found in Hospido *et al.* (2011). Four MBR systems were compared, taking into account the following impact categories defined by the Centre of Environmental Science (CML) of Leiden University: eutrophication, acidification, global warming, terrestrial ecotoxicity and freshwater aquatic toxicity.

3.4 RESULTS AND DISCUSSION

3.4.1 General observations

The results of 220 days of the combined UASB-MBR system operation are presented. Since in this work applicability of merged anaerobic, aerobic and MBR processes in psychrophilic conditions was considered, the system was operated at ambient temperature, and wastewater temperatures changed with seasons (23 – 17.5 °C, which corresponds to the end of summer and winter). On the other hand, high biogas production, sufficient for heating requirements of the system (i.e. to operate under mesophilic conditions) is not likely due to the low strength of treated wastewater. In this sense, the

system could be relevant in (semi-)tropical countries, for the treatment of low-strength wastewater, considering that typical COD concentration in municipal wastewater usually varies between 500 and 800 $mgCOD \cdot L^{-1}$ (Tchobanoglous et al., 2004) and may reach up to 1200 - 1500 $mgCOD \cdot L^{-1}$.

The average ORLs applied to the UASB stage and MBR stage (without taking into account the recirculation) were 0.95 and 0.75 $kgCOD \cdot m^{-3} \cdot d^{-1}$ for t-COD, and 0.61 and 0.29 $kgCOD \cdot m^{-3} \cdot d^{-1}$ for s-COD, respectively. The Hydraulic Retention Time (HRT) of the entire system varied between 13 and 21 h.

The average pH of the effluent from UASB was around 6.7. The pH of aerobic chamber and permeate varied from 6.7 to 7.7 and from 7.0 to 8.2, respectively, depending on the system performance. Moreover, the pH measured in the aerobic chamber was always higher than that of UASB effluent. This fact was caused mainly by the stripping of CO_2 from the bulk liquid by the aeration. Furthermore, during the periods where nitrification occurred, pH of aerobic chamber decreased, due to the production of hydrogen ions.

During the 220 operational days the calculated overall biomass yield was equal to 0.14 $gVSS \cdot gCOD^{-1} \cdot d^{-1}$, which is significantly lower than that of aerobic systems, fitting rather in the range of anaerobic bioreactors.

3.4.2 Organic matter removal

Variations of COD concentrations in the feeding, UASB effluent, recirculation and membrane effluent during the four operating periods are shown in figure 3-2. The total COD (t-COD) and soluble COD (s-COD) fed fluctuated from 200 to 1100 and from 150 to 1000 $mg \cdot L^{-1}$, respectively, and the average ratio between s-COD/t-COD fed to the reactor was around 0.75 (figure 3-3). The difference between t-COD and s-COD concentrations measured in the UASB effluent and recirculation from the first aerobic MBR chamber was attributed mostly to the presence of suspended solids. The s-COD measured in both, anaerobic effluent and recirculation, was similar, indicating that most of the soluble biodegradable COD was removed in the first anaerobic chamber. In general, the permeate COD concentration was as low as 5 $mg \cdot L^{-1}$.

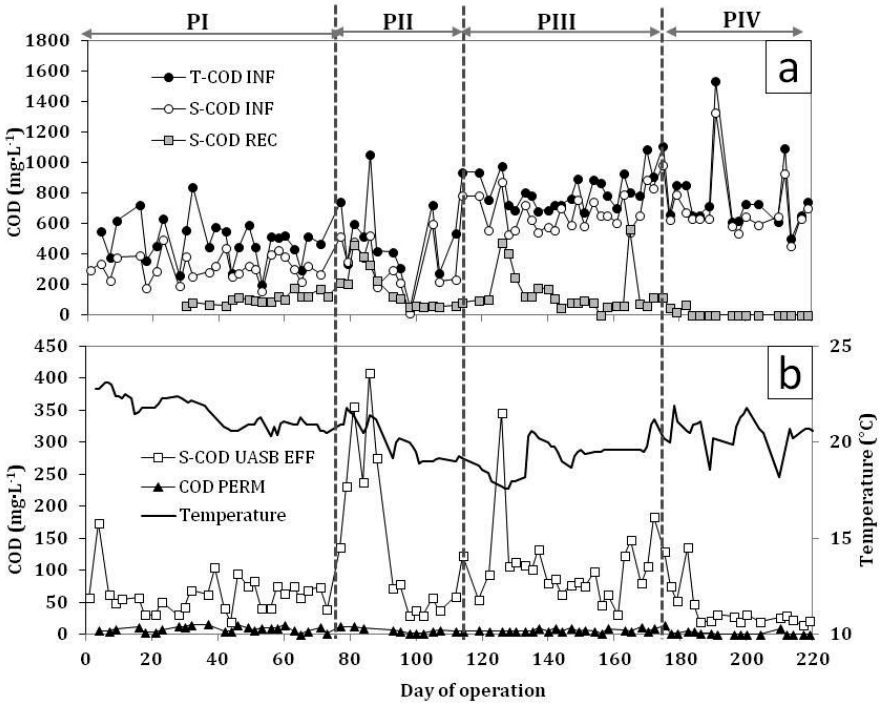

Figure 3-2 Variations of COD concentrations in the combine UASB-MBR during Periods I, II, III and IV. Figure a: (●) Total COD in the feeding, (○) Soluble COD in the feeding, (■) Soluble COD in the recirculation; Figure b: (□) Soluble COD in the UASB effluent, and (▲) Soluble COD in permeate, (—) temperature.

During the first 114 days of operation (Period I and II) large variations of the total COD fed to the reactor were observed, since the synthetic wastewater was kept at environmental temperature and subjected to a rapid and uncontrolled degradation. Therefore, from day 114 on, the feeding was stored in a fridge and unwanted degradation was not observed anymore. Nevertheless, the average COD concentration measured in the permeate in Periods I and II was very low, being around 10 $mg \cdot L^{-1}$.

In Period II strong fluctuations of the s-COD in the chambers were caused by various factors. Between operating days 78 and 98 of, additional 300 $mg \cdot L^{-1}$ COD as methanol were introduced into the system with the feeding, causing the increase of OLR fed to the UASB stage (figure 3-4). This additional load was not completely degraded anaerobically, therefore the OLR fed to the MBR also increased and accumulation of s-COD in the chambers (figure 3-2) and partial inhibition of the methanization stage

(figures 3-5 and 3-6) occurred. Consequently, the COD elimination, as well as overall system performance, were affected. As a result, the s-COD removal rate in anaerobic reactor decreased drastically, reaching values below 20% (figure 3-5), however no VFAs were detected in the UASB effluent (detection limit being 20 $mg \cdot L^{-1}$), indicating that no intermediate products accumulation occurred. On the other hand, the biogas composition analysis showed a diminution of methane percentage from more than 70% to less than 50% (see section 3.4.3).

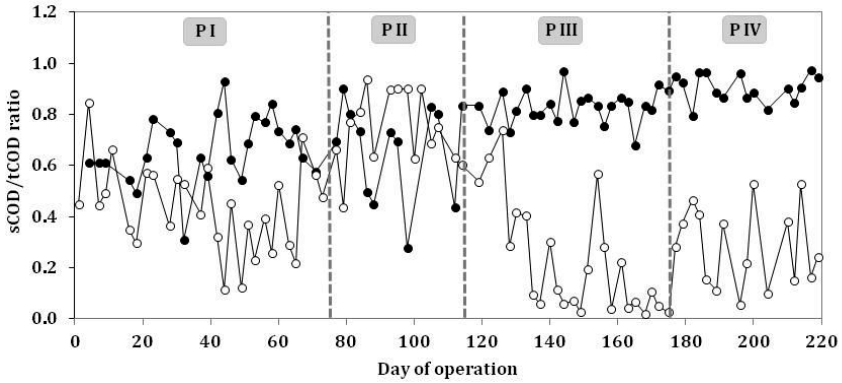

Figure 3-3 Soluble COD to total COD ratio during 220 days of operation of the combined UASB-MBR system. (●) s-COD/t-COD in the influent, (○) s-COD/t-COD in the UASB effluent

On the other hand, the above mentioned COD overload in MBR stage at the beginning of Period II caused a decrease of DO concentration (from 3.5 to less than 1.0 $mg \cdot L^{-1}$). Moreover, an accumulation of t-COD in MBR stage (data not shown) occurred, as a consequence of the elevated solids concentration in the UASB effluent and recirculation, caused mainly by a partial anaerobic biomass wash-out. Therefore, from the day 98 onwards the concentration of methanol was decreased to the tenth part, and the efficiency of the system was recovered, reaching s-COD removal of 70% in UASB stage and more than 95% in the entire UASB-MBR system. Methane reached more than 70% of the biogas composition.

In Period III the COD concentration in the feeding was increased from 900 to 1200 $mg \cdot L^{-1}$. The OLR fed to the UASB stage was 1.51 $kgCOD \cdot m^{-3} \cdot d^{-1}$ in terms of total COD. However, washed-out anaerobic biomass together with a fraction of excess aerobic sludge were continuously recycled to the UASB stage. If the COD load from recycled solids was taken into account, the OLR fed to the UASB stage would be much higher, reaching almost 8 $kgCOD \cdot m^{-}$

$^{3}\cdot d^{-1}$. As a consequence, the elevated concentrations of total and soluble COD were detected, first in the recirculation and then in the effluent from the UASB stage (figure 3-2). Moreover, s-COD concentration in the recirculation was higher than that measured in the UASB effluent. This fact was probably caused by the hydrolysis of particulate COD in the sludge accumulated at the bottom of the first aerobic chamber of the MBR stage.

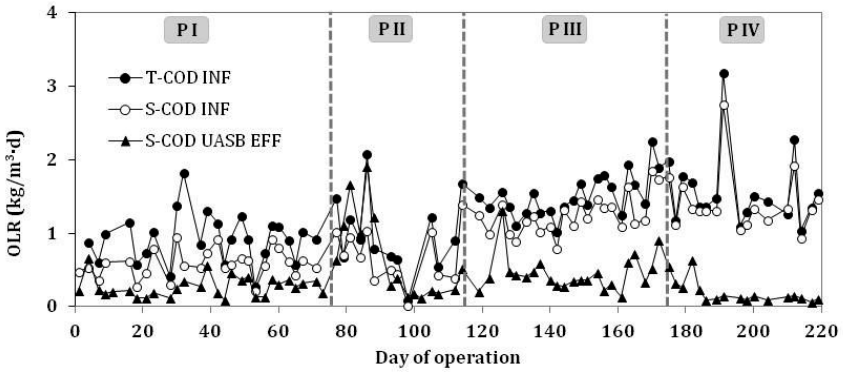

Figure 3-4 Organic Loading Rate fed to the UASB stage in terms of total COD (●), and soluble COD (○) and OLR in terms of soluble COD fed to the MBR (▲).

Nevertheless, from day 134 soluble COD concentration measured in UASB effluent gradually diminished to the values previously observed, indicating that the efficiency of the system was recovered and no further s-COD accumulation occurred (figure 3-2). However, t-COD measured in the recirculation and the UASB effluent kept increasing, so from day 141 on the system was purged again. The purges were realized from the 5th sampling port of the UASB (were the biomass concentration was around 31.0 gMLVSS$\cdot L^{-1}$) and the filtration chamber. Even so, the punctual appearance of suspended solids was still observed in both recirculation and the UASB effluent. Anyhow, the efficiency of the s-COD removal was recovered, reaching over 99% in the entire system, while around 80% was removed in the anaerobic bioreactor (figure 3-5). The t-COD removal during this phase of Period III was around 66% and more than 99% for the UASB stage and the entire system, respectively.

In Period IV the recirculation from the aerobic biofilm chamber to UASB stage was turned off in order to check the impact of the implementation of this strategy on the MBR stage performance. As a result, the t-COD concentration measured in the effluent form UASB stage diminished, with

occasional peaks of high COD concentration, observed usually after the weekend, when the UASB chamber was not purged. Moreover, occasional wash-out of biomass occurred. The average OLRs in terms of soluble COD applied to the UASB stage and MBR stage were 1.42 and 0.20 $kgCOD \cdot m^{-3} \cdot d^{-1}$, respectively.

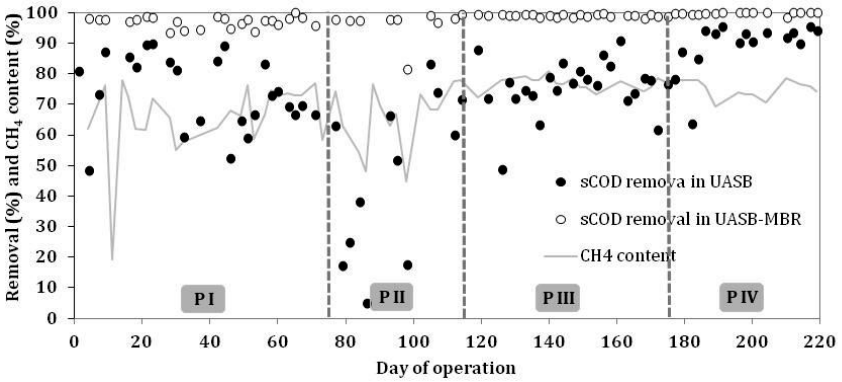

Figure 3-5 Elimination of COD in the UASB-MBR system during Periods I, II, III and IV. (●) Soluble COD removal efficiency in the first methanogenic UASB stage, (○) soluble COD removal in the whole system. (—) methane content in biogas produced in UASB reactor.

Very low concentrations of s-COD were measured in the effluent from UASB stage, being always below 10 $mg \cdot L^{-1}$. As it can be seen in figure 3-5, more than 95% of s-COD was eliminated in the anaerobic stage. COD measured in the permeate was usually below the detection limit and did not vary during the whole experimental period. The differences observed between s-COD in the chambers, and that observed in the permeate indicated that either further organic matter degradation occurred, or the membrane retained a fraction of colloidal matter present in the mixed liquor. However, this fraction was significantly lower in comparison with the previous operating periods.

Evolution of total Dissolved Organic Carbon (t-DOC) was similar to that observed for s-COD. The average removal rate in UASB reactor was around 77% during Period I. It decreased around 10% during Period II, most certainly due to the higher load caused by methanol addition and was recovered after diminishing to the tenth part the methanol fed. During the last period it reached 82%. Taking into consideration the whole operating system the t-DOC removal rate was constant during the whole operating period reaching values between 90 to 96%.

3.4.3 Biogas production

Biogas production in the UASB chamber was detected during the whole experimental period, but its production rate was not quantified with the required accuracy. Approximate amounts of biogas produced were estimated, being around 21.3 $L \cdot d^{-1}$ between day 100 and 140, and 13.6 $L \cdot d^{-1}$ between day 154 and 192.

Biogas production was observed within the whole experimental period. During Period I, especially at the beginning, strong variations in the biogas composition were observed (figure 3-6). Around day 10 high content of nitrogen and diminution of methane production were caused by the failure of the tubing with alimentation and introduction of air to the anaerobic chamber. On the other hand, strong variations on the composition of synthetic wastewater and recirculation from aerobic chamber might have led to the occurrence of denitrification in the UASB stage. This fact also influenced the methane production, since denitrifiers are strong competitors for methanogenic bacteria.

According to An *et al.* (2008) the C/N ratio required for complete NO_x^--N reduction to nitrogen gas by denitrifying bacteria depend on the nature of the carbon source and the bacterial species. A COD/NO_x^--N ratio 2.5 – 6.0 enables complete NO_x^--N reduction to elemental nitrogen. In the case of the present study, this ratio was much higher, since the concentration of nitrates in the feeding was between 10 and 15 $mg \cdot L^{-1}$ (section 3.4.4.1) and COD concentration varied from 200 to 1200 $mg \cdot L^{-1}$ (section 3.4.2). Even taking into account the nitrates introduced to the UASB chamber via recirculation, while nitrification in the aerobic chamber occurred, the COD/NO_x^--N ratio was at the range of 40. This fact confirms that denitrification could take place in the UASB chamber. However, apart from the presence of nitrogen gas in the biogas, no total nitrogen removal was observed in the system.

The diminution of methane content observed in Period II, could probably be explained by the toxic properties of methanol versus anaerobic bacteria (Enright *et al.*, 2005), especially considering that the system was operated under psychrophilic conditions. When the amount of methanol introduced to the system was significantly diminished, the activity of anaerobic

biomass was recuperated and methane content in biogas reached values observed previously, being more than 70%.

On the other hand the elevated nitrogen concentration in the produced biogas observed in periods I and II could be an effect of either desorption of N_2 present in the influent or denitrification of nitrates fed and recycled to the UASB stage.

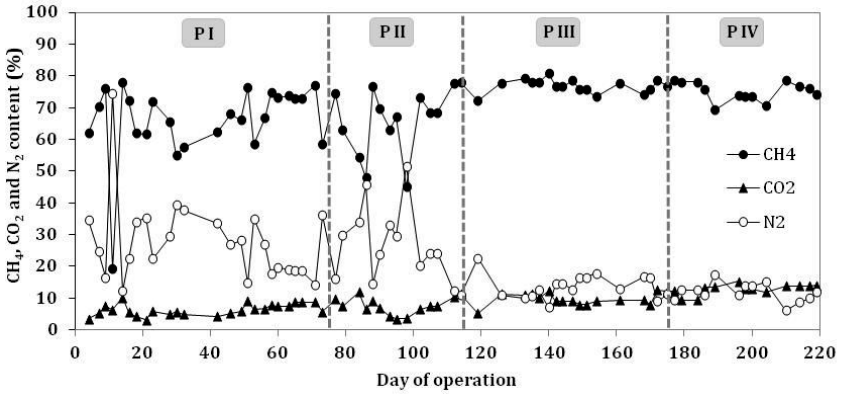

Figure 3-6 Biogas composition during 220 days of operation of the system. (●) CH_4 concentration, (○) N_2 concentration, (▲) CO_2 concentration.

During Period III and Period IV stable production of biogas was observed, with more than 70% of methane, in various points reaching 80% (figure 3-6).

3.4.4 Macronutrients removal efficiency

The bioreactor operated as indicated in section 3.3 is not suited for nutrients elimination. However, nitrogen conversion to either ammonia or nitrates is possible, depending on the effluent quality requirements.

3.4.4.1 Nitrogen conversion

During the first 114 days of operation (Periods I and II) the average concentration of NH_4^+-N in UASB effluent was between 10 and 20 $mg \cdot L^{-1}$, apart from the first days of Period I, when additional ammonia was fed to the system (up to 70 $mg \cdot L^{-1}$) . Almost all the ammonia produced due to protein hydrolysis in the anaerobic system was detected in the permeate

(figure 3-7). However, also a low fraction of nitrate, being around 5 $mg \cdot L^{-1}$, was present in the permeate. It would indicate, that although negligible, nitrification occurred in the system. Moreover, all nitrates introduced to the system with the feeding (5.81±3.01 and 7.94±6.79 $mgNO_3^-\text{-}N \cdot L^{-1}$ for Period I and Period II, respectively) and recirculation (3.27±2.08 and 4.01±3.05 $mgNO_3^-\text{-}N \cdot L^{-1}$, for Period I and Period II, respectively) were denitrified in the anaerobic UASB stage.

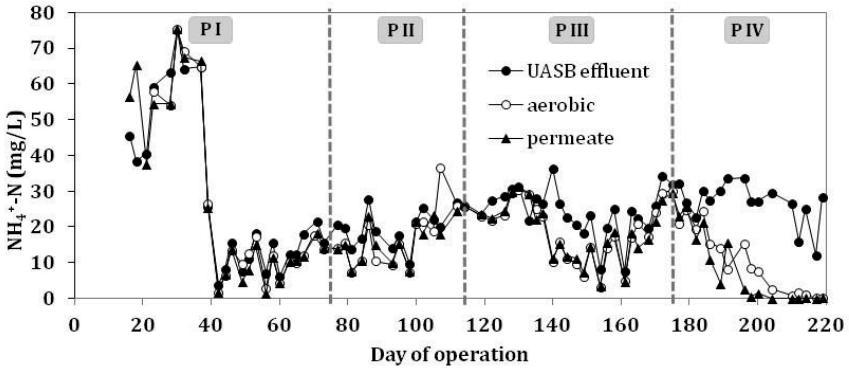

Figure 3-7 Ammonia concentrations during 220 days of the system operation in (●) influent, (○) aerobic chamber and (▲) permeate.

As mentioned previously (section 3.3.1), due to the PPCP elimination study methanol was introduced to the system. This fact caused a partial inhibition of methanogenesis, resulting in a decrease of methane production. This in turn favoured the domination of denitrifiers, which was reflected in the increase of nitrogen gas content in the biogas (section 3.4.3, figure 3-6).

From day 114 onwards (Periods III and IV), when the feeding COD was increased, ammonia concentration detected in the UASB effluent was between 20 and 35 $mg \cdot L^{-1}$ (figure 3-7).

During Period III nitrification in the aerobic chamber was observed, from day 140 onwards (figures 3-7 and 3-8). The concentration of nitrates measured in that chamber increased and the peak of 22 $mg \cdot L^{-1}$ can be observed (figure 3-8). It coincides with the increase of temperature from 18 to 20 °C. On the other hand, the appearance of nitrification had a strong influence on membrane performance (section 3.4.6). Therefore, from day 141 on the purges of biomass were restarted, resulting in the gradual

decrease of the NO_3^--N formation and increase of NH_4^+-N concentration in the aerobic chamber and permeate.

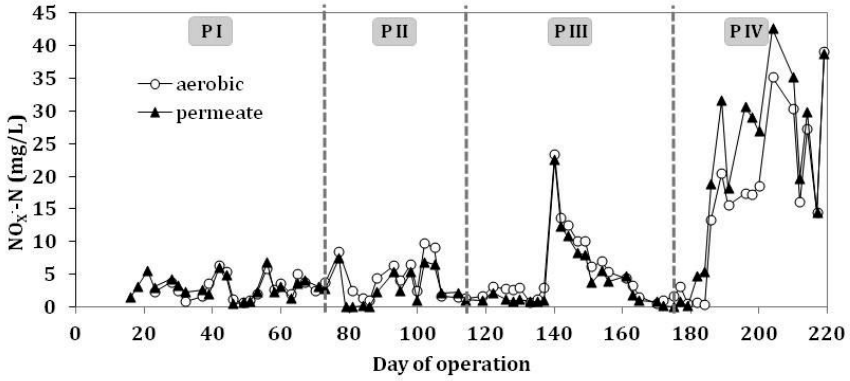

Figure 3-8 Nitrate concentrations during 220 days of the system operation in (○) aerobic chamber and (▲) permeate.

During Period IV, the recirculation from aerobic MBR chamber to UASB stage was turned off. As a result, the efficiency of UASB stage increased and thus the OLR fed to the MBR chamber (in terms of soluble COD) diminished to 0.20±0.16 kgCOD·m^{-3}·d^{-1}. Due to the combined effect of the SRT increase (no sludge recirculation) and low COD concentration in the MBR stage the nitrification process developed. Figure 3-7 shows that from day 203 negligible concentrations of ammonia were measured in the aerobic chamber and permeate. Taking into account that the average concentration of ammonia produced by the hydrolysis of proteins was between 25 and 35 mg·L^{-1}, equal amounts of NO_3^--N should be observed in the aerobic chamber (and permeate). However, the concentration of nitrates was slightly higher, which could indicate that additional nitrification of ammonia generated due to particulate COD hydrolysis occurred.

An *et al.* (2008) suggested that production of nitrite and nitrate is affected by the C/N ratio. Similar observation had been reported lately (Zhang *et al.*, 2005; Chiu *et al.*, 2007). Additionally, Yang *et al.* (2004) reported that relatively high substrate C/N ratios might favour growth of heterotrophic bacteria populations and hence affect nitrification performance (e.g. with C/N>3 the activity distribution of nitrifying bacteria is lower than 40%). In the case of present work, the average C/N ratios expressed as TOC/TN were 1.93, 2.38, 2.41 and 0.74 for Periods I, II, III and IV, respectively. As discussed

previously in this section, full nitrification could be obtained only in Period IV, when almost complete COD removal was realised in UASB chamber. This fact is in tune with the statement proposed by other authors.

3.4.4.2 Phosphorus conversion

During the whole operating period no phosphorus elimination was observed in the system (data not shown). Slightly higher concentrations of phosphates measured in the aerobic chamber and permeate were the effect of the hydrolysis of proteins in the anaerobic stage of the system. In the case of total phosphorous curious observation was made; significantly higher concentrations were measured when the supplier of the milk used for the synthetic feeding was changed.

3.4.5 Membrane performance

During 220 days of operation the flux was maintained between 12 and 15 $L \cdot m^{-2} \cdot h^{-1}$, with the only significant variations observed in Periods II and III, due to the higher membrane fouling (figure 3-9). Nevertheless, this worsening of the membrane performance occurred during these two periods due to overloading and nitrification start-up. In general, flux achieved in the proposed combined UASB-MBR system was higher than those observed in anaerobic membrane bioreactors, being between 5 and 10 $L \cdot m^{-2} \cdot h^{-1}$ (Spagni *et al.*, 2010; Zhang *et al.*, 2007; Lew *et al.*, 2009; Ho & Sung, 2010), but lower than those typically reported in aerobic membrane bioreactors operating with similar membrane modules, being between 20 and 25 $L \cdot m^{-2} \cdot h^{-1}$ (Judd, 2002; Wen *et al.*, 2004). On the other hand, fluxes observed were much lower than those referred by Leikness *et al.* (2007) of 50 $L \cdot m^{-2} \cdot h^{-1}$ working with a biofilm membrane bioreactor with a first Moving Bed Bioreactor (MBBR) followed by a filtration chamber connected in series.

Permeabilities between 100 and 200 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ were normally observed during the four operational periods (figure 3-10). These values were slightly better than those observed during the operation of similar membrane modules (Judd, 2002; Bouhabila *et al.*, 2001), and higher than permeabilities observed in anaerobic membrane bioreactors (Spagni *et al.*, 2010; Zhang *et al.*, 2007). After a chemical intensive cleaning on day 57, permeability rise up to 400 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ whereas the original permeability of the membrane

was only 250 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$. Therefore the behaviour of the membrane in Period I was possibly related with the lower initial permeability of the membrane module used.

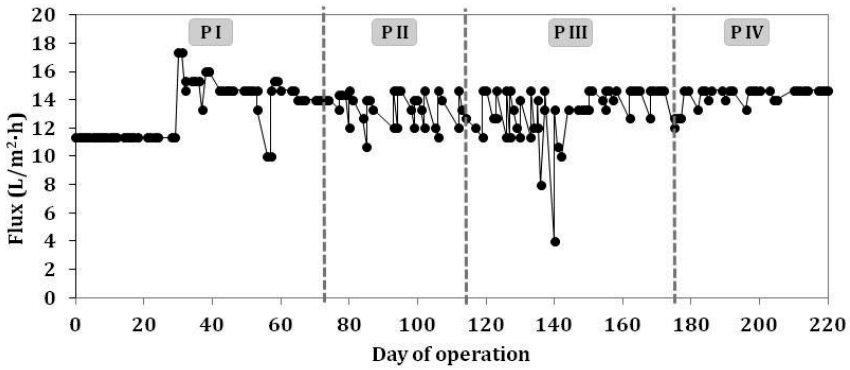

Figure 3-9 Membrane flux variations during 220 days of operation of the combined UASB-MBR system.

Carbohydrate fraction of the Soluble Microbial Products (SMPs) has been reported as the main contributor to membrane fouling because of its hydrophilic properties (Liao *et al.*, 2001). Recent studies have reported a pool of non-filterable organic matter in the liquid phase of the MBR sludge mixture much larger than SMP (Wang *et al.*, 2007; Wang & Li, 2008). These substances have been called biopolymer clusters (BPCs) and are suppose to be an important factor in the formation of the sludge fouling layer on the membrane surface and the increase of fouling potential (Wang & Li., 2008; Sun *et al.*, 2008). Therefore, not only carbohydrate fraction of SMP (data not shown) but also BPCs were followed in order to obtain a reliable fouling indicator.

Fouling rate was calculated as rate of permeability decline and expressed in terms of the increase of TMP with time (Pa/min) observed maintaining the flux constant. Fouling rate was calculated during period III and increased when either BPCs or SMPs increased. Nevertheless, this tendency was clearer when fouling rate was represented versus BPCs (3-10) than when it is versus SMPs (figure not shown). The higher BPC concentrations were observed between experimental days 130 and 145 of Period III, which overlapped with a worsening of the membrane performance. This period coincided with an increase of biomass concentration in the reactor due to the stopping of purges of biomass. On the other hand lower values of the fouling rate were observed when biomass purge was restarted. Broader

discussion on the SMP and BPC influence and correlations with membrane performance of proposed combined UASB-MBR system was presented by Sánchez (2013). Moreover, these influences with respect to lab-scale AnMBR will be revised in Chapter 5.

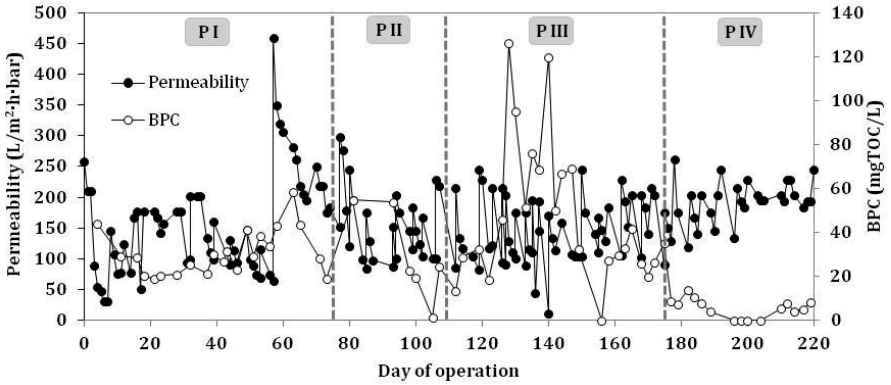

Figure 3-10 Membrane permeability evolution (●) and BPC concentration (○) in the filtration chamber of MBR stage of combined UASB-MBR system.

Membrane critical flux was determined and the value at which irreversible fouling occurred was 19 $L \cdot m^{-2} \cdot h^{-1}$. The criterion employed was that the increment of TMP with respect to time was higher than 10 Pa/min (Le-Clech *et al.*, 2003). During the whole experimental period, the flux applied was below critical flux, thus it was expected that predominant fouling was reversible. In fact, during all the operation permeability was almost fully recovered when a mechanical cleaning was carried out.

3.4.6 Life Cycle Assessment of combined UASB-MBR system

A brief ranking of MBR configurations studied by Hospido et al. (2011) is presented in table 3-1. Eutrophication has been identified as one of the most important impact categories to evaluate the efficiency of wastewater treatment of the plant. In comparison with the other three MBR reactors the combined UASB-MBR system took third position, with the pre-denitrification MBR being the best, and two stages aerobic MBR the worst contributor (table 3-1). Regardless of the reactor evaluated, the main impact in this category had the direct release of nutrients present in the treated water. Detailed information about the distribution of the substances released by the UASB-MBR system is presented in table 3-2. It can be seen

that ammonia and phosphorus contributed the most. The outstanding significance of ammonium release is likely to be due to the hydrolysis of the proteins present in the feeding in UASB stage, and the poor nitrification in the aerobic stage, which was taking place during the period inventoried. However direct release to the environment would not be advisable, this nutrient rich effluent could be suitable for e.g. agricultural reuse.

Table 3-1 Environmental ranking of MBRs under study (gray scale: black = worse configuration and white = best configuration).

	Two-stages aerobic MBR	Pre-denitrification MBR	Modified UCT1 system	Combined UASB-MBR
Eutrophication				
Global warming				
Acidification				
Terrestial ecotoxicity				
Freshwater ecotoxicity				

On the other hand, the UASB-MBR system had the lowest contribution in the emission of airborne acidifying chemicals, being 12 $gSO_2eq \cdot m^{-3}$, while the maximum value was noted for the two stages aerobic MBR (more than 30 $gSO_2eq \cdot m^{-3}$). The principal acidifying pollutants are mainly generated in a process of electricity production, therefore the proposed UASB-MBR has an advantage of energy recovery due to the generation of biogas. This benefit also contributed to the lower terrestrial and aquatic toxicity, compared to other MBRs evaluated. Nevertheless, the indirect impact associated with the energy production was pointed out as the main contributor for all the configurations.

Table 3-2 Distribution of the eutrophication impact among the substances released within the treated water

Parameter	Eutrophication impact
Phosphate (%)	58.4
Nitrate (%)	4.16
COD (%)	0.65
Ammonium (%)	36.8

In the case of global warming, compared with other MBRs evaluated, the UASB-MBR system occupied a second place (1.34 kg CO_2eq·m^3), right behind the modified UCT1 system with the lowest global warming impact (1.3 kg CO_2eq·m^3), followed by pre-denitrification MBR (2.2 kg CO_2eq·m^3). Two stages aerobic MBR presented an outstanding impact with values reaching 5.4 kg CO_2eq·m^3. Apart from energy use, the proposed UASB-MBR system presented negative values, which implies beneficial consequences (Hospido *et al.*, 2012). The reason behind that fact was the avoided production of energy from the generated biogas. However, this assumption does not take into account the impact of dissolved methane, which reaches the atmosphere due to the stripping from the UASB effluent. Methane has a global warming potential 25 times higher than carbon dioxide. For low strength wastewaters, which is the case for the proposed UASB-MBR system, dissolved methane might account up to 50% of the produced methane. Thus the use of anaerobic technology combined with aerobic post-treatment could increase greenhouse gas (GHG) emissions.

3.5 CONCLUSIONS

The combined UASB-MBR system achieved excellent COD removal performance, comparable with aerobic MBRs treating domestic wastewater. On average, the permeate COD was less than 6 mg·L^{-1} and the s-COD removal was above 95%, reaching 99% during the stable operation. Additionally, the effluent was free of suspended solids.

Biogas production was detected during the whole operating period, with average methane content of 75 – 80%.

Nutrient elimination was not observed during the operation of combined UASB-MBR system. However, ammonia conversion to nitrates is possible, if desired.

With respect to the performance of the membrane, the highest permeabilities were achieved in the periods when the biomass was purged of the system. The predominant fouling that took place in the membrane was reversible fouling, since permeability was recovered with mechanical cleaning. BPCs concentration was reported as a reliable parameter related with fouling, by decreasing permeability when BPC concentration increased. The membrane operated with fluxes of 15 $L \cdot m^{-2} \cdot h^{-1}$, lower than those achieved in other MBRs treating municipal wastewater, but higher than fluxes obtained in anaerobic MBRs.

In the case of LCA analysis the main contributor in eutrophication was the direct release of nutrients present in the treated water. For global warming the avoided production of energy from the generated biogas was the reason behind the negative values, which implies beneficial consequences. However, this assumption does not take into account the impact of dissolved methane stripped off the UASB effluent.

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Chapter 4

Application of combined UASB-MBR system in the treatment of dairy wastewater[1]

[1] Parts of this chapter were published in the following publications:

A. Sánchez, **D. Buntner** and J. M. Garrido (2013). *Impact of methanogenic pre-treatment on the performance of an aerobic MBR system*. Water Research, Vol 47 (3), pp 1229–1236.

D. Buntner, A. Sánchez and J. M. Garrido (2013). *Feasibility of combined UASB and MBR system in dairy wastewater treatment at ambient temperatures*. *Submitted to Journal of Chemical Engineering.*

SUMMARY

In this Chapter the feasibility of the combined UASB and MBR system for the treatment of dairy wastewater at ambient temperatures was investigated. As in **Chapter 3**, the system consisted of a methanogenic UASB stage and two-compartment post-treatment aerobic MBR stage, with a membrane ultrafiltration module. The objective of the system was to decrease the COD of dairy wastewater, producing a methane rich biogas, diminish overall sludge production, and to obtain high quality effluent due to the implementation of a membrane filtration stage. Since in **Chapter 3** the proposed UASB-MBR system was proved to be feasible for the treatment of low strength wastewater with the average organic loading rate of 1.25 $kgCOD \cdot m^{-3} \cdot d^{-1}$, in this Chapter higher ORLs were applied. The system presented a high tolerance to loading changes (up to 3.9 $kgCOD \cdot m^{-3} \cdot d^{-1}$) and temperature fluctuations (17 – 25 °C). Moreover, the impact of internal recirculation on MBR stage and an overall system performance was studied. The average total and soluble COD removals were above 95%, reaching 99% during the stable operation. The observed overall biomass yield was low, from 0.13 to 0.07 $gVSS \cdot gCOD^{-1}$. Biogas production yield reached 150 $L \cdot kg^{-1}$ of t-COD, with an average methane content of 73%. With respect to membrane performance, permeability values between 140 and 225 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ were obtained, similar to those reported for aerobic MBR systems. The average flux obtained was 13 $L \cdot m^{-2} \cdot h^{-1}$, reaching 19 $L \cdot m^{-2} \cdot h^{-1}$ in stable operation depending on operating conditions. These values were lower than those observed in aerobic MBR systems, but much higher than those referred for methanogenic AnMBRs

4.1 INTRODUCTION

4.1.1 Dairy sector and its characteristics

Dairy sector is among the most polluting of the food industries in terms of water consumption (BREF 2006). Waste water comes from various sources: as a result of water utilization during processing and cleaning and from the drying of dairy materials. In well managed facilities the generation of wastewater is in between 1 and 2 L per litre of processed milk (BREF 2006, Tawfik *et al.*, 2008), however it can reach even 10 L. Apart from high COD content, variable pH and temperature, dairy wastewater is characterised by the presence of solids (gross and finely dispersed/suspended), oil and grease, volatile substances (e.g. ammonia and organics), macronutrients such as phosphorus and/or nitrogen, pathogens (e.g. from sanitary waters), heavy metals and dissolved non-biodegradable organics (BREF 2006). Taking into account the complexity of dairy effluents it is crucial that they receive an efficient treatment, before reaching the environment.

Most on-site installations of dairy sector uses the following techniques as a primary treatment: screening, flow and load equalisation, neutralisation, sedimentation, Dissolved Air Flotation (DAF), centrifugation, and precipitation. As a secondary treatment, for lower strength waste water aerobic treatment is applied and for the streams with a BOD concentration greater than 1000 – 1500 mg/l, anaerobic treatment processes are used (BREF, 2006).

The majority of dairy industries situated in Galicia (Spain) dispose of a physical stage – most commonly DAF for removing fats and solids – followed by a Conventional Activated Sludge (CAS) systems (Arrojo *et al.*, 2002). The typical characteristics of such wastewater are the temperature between 17 and 32 ºC and an average COD around 2500 mg/L before, and 1350 mg/L after DAF. Nevertheless, even though the CAS effluents fulfil the legislation requirements, the application of this technology demonstrates a few significant disadvantages, such as high energy consumption, high sludge production and, consequently, high costs of sludge treatment and disposal.

4.1.2 Anaerobic treatment

During the last years the interest in anaerobic treatment methods for dairy wastewater has been increasing (Öztürk *et al.*, 1993; Tawfik *et al.*, 2008; Kushwaha *et al.*, 2011), because of their well-known advantages in treatment of effluents with relatively high concentrations of organic matter. There is no need for aeration equipment, energy investment is low and there are much lower quantities of excess sludge compared to aerobic processes (Ghaly & Pyke, 1991; Córdoba *et al.*, 1984). Moreover, the biogas generated during anaerobic fermentation may be used for the production of heat and/or power. The Up-flow Anaerobic Sludge Blanket (UASB) is by far the most widely used high rate anaerobic system for anaerobic sewage treatment, and a number of full-scale UASB systems have been installed worldwide (Fang *et al.*, 1990; Tran *et al.*, 2006). However, for the dairy sector, the application of anaerobic wastewater treatment is largely confined to relatively heavily polluted waste water with a COD between 3000 and 40000 mg/l (BREF, 2006).

For low strength wastewaters, as those generated in most of the dairies in Galicia, the heat gained by biogas combustion is insufficient for obtaining a significant temperature increase. Theoretically, a temperature increase of only 3 ºC is possible per g COD methanised. Thus, these systems should be operated at environmental temperatures (van Haandel & Lettinga, 1994) or as much as possible, at the temperature the wastewater is generated.

The main drawbacks of this kind of systems are related with the capacity loss, especially at low temperatures, and by the quality of the treated effluent that depending on the legal requirements, should be post-treated in order to diminish BOD, TSS and pathogens. An anaerobic system alone would not achieve a final waste water quality high enough for discharge to a watercourse. Therefore anaerobic installations are usually followed by an aerobic system. The energy gained from the anaerobic plant can be equivalent to that consumed by the aerobic step (BREF 2006). Aerobic process is particularly suitable for waste water with a low solid content and with relatively low COD levels (<2000 mg/l) and when small surface area is available. Anaerobic sludge-bed reactors followed by aerobic treatment are currently the most widespread combination used in the dairy sector (Kushwaha *et al.*, 2011).

4.1.3 Membrane technology as a post-treatment

Organic matter removal, although crucial in dairy wastewater treatment, is not enough if the discharge to the environment is considered. Membrane Bioreactors (MBRs) has been widely utilised as an efficient solution for wastewater treatment. The main advantages of the MBR process are the absolute control of solids and hydraulic retention time (HRT). The effluent quality is high, with very low microbial indicators, and MBRs are not affected by biomass wash out or bulking (Stephenson, 2000). Limitations inherent to MBR processes are the cost of membranes and operative costs due to fouling and higher energy consumption compared to traditional Wastewater Treatment Plants (WWTPs). Over the last decade, the adaptation of membranes coupled with anaerobic biological processes has made Anaerobic Membrane Bioreactors (AnMBR) a promising alternative to conventional wastewater treatment, allowing to achieve a similar results to aerobic MBRs (Hu & Stuckey, 2006). However, in the case of AnMBRs the fluxes tend to be lower than those reported for aerobic MBRs – most of the authors reported fluxes in the range of 5 - 15 $L \cdot m^{-2} \cdot h^{-1}$ at temperatures above 30 °C (Zhang *et al.*, 2005; Saddoud *et al.*, 2007; Trzcinski & Stuckey, 2009). Moreover, the flux diminishes with the temperature decrease of the anaerobic process. Feasible flux has a strong influence on both the capital and operation costs of the process.

Implementing the membrane technology to obtain high quality effluent opens the door to the possible reuse of generated streams. Some dairy production activities have special requirements of the natural resources, such as the need for receiving waters for the discharge of large volumes of treated wastewater (BREF 2006). The treatment of these selected dairy wastewaters with the aim of water reuse could simultaneously lower the total water consumption and the effluent production of the dairy plant (Vourch *et al.*, 2008). Thus, the purified water produced by membrane treatment could be reused in the dairy factory as heating or cooling water or for cleaning purposes, depending on the quality standards.

4.2 OBJECTIVES

The aim of this study was to investigate the feasibility of a combined UASB-MBR system for the treatment of dairy wastewater at ambient temperatures. The proposed system consisted of: a first methanogenic UASB stage, and a second MBR stage. MBR stage was composed of two chambers: aerobic chamber with biofilm growing on small carrier elements maintained in suspension, and membrane filtration chamber. The objective of the first methanogenic stage was to diminish the COD of the dairy wastewater, producing a biogas with high methane content. In the second stage, the remaining soluble biodegradable COD was oxidized by the heterotrophs and finaly, the membrane filtration assured high quality effluent. Moreover, the membrane module could be operated at higher fluxes than those reported for AnMBRs, and closer to those obtained in aerobic MBRs treating anaerobic wastewater. In general, the concept of proposed UASB-MBR system was to join the advantages of the methanogenic and aerobic membrane bioreactor processes, by significantly reducing the incoming COD load, producing biogas rich with methane, that could serve as an additional energy source, and a high quality permeate, feasible for reuse.

Two different operation strategies of combined UASB-MBR were studied: First, the system was operated as two reactors connected in series, with the first UASB stage, and second MBR stage. The main idea was to check if anaerobic treatment followed by the aerobic polishing step would be sufficient to obtain high quality effluent. After that, the sludge recirculation from aerobic chamber of MBR stage to the UASB reactor was turned on, in order to study the benefits and inconveniences of this strategy. By this way, the washed-out anaerobic biomass could be returned to the UASB stage. Moreover, surplus aerobic sludge was subjected to anaerobic digestion and thus the overall sludge generation could be diminished. This second strategy was implied in order to check the effectiveness and the possible advantages over the previous one.

Furthermore, the impact of sludge recirculation on the membrane performance was also analysed.

4.3 MATERIALS AND METHODS

4.3.1 Bioreactor and the strategy of operation

A 176 L combined UASB-MBR system consists of 120 L methanogenic UASB stage and two-compartment post-treatment aerobic MBR stage, with first 36 L aerobic chamber where biofilm is growing both onto plastic support and in suspension, and a second 20 L membrane filtration chamber (figure 3-1). The effluent of the UASB stage was led to the aerobic biofilm stage filled with 18.5 L (50 % of the effective volume) of Kaldnes K3 filter media. Two peristaltic pumps were located in the aerobic chamber, the first was continually pumping the mixed liquor to the membrane filtration chamber, the second was used during part of the experimentation to recycle the suspended biomass to the anaerobic UASB chamber. Finally, in the filtration stage membrane module Zenon ZW10 with a surface area of 0.9 m^2 was employed. This module consists of PVDF hollow-fibre membrane, with a pore size of 0.04 μm. The membrane was operated in cycles of 7.5 min with a permeation period of 7 min and a backwashing period of 0.5 min. The filtration chamber was aerated in order to minimize membrane fouling. The operation of the system was controlled by a PLC (Siemens S7-200) connected to a PC. Trans-membrane pressure (TMP) data was measured with an analogue pressure sensor (Efector500 PN-2009) and collected in the PC via an analogue PLC module Siemens EM 235.

With the objective of simulate dairy effluents generated after a DAF stage, the reactor was fed using semi-synthetic wastewater composed of diluted skimmed milk, $NaHCO_3$ and trace elements. A comparison of the main parameters of dairy wastewater and synthetic wastewater used in UASB-MBR is presented in table 4-1.

The combined UASB-MBR system was operated for 220 days prior to the start of this research. Before day 0 the system was fed with low-strength synthetic wastewater and the results were presented in Chapter 3. The present study was performed during 292 days and the operation could be divided in three different periods:

Table 4-1 Comparison of typical parameters for dairy wastewater (before and after DAF) and synthetic wastewater used as a UASB-MBR system feeding.

Parameter	Unit	Raw wastewater	DAF effluent	UASB-MBR
COD	$mg \cdot L^{-1}$	2500	1350	2000
BOD	$mg \cdot L^{-1}$	1500	810	800
SS	$mg \cdot L^{-1}$	1000	100	n.d.
P_{total}	$mg \cdot L^{-1}$	40	24	14
N_{total}	$mg \cdot L^{-1}$	90	77	24
Temperature	°C	25 - 38	17 - 32 (26)	17 - 25 (21)

Period I (From day 0 until day 32)

During this period, the recirculation pump from the aerobic MBR chamber to UASB stage was turned off and the system worked as two reactors connected in series. The average HRT of the UASB was 10h, being 14h in the case of the entire system. The COD concentration in the feeding was maintained around 1000 $mg \cdot L^{-1}$.

Period II (From day 33 until day 194)

During this period the recirculation pump from aerobic MBR chamber to UASB stage was turned on, converting the two reactors connected in series into one. This strategy was assayed in order to avoid the anaerobic biomass losses from the UASB stage, as well as to maintain low biomass production in the system, since part of the excess aerobic biomass was hydrolysed in the anaerobic stage. Initially, the recirculation ratio was 0.15, but form day 97 it was set at 0.075. HRT was 15 and 20h in the case of UASB chamber and the entire system, respectively. COD concentration in the feeding was maintained around 1000 $mg \cdot L^{-1}$. In order to check the possibility of nutrient elimination from day 90 onwards anoxic cycles in the aerobic stage were implemented, with the on/off aeration periods of 20/30 min.

Period III (From day 195 until day 292)

The COD concentration was increased step-wisely during this period (by reducing the dilution), in order to check the system capacity. During this period, the recirculation pump from the aerobic chamber to UASB was on. HRT was 11h for UASB and 15h for the entire system. Additionally, two

on/off aeration periods were studied: 40/10 (days 199 – 243) and 30/20 min (days 244 – 292).

4.3.2 Analytical methods

Temperature, pH, alkalinity and the concentrations of Dissolved Oxygen (DO), Volatile Suspended Solids (VSS), total and soluble Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), ammonium, nitrite, nitrate, phosphate and total phosphorous were determined according to the Standard Methods (APHA-AWWA-WPCF, 1999). Concentrations of total Dissolved Organic Carbon (DOC) and total dissolved Inorganic Carbon (IC) were measured with a Shimadzu analyzer (TOC-5000). Biogas composition was measured in a gas chromatograph HP 5890 Series II with the column of Porapack Q 80/100 2m x 1/8" (SUPELCO).

4.4 RESULTS AND DISCUSSION

4.4.1 General observations

The system was operated during 292 days at ambient temperature, (wastewater temperature changed with seasons from spring to winter) varying in the range of 17.5 – 24.5 °C. The average pH values measured in the effluent from UASB stage, aerobic chamber and permeate were 6.7, 7.4 and 7.9, respectively. Alkalinity of the UASB stage was maintained at the approximate level of 500 $mgCaCO_3 \cdot L^{-1}$.

4.4.2 Organic matter removal

Since dairy wastewater is characterised by the relatively high COD content it is important to obtain significant organic matter removal before the effluent could be subjected to the further utilization. In the case of present work, the major fraction of the incoming COD was degraded in the first methanogenic UASB stage, while the subsequent aerobic and membrane filtration chambers served as a polishing step. The mean value of organic

loading rate fed to the UASB stage (in terms of total COD) was 1.95 $kg \cdot m^{-3} \cdot d^{-1}$. The average HRT values were 13 and 18 h for the UASB stage and entire UASB-MBR system, respectively, with variations in the range of 8–28 h for the methanogenic stage and 11–37 h for the entire system. During the whole operation most of the biodegradable soluble COD (s-COD) was removed in the first UASB stage (figure 4-1), with the average elimination efficiency of 95%. However, total COD removal in the UASB stage had a different pattern, since its calculation depended not only on the organic load applied, but also on the presence of solids in the UASB effluent. The overall COD removal efficiency in the combined UASB-MBR system was close to 99% during the 292 days of operation, with the final COD concentrations below 6 $mg \cdot L^{-1}$ in the permeate.

4.4.2.1 Period I – Without biomass recirculation from the MBR to UASB

During this period the recirculation between the aerobic chamber of MBR stage and UASB stage was off. The major fraction of the incoming COD was degraded in the first UASB reactor, and second two-compartment MBR, consisting of aerobic biofilm/suspension chamber and membrane filtration chamber, was used as a polishing step. The main idea was to check if anaerobic treatment followed by the aerobic post-treatment would be sufficient to obtain high COD removal, good quality effluent and lower biomass production than that reported for aerobic treatment.

The average values of the t-COD and s-COD fed into the system during this period were around 960 and 840 $mgCOD \cdot L^{-1}$ (figure 4-1), respectively, with the average s-COD/t-COD ratio being around 0.84 (figure 4-2). This period was characterized by a quite stable operation, with the average removal rates in UASB stage of 66.7 and 88.2% for total and soluble COD, respectively. As a consequence, the organic load introduced to the subsequent MBR stage (taking into account both biofilm and membrane filtration chamber) was very low, being around 0.29 $kg \cdot m^{-3} \cdot d^{-1}$ in terms of soluble COD. Permeate COD concentration was always lower than 6 $mg \cdot L^{-1}$. Therefore, the entire UASB-MBR system reached the average COD removal efficiency of 99.4%.

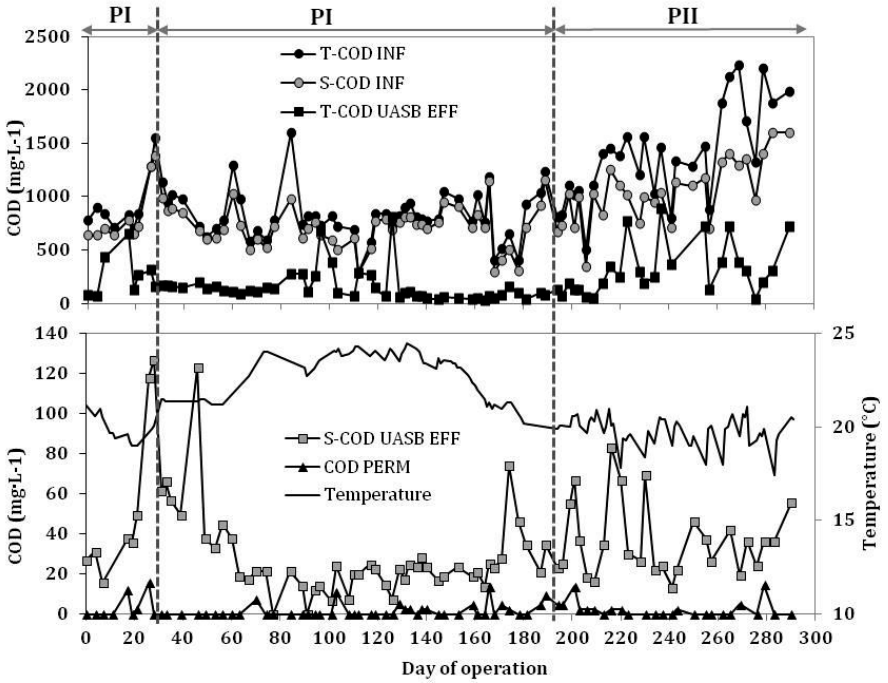

Figure 4-1 COD concentration during the three periods of operation: t-COD in the influent (●), s-COD in the influent (●), t-COD in the UASB effluent (■), s-COD in the UASB effluent (■), COD in the permeate (▲) and the temperature (—).

On day 7 of the operation of the system the HRT of the system was decreased to 8.8 h what caused some anaerobic biomass washout. As a consequence, the t-COD loading rate of the aerobic chamber increased to almost 2.78 $kg \cdot m^{-3} \cdot d^{-1}$ (figure 4-3). Because this tendency was not observed for soluble COD (0.10 $kg \cdot m^{-3} \cdot d^{-1}$), the increase of t-COD concentration was attributed only to the appearance of biomass in the UASB effluent. Moreover, on account of the implementation of membrane filtration the efficiency of the organic matter elimination in the entire UASM-MBR system was not affected, which demonstrates the flexibility and robustness of the system in the case of biomass washout. Nevertheless, due to the occasional appearance of granules in the effluent from UASB stage, on day 13 of operation the HRT was increased to 14.7 h to avoid further loss of anaerobic biomass and diminution of methanogenic capacity of the UASB. This capacity loss could be significant, since during this period the recirculation between MBR and UASB was turned off and the washed-out biomass did not return to the UASB.

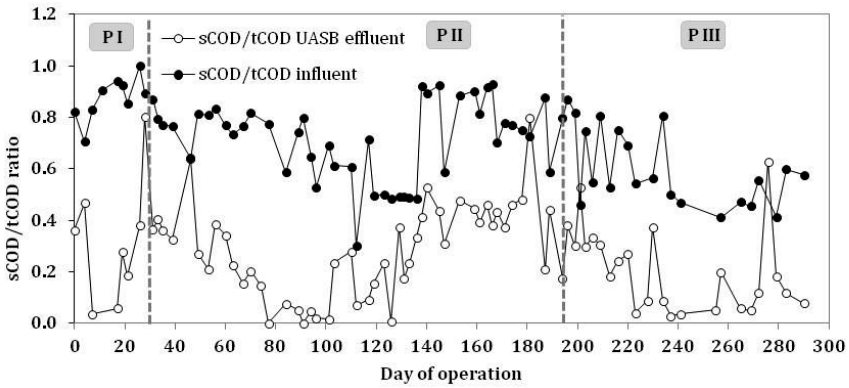

Figure 4-2 Soluble COD to total COD ratio during three periods of UASB-MBR system operation: (●) in the influent, taking into account the COD from the recirculation, and (○) in the effluent form the UASB stage.

The overall biomass yield calculated in Period I was 0.13 kgMLVSS·kgCOD^{-1}, which is in the range of anaerobic systems rather than aerobic (van Haandel & Lettinga, 1994).

To check the resistance of the system to overloads, on day 28 the t-COD concentration fed to the UASB reactor was increased to around 1500 mg·L^{-1}, giving an approximate loading rate of 3.34 kg·m^{-3}·d^{-1} (figure 4-3). This strategy caused a peak of s-COD in the effluent from anaerobic step, however, the overall efficiency of the system was not affected and therefore, the resistance and reliability of the UASB-MBR was confirmed.

4.4.2.2 Period II – Combined UASB-MBR system with recirculation

During this period the recirculation from aerobic chamber of MBR stage to the UASB stage was turned on maintaining the ratio equal to 0.15. This strategy could be used to treat anaerobically the surplus aerobic biomass and in order to return the fraction of washed-out anaerobic biomass to the UASB stage. The HRT was increased and the OLR fed to the UASB diminished to the mean value of 1.35 kg·m^{-3}·d^{-1} and 1.16 kg·m^{-3}·d^{-1} in terms of total and soluble COD, respectively. The average values of the t-COD and s-COD fed into the system were around 1050 and 715 mgCOD·L^{-1}, respectively, with the s-COD/t-COD ratio being around 0.87 (figure 4-2).

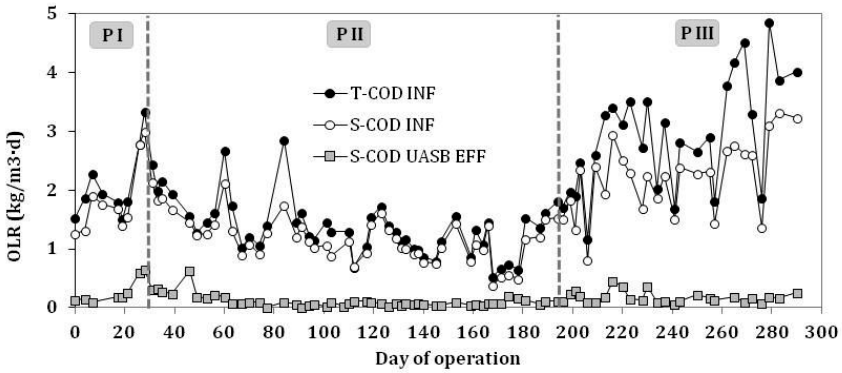

Figure 4-3 Overall Organic Loading Rate during the three periods of UASB-MBR system operation: t-COD in the influent (●), s-COD in the influent (○), s-COD in the UASB effluent (■).

On day 46 of Period II, due to the accidental washout of the anaerobic biomass, the s-COD removal efficiency in UASB stage decreased to 70%, with no influence on the overall system performance. In this case, the aerobic MBR stage served as a buffer, oxidizing the extra load of organic matter. Shortly after this incident the UASB stage reached stable operation with removals of more than 80 and 95% in terms of total and soluble COD, respectively (figure 4-4). This high COD removal led to the insufficient organic matter supply to the aerobic stage - the OLR fed to the MBR diminished to values of only 0.5 kg t-COD·m^{-3}·d^{-1} and caused a decrease of MLVSS concentration to around 0.5 gMLVSS·L^{-1}. This low biomass concentration caused in turn an increase of the fouling of the membrane. Sánchez *et al.* (2013) explain further details of how the applied food to microorganism (F/M) ratio or MLVSS concentration influence the membrane performance in combined UASB-MBR system. Therefore, starting from day 97, the recirculation ratio was decreased to 0.075.

Period II of operation of the system took place during the summer, when the UASB reactor operated at the highest temperatures observed during this research (20 – 25 °C). This was the reason of the higher COD removal efficiency observed in the UASB and the low OLR fed to the MBR. For wastewater treatment using combined UASB and MBR systems, it would be advisable to maintain a minimum biodegradable OLR in MBR stage, and thus F/M ratio, in order to guarantee biomass development and limit membrane fouling. It could be done by feeding a part of the influent to the aerobic chamber, maintaining the minimum biodegradable COD

concentration and allowing suspended biomass development. This strategy could be applied also if the nitrogen removal is desired.

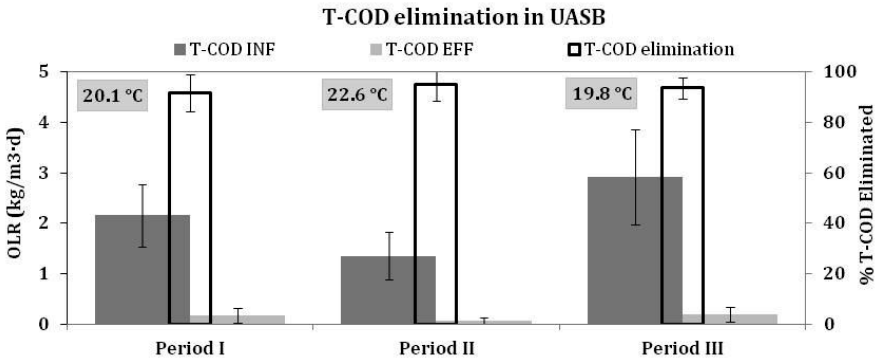

Figure 4-4 The average influent OLR in terms of t-COD and its elimination in UASB stage. The average temperature is indicated in grey box in the left corner of corresponding period.

4.4.2.3 Period III – The influence of organic load on the performance of UASB-MBR system

During this period the average COD concentration was increased step-wisely to approximately 2000 $mg \cdot L^{-1}$ t-COD and 1600 s-COD (with s-COD/t-COD ratio of 0.77, see figure 4-2) in order to check the robustness of the system to overloads, which might be a case for dairy wastewater treatment facilities. The OLR fed into the reactor in terms of total COD was around 1.83 $kg \cdot m^{-3} \cdot d^{-1}$ at the beginning of the period and reached the maximum values of 4.85 $kg \cdot m^{-3} \cdot d^{-1}$ (figure 4-3). The t-COD removal in UASB stage varied from 60 to 99% and in the case of the entire system the COD removal efficiency was always above 99% (figure 4-4). In general, the average soluble COD removal in UASB stage was around 96% along Period III, even considering the applied load.

On day 201 due to the increase of organic load, the soluble COD content in the effluent from UASB stage increased (figures 4-1 and 4-3). Despite causing a decrease of DO concentration in the aerobic chamber (from 3.5 to 0.5 $mg \cdot L^{-1}$), additional biodegradable organic matter enhanced the heterotrophic biomass development and the overall UASB-MBR system performance was not affected.

Around day 222 the system suffered from some operational problems (the recirculation tube was broken), due to which an anaerobic and especially

aerobic biomass loss occurred. To avoid the failure of the system the COD load was diminished by increasing the HRT. As a consequence, almost all the incoming fraction of COD was transformed in the methanogenic stage. Thus, the aerobic biomass was subjected to negligible organic matter load which caused diminution of the MLVSS concentration in the MBR stage.

During the whole Period III occasional anaerobic biomass washout occurred, without any visible influence on the performance of the UASB-MBR system. Due to the implementation of the internal recirculation anaerobic granules were directed back to the UASB stage, allowing to avoid methanogenic capacity loss. Higher ORL guaranteed the minimum organic matter load to the aerobic chamber, which in turns allowed to maintained the optimal MLVSS concentration of approximately 3 $g \cdot L^{-1}$. The efficiency of the system in organic matter removal was very high and combined UASB-MBR system was proved to be flexible and resistant to changes such as OLR picks or biomass washout. Moreover, it was operated at temperature range from 17 to 25 °C, which is lower than that reported for dairy wastewater treatment facilities in Galicia (Spain).

In general, the elimination of organic matter by combined UASB-MBR system was very efficient, being more than 99% for both soluble and total COD, while most of the organic matter was removed in UASB stage alone. Highly efficient UASB systems treating municipal or industrial wastewater at ambient temperatures were reported previously. At 21 °C Lettinga *et al.* (1983) achieved 60 - 80% COD removal, with OLR of 1.6 $kg \cdot m^{-3} \cdot d^{-1}$. At 10 - 18 °C de Man *et al.* (1986) achieved COD removal efficiency between 45 and 60%, working with an UASB reactor treating similar OLR. At the temperature range from 12 to 20 °C the COD removal efficiency was between 30 and 75% (de Man *et al.*, 1988), treating higher OLRs, being approximately 3.5 $kg \cdot m^{-3} \cdot d^{-1}$, and 55% at 20 °C (Tang *et al.*, 1995). The results of COD removal in UASB stage of the combined UASB-MBR system proposed in this work are much better. Moreover, by the addition of the post-treatment MBR stage, the system guarantees high effluent quality together with total solid retention at ambient temperatures. In this sense, the proposed system could be a solution for retrofitting those WWTPs treating low-strength wastewaters or sewage, or some industrial wastewater streams, which relies in the use of the UASB technology at environmental temperatures.

Additionally, a very good linear correlation between ORL fed to the UASB stage of the system and ORL eliminated by this stage was found (figure 4-5), which indicates that the maximum capacity of UASB have not been reached yet. It can also be seen that anaerobic stage of the system was highly efficient regardless of ORL applied, showing its robustness and flexibility.

On the other hand, the employment of internal recirculation from aerobic chamber of MBR stage to the UASB allowed to diminish solids generation in the system and maintain low biomass production, since part of the excess aerobic sludge was hydrolyzed in the methanogenic chamber. Due to the application of this strategy the overall biomass yield calculated for the combined UASB and MBR system in Period III was 0.07 gMLVSS·gCOD^{-1}·d^{-1}, that was lower than that of 0.13 gMLVSS·gCOD^{-1}·d^{-1} calculated for Period I, in which the surplus aerobic biomass was not recycled to the UASB system.

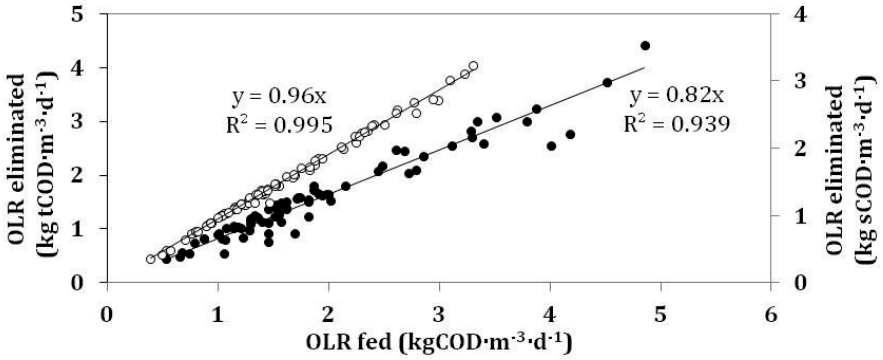

Figure 4-5 The correlation between ORL fed to the UASB stage and ORL eliminated in UASB stage in terms of total COD (●) and soluble cod (○).

4.4.3 Nitrogen conversion

Originally, the proposed system was not designed for nitrogen removal, however during its operation some nitrogen conversions were observed (figure 4-6). This fact allows to assume, that depending on the requirements of the effluent quality, combined UASB-MBR system could be suitable for nitrogen elimination, with slight operational modifications, which will be discussed below.

4.4.3.1 *Nitrification (Days 0 – 90)*

During Period I, as mentioned in section 4.4.2 "Organic matter removal", the system operation was stable. The residual COD was fed to the aerobic chamber, which in turn allowed to obtain low C/N ratio and stable nitrification, with the complete oxidation of approximately 30 mgNH_4-N·L^{-1} (figures 4-6, 4-7 and 4-8). Taking into account, that the average concentration of ammonia produced in UASB stage by the hydrolysis of proteins was between 25 and 35 mg·L^{-1}, similar amounts of NO_x-N should be observed in the aerobic chamber (and/or permeate). However, that concentrations were always slightly higher, which could indicate that during this period additional ammonia, produced by the hydrolysis of particulate fraction of COD, was oxidized. During the first 32 operating days (Period I) there was no recirculation from aerobic chamber to UASB stage, nitrifying bacteria could grow both in suspension and in the form of biofilm. This fact explains why most of the TN in permeate was present as N-NO_X, while the N-NH_4 was very low.

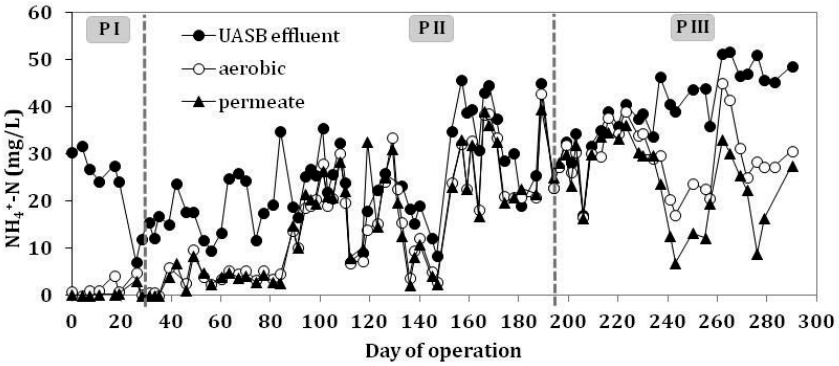

Figure 4-6 Concentrations of ammonia nitrogen during three periods of operation measured in: (●) UASB effluent, (○) first aerobic chamber of MBR stage, (▲) permeate.

From day 33 till day 90 (beginning of Period II), when the recirculation was turned on, around 4 mg·L^{-1} of ammonia appeared in the aerobic chamber of MBR stage and permeate. This fact indicates a decrease of nitrification capacity, which could be caused by the gradual wash-out of suspended nitrifying bacteria with recycled sludge. Since the recirculation ratio in this period was 0.15, with the average flow of 225 L·d^{-1}, the SRT of biomass of the MBR stage was around 1.6 day, which is not sufficient to maintain stable nitrification. This also explains why there was no difference between the

ammonia concentration in aerobic and filtration chamber (figure 4-6) – all the ammonia was oxidized probably by the nitrifying biomass in biofilm growing on plastic support present in the aerobic chamber.

4.4.3.2 Anoxic cycles (Days 90 – 220)

From day 90 of Period II onwards, anoxic cycles (30 min no aeration/20 min aeration) were implemented in the aerobic support chamber, to stimulate the process of denitrification and enhance nitrogen removal in the system. However, in the case of proposed UASB-MBR system introduction of the anoxic cycles caused a sharp DO concentration depletion (from more than 4 to below 0.5 $mg \cdot L^{-1}$) and thus nitrification was strongly affected due to the competition between heterotrophs and nitrifiers for the oxygen.

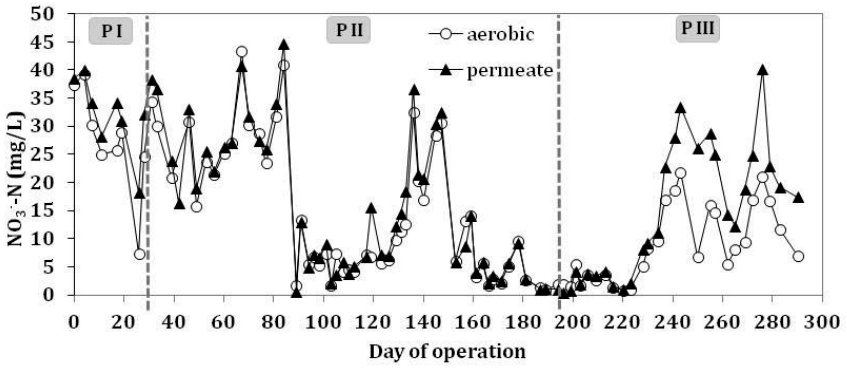

Figure 4-7 Concentrations of nitrate during three periods of operation measured in: (●) UASB effluent, (○) aerobic stage, (▲) permeate.

According to An *et al.* (2008) the C/N ratio required for complete NO_x-N reduction to nitrogen gas by denitrifying bacteria depend on the nature of the carbon source and the bacterial species. A COD/NO_x-N ratio 2.5 – 6.0 enables complete NO_x-N reduction to N_2. When anoxic cycles were applied the C/N ratio did increased, but since poor nitrification was observed, no denitrification occurred (figures 4-6 and 4-7). Negligible concentrations of ammonia were oxidized in the aerobic support chamber during the whole operation with anoxic cycles except form days 120 – 150. During this episode nitrification in the aerobic chamber of MBR occurred (figure 4-6) and a peak of nitrates could be observed (figure 4-7). One of the reasons might be a temporary turn-off of the recycle pump for maintenance

purposes, and therefore changing the rate of the competition between heterotrophic and nitrifying bacteria. Moreover, a DO concentration was higher during these days.

The diminution of the biomass concentration in the aerobic chamber (as an effect of sludge recirculation) and the temperature drop observed from day 142 onwards (from 24 to 20 °C) also had an influence on the nitrification failure. On the other hand, although negligible ammonia removal was observed in the aerobic chamber of the MBR stage, there was a certain concentration of nitrates present in this chamber and the permeate (figure 4-7 and 4-8). All those factors, low biomass concentration, temperature drop and nitrification in the filtration chamber significantly influenced the membrane behaviour, causing the increase of fouling and worsening the flux, which reached values down to 8 $L \cdot m^{-2} \cdot d^{-1}$. At this point, since the system was operated for more than 1 year, the membrane module was subjected to the chemical maintenance cleaning.

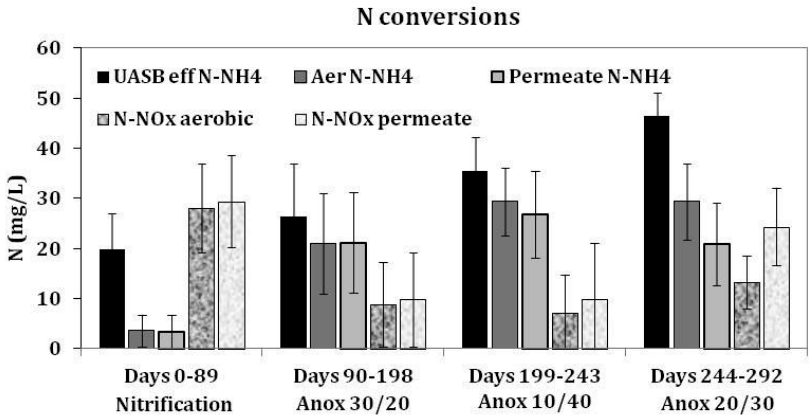

Figure 4-8 Nitrogen conversions in combined UASB-MBR system during 292 days of operation. In black, $N\text{-}NH_4$ concentration in UASB effluent; in dark grey $N\text{-}NH_4$ concentration in the first aerobic chamber of MBR stage; in pale grey $N\text{-}NH_4$ concentration in the permeate; in dark marble $N\text{-}NO_X$ concentration in aerobic chamber and in pale marble $N\text{-}NO_X$ concentration in the permeate.

To avoid the DO concentration deficiency and manipulate N conversion to $N\text{-}NO_X$, the length of anoxic cycles was modified (10 min no aeration/40 min aeration). However, it took more than a month to recuperate nitrification process in the aerobic MBR chamber (figure 4-8).

4.4.3.3 Nitrification regression (Days 222 – 292)

Around day 222 of Period III, as commented in the "Organic matter removal" section, due to the accidental biomass wash-out, the COD load to the system was decreased by increasing HRT. As a result, almost all the incoming fraction of COD was transformed in the anaerobic UASB stage. Thus, aerobic biomass was subjected to negligible organic matter load which caused its starvation. On the other hand the drop of available COD load caused an increase in the DO concentration in the aerobic MBR chamber, giving a nitrifiers growing in biofilm a chance, and the nitrification process was set off. This in turn strongly affected membrane operation (section 4.4.6 "Membrane performance" ; Drews *et al.*, 2007). The difference in the concentration of nitrates in the aerobic chamber and permeate (figure 4-7 and 4-8) indicated that part of the incoming ammonia was further oxidized in the filtration stage, again negatively influencing the membrane operation. After day 232, when the nitrification appeared again in the aerobic chamber, the COD/NO_x-N ratio was in the required range, being 2.5 (An *et al.*, 2008). However, no nitrogen elimination was observed taking into account the entire system. If denitrification occurred, it was negligible and NO_x-N concentration was probably compensated by the oxidation of ammonia generated by the hydrolysis of accumulated solids.

4.4.4 Biomass behaviour

According to Lettinga *et al.* (1998) the UASB inoculated with flocculent sludge would be more adequate for the treatment of raw dairy wastewater, mostly due to its higher resistance to solids content in the feeding. The accumulation of solid fraction may cause some operational problems, such as clogging, accumulation of organic matter inside the reactor, or, especially in the case of dairy wastewater, sludge flotation and wash-out of active biomass (Córdoba *et al.* 1984; Nadais *et al.*, 2005). However, in the case of present work, the incoming wastewater was semi-synthetic, simulating dairy wastewater after DAF pre-treatment and therefore free of large solids and fats. The organic matter built-up, as mentioned by Lettinga *et al.* (1998), did not occurred. Nevertheless, accumulation of flocculent excess sludge between the anaerobic granules took place, causing the flotation of biomass in the UASB stage. This layer was formed by the singular whitish anaerobic granules and some fraction of flocculent sludge.

Similar problem was also reported by Nadais *et al.* (2005), who studied the UASB reactor treating dairy wastewater at mesophilic conditions. The authors described the formation of a significant amount of floating whitish organic matter, oily to the touch, which could cause the clogging of the three-phase separator and wash-out of active biomass.

Accumulation of floating layer of flocculent sludge and its occasional wash-out could also explain some punctual variations in t-COD concentration measured in UASB effluent. However, in the case of present work biomass flotation and wash-out did not endanger the effectiveness of the UASB stage due to the application of internal recirculation. In these sense the proposed combined UASB-MBR system is a good solution for the treatment of dairy wastewater at lower temperatures.

4.4.5 Biogas production

During 292 days of operation constant production of biogas was observed, with more than 70% of methane, in various points reaching almost 80% (figure 4-9). The average biogas production rate was 58 $L \cdot d^{-1}$, being the highest in Period III (table 4-2), where the OLR was increased. The average methanization rate, referred to the t-COD and s-COD fed to the UASB stage, was 56 and 66%, respectively, while the COD elimination was in both cases higher, being 82 and 95% for total and soluble COD, respectively. This fact could be probably explained by two causes: the accumulation of a aerobic fraction of biomass recycled to the UASB stage, or underestimation of methane dissolved in the effluent. The first phenomena was also reported by Nadais *et al.* (2005), who attributed the lower methanization rate to the accumulation of solids in the UASB reactor. Underestimation of dissolved methane concentration was explained by van Haandel & Lettinga (1994) indicating, that the concentration of methane dissolved in the effluent will obey Henry´s law. In this sense, if the digested COD concentration is high, dissolved methane is insignificant in relation with methane in the gas phase. However, if the digested COD concentration is low (not much greater than 64 $mg \cdot L^{-1}$), dissolved methane will from a considerable fraction of produced COD. As was observed later (Sánchez *et al.*, 2013) the UASB effluent was oversaturated with methane and therefore the mass balances were never closed.

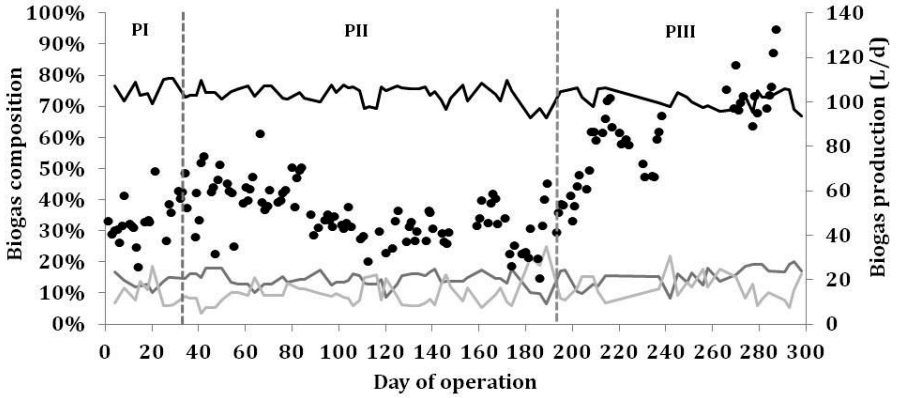

Figure 4-9 Biogas production and composition during Period I, II and III of operation of the combined UASB-MBR system. (black) methane, (dark grey) carbon dioxide, (pale grey) nitrogen gas, (●) biogas production.

The highest methane conversion rate was observed in Period II, when the HRT was longer, reaching the average values of 63 and 72% of methanization of total and soluble COD, respectively (table 4-2, figure 4-10). At the end of Period II, higher production of biogas was observed, with a rising tendency within the Period III, when the organic load was increased step-wisely. The system was able to produce up to 130 $L \cdot d^{-1}$ of biogas, which corresponds to the maximum biogas yield of 260 $L \cdot kg^{-1}$, taking into account the t-COD fed to the UASB stage. However, during Period III the methanization rate was lower than during the previous periods, being 43 and 55% for total and soluble COD, respectively (table 4-2, figure 4-10).

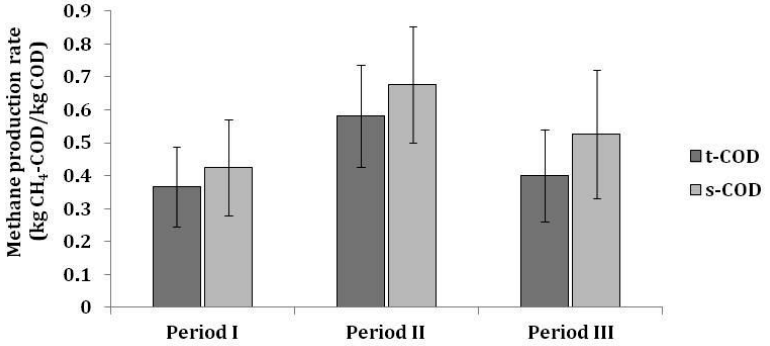

Figure 4-10 Methane production rate in terms kg CH4-COD referred to total and soluble COD.

Consequently, the average biogas yield determined during Period III, being 207 $L \cdot kg^{-1}$, was lower than those observed in Periods I and II, being 183 and

230 L·kg^{-1}, respectively (figure 4-10). Detailed comparison of the system efficiency in biogas production is presented in table 4-2.

Table 4-2 Performance data of combine UASB-MBR system in terms of biogas production efficiency.

Parameter	Unit	Period I	Period II	Period III
Biogas production rate	L/d	46.0±9.1	48.2±11.0	78.9±21.9
CH_4 content	%	75.0±2.8	73.8±2.92	72.3±2.37
Biogas production yield	L/kg t-COD	182.6±55.4	229.3±81.2	206.6±69.5
	L/kg s-COD	211.4±68.67	349.1±94.9	272.1±96.7
Methane production yield	L/kg t-COD	137.6±45.2	220.1±58.4	149.7±52.5
	L/kg s-COD	159.2±54.8	256.2±66.6	196.9±72.6
COD reduction in UASB stage	% t-COD	67	84	83
	% s-COD	88	95	96
COD methanization ratio	% t-COD	44.7±13.6	68.5±24.8	45.9±15.8
	% s-COD	51.6±16.5	79.8±32.2	60.4±21.7

4.4.6 Membrane performance

4.4.6.1 Feasible flux and permeability

The average flux obtained was 13.1 ± 3.0 L·m^{-2}·h^{-1} which was higher than those previously observed in anaerobic membrane bioreactors, between 5 and 10 L·m^{-2}·h^{-1} (Zhang *et al.*, 2007; Ho & Sung, 2010; Spagni *et al.*, 2010) but lower than those typically reported in aerobic membrane bioreactors operating with similar membrane modules, between 20 and 25 L·m^{-2}·h^{-1} (Judd, 2002; Wen *et al.*, 2004). Nevertheless, the flux obtained was similar to that obtained in other dairy applications, between 8 and 13 L·m^{-2}·h^{-1} (Bick *et al.*, 2009; Judd, 2006).

The highest stable flux observed, 19 L·m^{-2}·h^{-1}, and the lowest fouling rate were obtained in Period I, during which recirculation between aerobic and anaerobic stages was turned off and biomass concentration in filtration chamber was 1.71 ± 0.64 g VSS·L^{-1} (table 4-3). On the other hand, the highest fouling rates and the lowest fluxes were obtained during Period II,

when the recirculation was turned on and MLVSS in the filtration chamber was very low (below 0.5 $g \cdot L^{-1}$).

Permeability values of 168 ± 75 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$ were observed during three operational periods (figure 4-11). These values were slightly better than those observed during the operation of similar membrane modules (Bouhabila *et al.*, 2001; Judd, 2002), and higher that permeabilities observed in anaerobic membrane bioreactors (Zhang *et al.*, 2007; Spagni *et al.*, 2010). The highest permeability values were observed in Period III (table 4-3).

Recovery cleanings were performed in periods II and III, when recirculation was on. The average membrane critical flux measured during the whole operation of the UASB-MBR system was 20.2±2.8 $L \cdot m^{-2} \cdot h^{-1}$. The highest value of critical flux was obtained during Period I, reaching 28.0 $L \cdot m^{-2} \cdot h^{-1}$, when aerobic sludge recirculation was off.

4.4.6.2 Membrane fouling

During the periods I to III the impact of aerobic sludge recirculation to methanogenic stage with was studied. In this sense, biomass from the aerobic stage was recycled to the UASB reactor. The lowest values of colloidal BPC concentration (cBPC) and the highest stable permeabilities were obtained in Period I, when recirculation was turned off (figure 4-11). Moreover, higher fluxes were also applied during this period (table 4-3).

One of the advantages of the studied MBR configuration is the possible recovery of washed out anaerobic biomass from the second aerobic stage. This might avoid the loss of capacity of the methanogenic system, especially when operated at lower temperatures. However, altogether with the anaerobic sludge, aerobic biomass was also recycled to the methanogenic stage. Hydrolysis of complex substrates might be the limiting step of methanogenic process, especially at ambient temperatures (van Haandel and Lettinga, 1994). Thus, cBPC increase might be caused by the partial degradation of aerobic MLVSS recycled to the methanogenic stage in periods II and III.

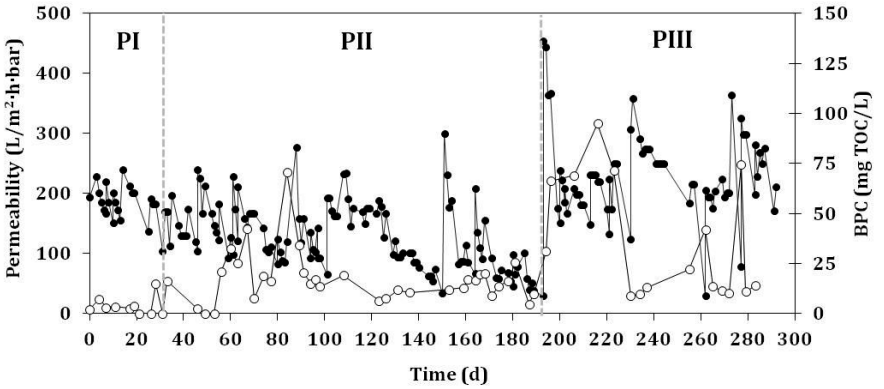

Figure 4-11 Permeability (●) and cBPC (○) concentration during 292 days of UASB-MBR system operation.

The applied food to microorganism (F/M) ratio, or SRT, might influence the membrane performance. F/M referred to soluble COD, and applied to the aerobic and filtration stages was very low during 292 days of operation of the UASB-MBR system. During Period I the F/M was around 0.025 $kgCOD \cdot kgMLVSS^{-1} \cdot d^{-1}$, while during periods II and III, when the recirculation was on, it increased to 0.036 $kgCOD \cdot kgMLVSS^{-1} \cdot d^{-1}$. SRT, calculated in Period I (no recirculation) was between 12 and 16 d. In periods I and III (with suspended biomass recirculation from the MBR to the UASB system) it was difficult to define a SRT, since a fraction of aerobic biomass was continually recycled between the UASB and MBR systems. Nevertheless, the amount of aerobic biomass purged from the system was similar to that Period I. Thus, variations of SRT or F/M could be discharged to be the main cause of the observed MBR behaviour.

Despite operating with very low MLVSS concentrations a strong correlation between this parameter and membrane performance was reported. As can be observed in table 4-3, the lowest MLVSS concentration in Period II (around 0.4 $g \cdot L^{-1}$) led to lower fluxes and lower permeabilities than those observed in Periods I and III, where MLVSS concentration was around 1.7 and 2.7 $g \cdot L^{-1}$, respectively. OLR applied to the aerobic stages had a great impact on MLVSS concentration. Lower OLR observed in Period II led to a decrease of MLVSS concentration, what had a negative effect on membrane performance (table 4-3). This effect was accentuated by the recirculation between the aerobic and the methanogenic stage that started in this

period. The details of the influence of MLVSS concentration on membrane performance was further studied by Sánchez *et al.* (2013).

Table 4-3 Membrane performance and relationship with MLVSS.

Parameter	Unit	Period I	Period II	Period III
MLVSS	$g \cdot L^{-1}$	1.7 ± 0.6	0.4 ± 0.2	2.7 ± 1.3
Flux	$L \cdot m^{-2} \cdot h^{-1}$	16.7 ± 2.3	11.4 ± 2.3	14.7 ± 1.8
Permeability	$L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$	188 ± 30	134 ± 68	225 ± 63
OLR	$kg \cdot m^{-3} \cdot d^{-1}$	1.4 ± 1.1	0.6 ± 0.6	1.6 ± 1.2
Temperature	ºC	20.1 ± 0.8	22.6 ± 1.3	19.8 ± 0.8
Recirculation	-	No	Yes	Yes

4.4.6.3 Temperature influence on membrane performance

Temperature also played an important role in the membrane performance, since it varied between 17 ºC (winter) and 25 ºC (summer). COD removal efficiency in the first methanogenic chamber increased with temperature, causing a diminution of the biodegradable COD supplied to the aerobic stages, and, as a consequence, leading to a lower MLVSS concentration and a higher fouling rate of the membrane. Also higher temperatures observed in Period II provoked an improvement of COD removal in the methanogenic stage and hence, a decrease in the OLR applied to the aerobic stage of the MBR (table 4-3). Thus, in this kind of anaerobic/aerobic MBR systems it is important to supply a minimum OLR in the aerobic stage in order to maintain MLVSS, and hence control the fouling rate. In this sense, the system could be modified by feeding a small fraction of the raw influent directly into the aerobic stage, in order to assure a minimum biodegradable COD supply, especially when operating at higher temperatures.

4.5 CONCLUSIONS

The proposed system was proved to be highly efficient in the treatment of dairy wastewater at ambient temperatures. Moreover, it presents a high

tolerance to organic loading changes (up to 3.9 $kgCOD \cdot m^3 \cdot d^{-1}$) and temperature fluctuations (17 – 25 °C). This fact should guarantee a stable operation of the proposed combined UASB-MBR system when the real dairy wastewater at environmental temperatures will be treated. It is important to highlight that the system may lose its efficiency when solids accumulation occurs. Therefore, appropriate sludge purges and appropriate recirculation ratio should be implemented to control the amount of biomass in the system.

The average total and soluble COD removal was above 95%, reaching 99% during the stable operation. Nutrient removal was not observed, however its conversion is possible, if necessary. In this case some modifications, such as by-passing a part of the influent directly to the aerobic chamber to improve denitrification process has to be studied. Regarding phosphorous removal the BAT in the case of dairy wastewater is chemical precipitation (BREF, 2006).

High biogas production was detected during the whole operation of the system, with an average methane content of 73% and the highest production rate, measured during Period III was 130 $L \cdot d^{-1}$. Average biogas yields, expressed as L of methane per ton of incoming t-COD were 138, 220 and 150 for Periods I, II and III, respectively.

The application of the internal recirculation allows to avoid a loss of methanogenic biomass in the case of its wash-out from UASB stage of the system. On the other hand, it assures lower overall sludge production, since part of the surplus aerobic sludge is hydrolysed in anaerobic stage. Moreover, non-readily biodegradable compounds can be subjected to further degradation.

Aerobic MBR post-treatment of the UASB effluent in general serves as a buffer; in the case when the anaerobic COD removal efficiency decreases, the remaining organic matter is oxidized in the aerobic MBR chamber. However, longer HRT assures almost complete elimination of COD in the methanogenic step.

With respect to the membrane performance, both MLVSS concentration and recirculation between aerobic and anaerobic stages are the main factors affecting membrane fouling. Therefore, in this system it would be necessary to assure a minimum OLR in the aerobic stage in order to minimize fouling rate.

Very low COD concentration and the level of nutrients in the effluent allows reusing purified wastewater (e.g. in agriculture). Moreover, application of the membrane module guarantees that the permeate is free of pathogens.

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Chapter 5

The influence of hydrolysis induced biopolymers from recycled aerobic sludge on specific methanogenic activity and sludge filterability in an anaerobic membrane bioreactor [1]

[1] Parts of this chapter were submitted to be published in the following publication:

D. Buntner, H. Spanjers, J.B. van Lier (2013). *The influence of hydrolysis induced biopolymers from recycled aerobic sludge on specific methanogenic activity and sludge filterability in an anaerobic membrane bioreactor*.

SUMMARY

The objective of the present study was to evaluate the impact of excess aerobic sludge on the specific methanogenic activity (SMA), in order to establish the maximum allowable aerobic sludge loading. Moreover, the potential influence of biopolymers and extracellular substances, that are generated as a result of excess aerobic sludge hydrolysis, on membrane performance was determined by assessing the fouling potential of the

liquid broth, taking into account parameters such as specific resistance to filtration (SRF) and sludge filterability. These assays were performed to assess the impact on SMA of different ratios of aerobic sludge, i.e. 0.03, 0.05, 0.10 and 0.15. It means that to 2.5 $gVSS \cdot L^{-1}$ of anaerobic seed sludge 0.075, 0.125, 0.250 and 0.375 $gVSS \cdot L^{-1}$ of aerobic sludge were added, respectively. It was found that a low amount of aerobic sludge leads to an increased SMA and a high membrane fouling potential. Results showed that addition of 15% of aerobic sludge caused more than 20% SMA decrease.

The increase in biopolymers, characterized as biopolymeric cluster (BPC), extracellular polymeric substances (EPS), and soluble microbial products (SMP) could be ascribed to aerobic sludge hydrolysis. A clear positive correlation between the concentration of colloidal fraction of BPC (cBPC) and specific resistance to filtration (SRF) and negative correlation between cBPC and supernatant filterability (SF) measured at the end of SMA tests (in relation with aerobic sludge ratio) was observed, indicating that sludge filtration resistance increases when more aerobic sludge is hydrolyzed, and thus more cBPC is released.

During AnMBR operation, proteins significantly contributed to sludge filterability decrease expressed as SRF and filterability, whereas the carbohydrate fraction of SMP was of less importance due to low concentrations. On the contrary, carbohydrates seemed to improve filterability and diminish SRF of the sludge. Albeit, BPC increase caused an increase in mean TMP during the AnMBR operation, confirming that BPC is positively correlated to membrane fouling.

5.1 INTRODUCTION

5.1.1 Foulants in MBR technology

Foulants characterization has been a major research topic related to membrane bioreactor (MBR) technology in recent years. Although many studies claim that extracellular polymeric substances (EPS) are responsible for fouling, no clear relationship between EPS concentration and fouling

was found (de la Torre *et al.*, 2008). Taking into account that the term EPS covers a large range of compounds of different nature, such as microorganisms´ secretions, cell lysis products and compounds already present in the influent and adsorbed to EPS matrix, its composition varies depending on the reactor application and operation. In general, these compounds are mainly polysaccharides (PS), proteins, nucleic acids, and humic substances (Drews *et al.*, 2006; Sheng *et al.*, 2010). However, in practice EPS are measured as a sum of the PS and proteins in the sludge. The perceived role of EPS in sludge is twofold. First, the bound or strongly bound EPS is generally linked to binding bacterial cells together, facilitating floc or granule formation, facilitating substrate and product transfer, enhancing resistance to toxins, and facilitating inter-cell communication. Second, EPS that is detached from cells and which is dissolved into the water phase of the mixed liquor is referred to as loosely bound or soluble EPS (Wingender *et al.*, 1999, Laspidou & Rittmann, 2002; Sheng *et al.*, 2010). Both forms of EPS either accumulate on the membrane surface as an effect of filtration, increasing fouling, and/or are decomposed by bacterial cells present in the mixed liquor and membrane cake (Nagaoka & Akoh, 2008).

Another set of compounds commonly used to describe fouling potential are soluble microbial products (SMP). SMP are soluble organic compounds that are released during biomass metabolism and decay. Like EPS, SMP is complex, consisting of proteins, polysaccharides, and some humic-like materials (Azami *et al.*, 2012). Usually this fraction is considered equal to soluble EPS and/or loosely-bound EPS, although some differences were reported that basically derive from the extraction method (Ramesh *et al.*, 2006).

Another category of organic compounds that has been identified in the liquid phase of MBR sludge and in the cake sludge on membrane surfaces consists of biopolymer clusters (BPC) ranging from 2.5 to 60 μm in size. Based on confocal laser scanning microscopy (CLSM) examination, BPC are free and independent organic solutes that are different from biomass flocs and EPS and much larger than SMP (Wang & Li, 2008; Sun *et al.*, 2008). Compared to EPS, BPC contain more polysaccharides and proteins and less humic substances. Wang & Li (2008) state that BPCs are an important foulant that interacts with biomass flocs to form the sludge cake fouling layer on the membrane).

Recent studies showed a clear correlation between the BPC concentration and fouling (Sánchez *et al.*, 2013; Le-Clech *et al.*, 2006; Sun *et al.*, 2008; Wang *et al.*, 2007; Wang & Li, 2008). On the other hand, the source of BPC remains unclear. In order to further elucidate the origin of BPC, Sánchez *et al.* (2013) studied the influence of excess aerobic sludge recirculation to the anaerobic stage of an integrated UASB-MBR system on the presence of colloidal (less than 0.45 μm) fraction of BPC (cBPC) and membrane performance. The cBPC was measured as the difference between total organic carbon (TOC) of the mixed liquor supernatant and permeate TOC (see section 2.3). Excess aerobic sludge generated in an aerobic chamber of the MBR stage (also containing a fraction of washed-out anaerobic biomass) was directed to the UASB stage, with the purpose to reduce overall sludge production (and avoid capacity loss of the UASB stage). It was found that apparently due to the hydrolysis of the recycled aerobic biomass, the concentration of cBPC in the effluent of the UASB stage increased, which in turn strongly affected membrane operation. However, the possible effects of adding excess aerobic sludge on the methanogenic capacity of the integrated UASB-MBR system and its relation with fouling properties of sludge were not studied.

Various authors state that the excessive generation of EPS and BPC in anaerobic membrane bioreactors can lead to poor membrane performance (Rosenberger *et al.*, 2006; Drews *et al.*, 2006; Nagaoka & Akoh, 2008). However, in addition to membrane fouling the presence of EPS may also affect the biomass activity (Mu *et al.*, 2006; Zheng & Yu, 2007).

5.2 OBJECTIVES

The objective of the present study is to evaluate the impact of aerobic sludge addition on the specific methanogenic activity (SMA). Moreover, the potential influence of cBPC and EPS, generated as a combined effect of aerobic sludge addition and its hydrolysis, on membrane performance was assessed in terms of fouling potential, taking into account parameters such as specific cake resistance (SCR) and filterability.

5.3 MATERIALS AND METHODS

5.3.1 Bioreactor and the strategy of operation

An anaerobic MBR (AnMBR) with an effective volume of five litres was used in this study. Inoculum sludge was taken from a black water treatment plant (Sneek, The Netherlands) and passed through a 10-mesh (2 mm) sieve to avoid potential clogging of the system. Total suspended solids (TSS) concentration was about 6 $g \cdot L^{-1}$. The same sludge was also used for batch experiments. The reactor was operated at ambient temperature (18.5 – 22.5 °C). A multi-blade stirrer was used for mixing. The rotation speed of the stirrer was fixed at 30 rpm. A tubular PVDF ultrafiltration membrane with an average pore size of 0.03 µm, was used as side stream membrane module (Norit X-Flow). Its length and diameter were 0.74 m and 5.2 mm, respectively. The membrane was operated in a gas-lift mode. Biogas from the head space of the reactor was recycled and injected into the bottom of the membrane tube by using a gas pump (Watson Marlow 323 D). Sludge was introduced into the membrane by means of gas motion. The gas velocity and the liquid velocity in the tubular membrane were 0.74 $m \cdot s^{-1}$ and 0.34 $m \cdot s^{-1}$, respectively. The operating flux was set at 8 $L \cdot m^{-2} \cdot h^{-1}$. TMP was measured by means of a pressure sensor (AE sensor 261920). Labview was used to record the pressure signal from the pressure sensor and to control the operation of the membrane module. Back flush was carried out automatically for 6 seconds per each 10 minutes of filtration and the back flush flux was set at 200 $L \cdot m^{-2} \cdot h^{-1}$. A schematic view of the setup is shown in figure 5-1. The AnMBR was fed with synthetic low-strength wastewater (table 5-1) and, after reaching stable operation, aerobic sludge originating from the sludge recirculation line of the Harnaschpolder activated sludge wastewater treatment plant (WWTP) (Midden-Delfland, The Netherlands) was also fed.

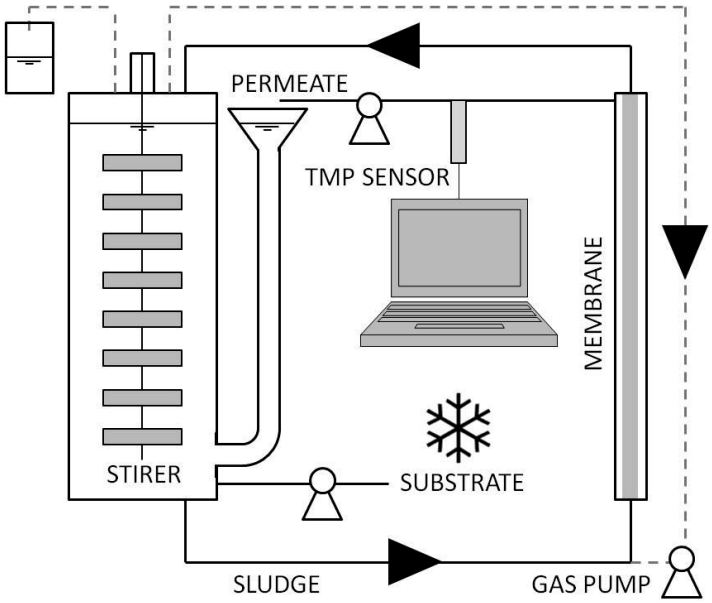

Figure 5-1 Schematic view of the AnMBR reactor with tubular PVDF ultrafiltration membrane. The substrate was stored in the fridge at 4 °C to avoid its degradation.

Table 5-1 Composition of the synthetic feeding used for AnMBR operation.

	Compound	Concentration	Unit
Micronutrients	$FeCl_3 \cdot 6H_2O$	1.2	mg/L
	$CoCl_2 \cdot 6H_2O$	1.2	mg/L
	$MnCl_2 \cdot 4H_2O$	0.3	mg/L
	$CuCl_2 \cdot 2H_2O$	0.018	mg/L
	$ZnCl_2$	0.03	mg/L
	HBO_3	0.03	mg/L
	$(NH_4)_6Mo_7O_2 \cdot 4H_2O$	0.054	mg/L
	$Na_2SeO_3 \cdot 5H_2O$	0.06	mg/L
	$NiCl_2 \cdot 6H_2O$	0.03	mg/L
	EDTA	0.6	mg/L
	HCl 36%	0.6	µl/L
	Resazurin	0.3	mg/L
Macronutrients	NH_4Cl	1.02	g/L
	$CaCl_2 \cdot 2H_2O$	0.048	g/L

	Compound	Concentration	Unit
	$MgSO_4 \cdot 7H_2O$	0.054	g/L
Acetate	$NaC_2H_3O_2 \cdot H_2O$	2.7	g/L
		1.27	gCOD/L
Buffer	K_2HPO_4	1.063	g/L
	$NaH_2PO_4 \cdot 2H_2O$	0.608	g/L

5.3.2 Batch SMA tests

Prior to the introduction of aerobic sludge to the AnMBR, batch SMA tests were carried out in an Automatic Methane Potential Test System (AMPTS, Bioprocess Control, Sweden) to evaluate the influence of the aerobic sludge on anaerobic biomass activity. As in the case of AnMBR, inoculum sludge was taken from a black water treatment plant (Sneek, The Netherlands) and equally pre-treated. Aerobic sludge was taken from Harnaschpolder WWTP, similar to the AnMBR experiment. Different ratios of aerobic excess sludge /anaerobic seed sludge were tested, i.e. 0.03, 0.05, 0.10 and 0.15 based on VSS. It means that to 2.5 $gVSS \cdot L^{-1}$ of anaerobic seed sludge 0.075, 0.125, 0.250 and 0.375 $gVSS \cdot L^{-1}$ of aerobic sludge were added, respectively. During the SMA tests, acetate with an initial concentration of 1.5 g $COD \cdot L^{-1}$ in the test vial was used as the main substrate and anaerobic sludge concentration was 2.5 $gVSS \cdot L^{-1}$. Micro and macro nutrient addition as well as pH control was performed as described by Jeison (2007). The total volume of the mixture of sludge and medium was 400 ml. The SMA test was performed at 20 °C, similar to AnMBR reactor operation. Each test lasted approximately 10 days and the maximum slope was taken for calculating SMA activity.

Since the anaerobic seed sludge was stored in the fridge, the SMA batch tests were performed in 2 sets: Set 1 was carried out with the anaerobic seed sludge without pre-activation and without any acclimation period, in Set 2 a new portion of the anaerobic seed sludge was previously activated (by adding around 1 $g \cdot L^{-1}$ of sodium acetate) and incubated for 3 days at ambient temperature prior to the test. In both runs 2 types of control vials were included for assessing background SMA of both anaerobic sludge and anaerobic sludge plus the corresponding fraction of aerobic sludge. Background SMA was taken into account in final SMA value calculations.

Each set of test vials consisted of i. anaerobic sludge , ii. anaerobic sludge with acetate, iii. anaerobic sludge with corresponding fraction of aerobic sludge and iv. anaerobic sludge with corresponding fraction of aerobic sludge and acetate.

5.3.3 Analysis

5.3.3.1 Chemical analysis

Chemical Oxygen Demand (COD) and Total Organic Carbon (TOC) for BPC analysis were determined using test kits (Merck Millipore). cBPC in the liquid phase of the sludge (Sun *et al.*, 2008) was assessed as follows:

$$cBPC = TOC_{ML} - TOC_P \quad \text{eq. 1}$$

Where TOC_{ML} is the TOC concentration of mixed liquor sample (taken form batch vial and the reactor) after centrifugation at 4000g for 15 min and filtration through a 0.45 µm nitrocellulose membrane filters (HA, Millipore), and TOCP is the TOC of the permeate of the reactor membrane module.

EPS (bound EPS) and SMP (soluble EPS) analysis were carried out as follows: for SMP determination the biomass sample (taken form batch vial and the reactor) was centrifuged at 12000g for 10 min (Heraeus, Labofuge 200). Hereafter the supernatant was filtered through 0.45 µm nitrocellulose membrane filters (HA, Millipore) and analyzed for carbohydrate and protein fraction. EPS were determined by re-suspension of centrifuged biomass sample with demi-water. Hereafter the sample was placed in the oven at 80 °C for 1 hour after which the sample was centrifuged for 20 min at 12000g. The supernatant was filtered through 0.45 µm nitrocellulose membrane filters and analyzed for carbohydrate and protein fraction. Carbohydrate and protein fraction of SMP and EPS were determined following the methods of Dubois *et al.* (1956) and Lowry *et al.* (1951), respectively.

5.3.3.2 Sludge filtration measurements

The specific resistance to filtration (SRF) of sludge samples taken from the reactor and sludge samples taken at the end of SMA batch tests was determined following the method of Wisniewski & Grasmick (1998). The test was conducted in a 50-mL cell (Model 8050, Amicon) using a 0.7- µm

glass filter (GF/F 1825-047, Whatman). The cell was filled with 40 mL of the mixed liquor sample, and a constant pressure (0.5 bar or 50 kPa) was applied by means of pressurized air. The production of filtrate under pressure was continuously recorded by an electric balance that was connected to a PC data logger. The test lasted for 30 min. The SRF ($m \cdot kg^{-1}$) can be calculated as follows:

$$SRF \times E^{12} = \frac{2 \times A^2 \times \Delta P \times b}{\mu \times C} \quad \text{eq. 2}$$

where ΔP (50 kPa) is the pressure applied, A (0.00134 m^2) the filtration area, μ is the viscosity ($kg \cdot m^{-1} \cdot s^{-1}$), C the total suspended solids ($kg \cdot m^{-3}$), and b is the time-to-filtration ratio ($s \cdot m^{-6}$), which is the slope of the curve that is obtained by plotting the time of filtration to the volume of filtrate ratio ($t \cdot V^{-1}$) versus the filtrate volume (V). C of the sludge sample should not exceed 10 $gTSS \cdot L^{-1}$, otherwise the permeate (or tap water) should be used to dilute the sample.

Sludge samples for supernatant filterability (SF) measurement (taken from the AnMBR and sludge samples taken at the end of SMA batch tests) were previously centrifuged at 14000 rpm for 10 minutes and a volume of 35 – 40 mL of the supernatant of the sample was taken. The SF procedure was similar to that for SRF evaluation, except for the application of stirring 5 min before and during the filtration (to avoid polarization concentration) in the case of SF, and that during the test the sample was filtered through a 0.22-μm cellulose membrane filter (GSWP 04700, Millipore). The test lasted 10 min and the slope when the flow was stabilized was taken into account (usually between 300 and 600s). SF is expressed in $mL \cdot min^{-1}$.

5.4 RESULTS AND DISCUSSION

5.4.1 Impact of aerobic sludge on SMA

Figure 5-2 shows that there was a small, significant difference between sensitivity of SMAs towards aerobic sludge obtained in Set 1 and Set 2, which indicates that pre-activation of anaerobic biomass is necessary prior to the SMA test. As shown in figure 5-2 for both sets, aerobic sludge up to

a fraction of 0.10 had no negative impact on methanogenic activity. On the contrary, a slight stimulation of SMA was observed (figure 5-2b) with a maximum of 8% and 22% increase for the 0.10 fraction of aerobic sludge during Sets 1 and 2, respectively. The observed activity increase might be attributed to a partial hydrolysis of the aerobic sludge and the subsequent use of compounds like hydrogen or formate as an additional substrate for methanogenesis. However, the addition of 0.15 of aerobic sludge caused a decrease in anaerobic activity: SMA values diminished about 24 and 20% in Set 1 and 2, respectively.

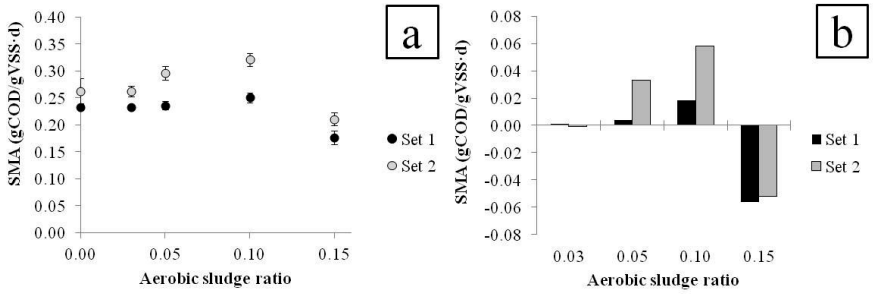

Figure 5-2 (a) Specific Methanogenic Activity (SMA) for different fractions of aerobic sludge; (b) increase/decrease of SMA for different fractions of aerobic sludge in relation with SMA of anaerobic sludge without aerobic sludge addition.

The assessed SMA values without any aerobic sludge addition were 0.23±0.02 and 0.26±0.01 $gCOD \cdot gVSS^{-1} \cdot d^{-1}$, which corresponds to 87 and 98 $mLCH_4 \cdot gVSS^{-1} \cdot d^{-1}$, for Set 1 and 2, respectively. However, since acetate was used as the sole substrate, only acetotrophic methanogenic activity was reflected. It should be noted that when more complex substrates are used, it is not always clear whether methane formation is the rate-limiting step in substrate conversion. In such case 'a' methanogenic activity is measured and not the SMA.

5.4.2 Evaluation of fouling potential in short-term experiment – EPS, SMP, cBPC and SRF

The samples used for the SMA test (Set 2) were analyzed for concentrations of EPS, SMP, and cBPC. The bound fraction (represented by EPS) of both carbohydrates and proteins was predominant, whereas the concentration of both fractions of SMP was constant and independent of the

aerobic/anaerobic sludge ratio (data not shown). Moreover, the concentrations of proteins were much higher than those of carbohydrates and increased with the increase in the aerobic/anaerobic sludge ratio (figure 5-3a). One of the key functions of extracellular proteins is enzymatic conversion of macromolecules and particulate material in the microenvironment of the embedded cells. Organic matter sorbed to the bacterial cell can therefore be hydrolyzed and low-weight hydrolysis products can be easily used by the bacteria without diffusion loss of products to the surrounding water (Laspidou & Rittmann 2002). This indicates that the presence of proteins bound to bacterial cells could either be an effect of release of enzymes by anaerobic bacteria in order to hydrolyze aerobic sludge, or due to cell lysis of the latter. The increase in exopolymers concentration related with the increase in aerobic sludge fraction is even more clear when evaluating the carbohydrate fraction of EPS (figure 5-3a). The observed increase in concentration of soluble proteins and more or less equal concentration of carbohydrates with increasing aerobic sludge addition suggests that the proteins were secreted by anaerobic sludge as hydrolytic enzymes rather than being products from aerobic biomass hydrolysis. These results would then confirm our hypothesis that increase in EPS concentration is an effect of aerobic sludge hydrolysis by anaerobic microorganisms. On the other hand, the abundance of bound proteins is typical for aerobic flocculent sludge (Dignac *et al.*, 1998), since these compounds have a predominant role in the floc formation.

Additionally, a positive correlation between EPS (protein fraction) and SRF was found (figure 5-3b), indicating that the increase in EPS concentration has a negative impact on fouling, as expressed by the specific cake resistance increase. This is in agreement with other authors (e.g. Nagaoka & Akoh, 2008; Drews *et al.*, 2006).

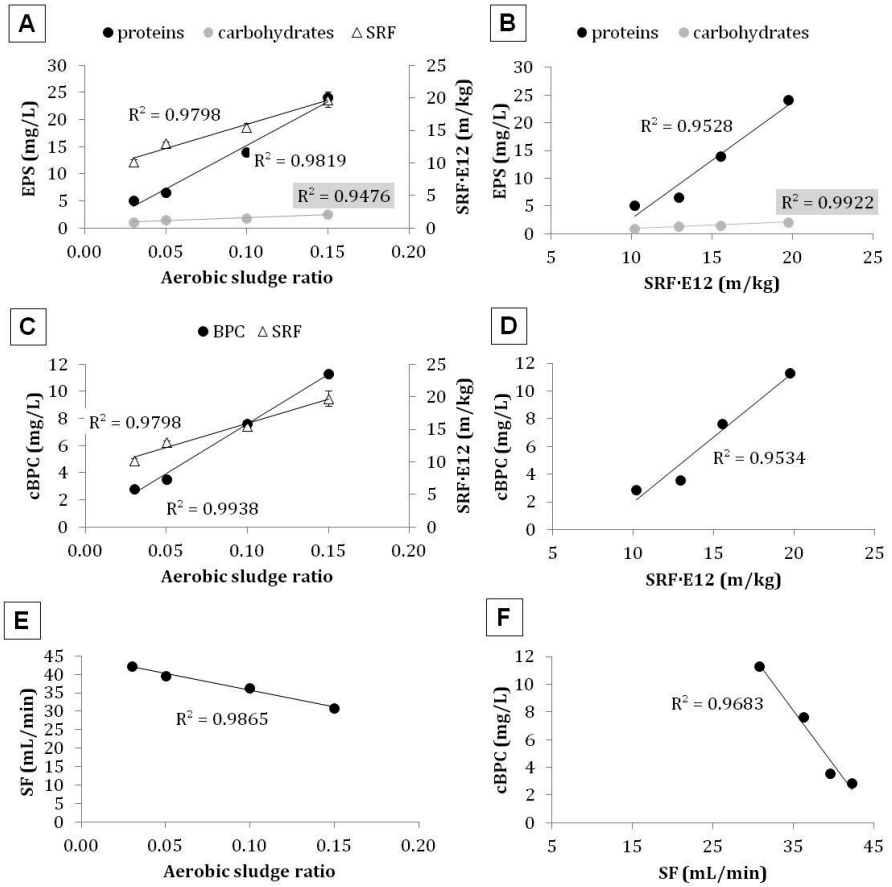

Figure 5-3 Correlations observed at the end of SMA test: (A) between EPS concentrations and the ratio of aerobic sludge and SRF; (B) between EPS concentration and SRF; (C) between cBPC concentration and the ratio of aerobic sludge and SRF; (D) between cBPC concentration and SRF; (E) between SF and aerobic sludge ratio; and (F) between cBPC concentration and SF.

As in the case of EPS, a clear positive correlation was found between the aerobic/anaerobic sludge ratio and cBPC concentration (figure 5-3C, in black) and SCR (figure 5-3C, in grey). Our present findings are in agreement with results obtained by Sánchez *et al.* (2013) who demonstrated an apparent correlation between cBPC concentration and fouling rate during the operation of an AnMBR. On the other hand, deflocculation of activated sludge flocs under anaerobic conditions was also mentioned by Wilén *et al.* (2000) who observed that, and although deflocculated particles were mainly bacteria and floc fragments, release of some soluble EPS was reported. A negative impact of activated sludge deflocculation on activated sludge

filterability is also observed in aerobic MBR systems (Meng & Yang, 2007; van de Broek *et al.*, 2010; Krzeminski *et al.*, 2012).

Finally, a clear positive correlation between cBPC and SRF (figure 5-4D) and negative correlation between cBPC and SF (figure 5-4F) were observed, indicating that with an increase in aerobic sludge hydrolyses, more cBPC is released leading to more resistance to filtration. Therefore, increased fouling properties of the sludge mass would be expected if the aerobic sludge load applied to the AnMBR would increase.

Although both parameters, EPS and cBPC, seem to describe well the fouling potential of the sludge our results show that cBPC is a more convenient parameter for sludge fouling potential than EPS, owing to the simplicity and reliability of its measurement.

5.4.3 Influence of aerobic sludge on AnMBR performance – long-term experiment

In order to check if the results obtained in short-term experiment will be also observed during long-term operation., an AnMBR reactor was operated for 65 days and the operation period was divided into five stages: In Stage I (days 0-15) the reactor was started up; basic parameters were measured to assure proper operation of the system. Stage II (days 16 – 30) was characterized by stable operating conditions, e.g. reflected by constant COD elimination characteristics. This stage served as an acclimatization period for the anaerobic bacteria, since the inoculum was adapted to black water whereas in our present work acetate was used as the substrate. During this period the average COD elimination was limited to 50%. In stage III (days 31 – 46) excess aerobic sludge was introduced to the reactor as an additional feed in a continuous mode. The load of aerobic sludge was maintained at 22.5±3.6% of the incoming COD. In the case of SMA batch test the load of aerobic sludge up to around 60% (which corresponds to the COD load introduced with 0.10 ratio of aerobic sludge) did not negatively influence SMA activity.

Stage IV (days 47 – 53) started with a system failure and therefore was used for the recuperation to stable AnMBR operation. No aerobic sludge was introduced during this stage.

As can be seen in figure 5-4 the introduction of aerobic sludge resulted in a COD elimination exceeding 90% in stage III. Moreover, BPC concentration increased significantly when the aerobic sludge was introduced (figure 5-4).

Wang *et al.* (2007) highlights the fact that the COD concentration in an MBR effluent is always lower than the soluble COD of the bulk sludge supernatant, which is probably caused by the adsorption and interception of SMP and other organic macromolecules by the sludge layer on the membrane. In addition, SMP and colloidal matter penetrating the cake layer are partly entrapped as well in the cake layer forming large-sized BPC, resulting in low permeate cBPC concentration (Sánchez *et al.*, 2013).

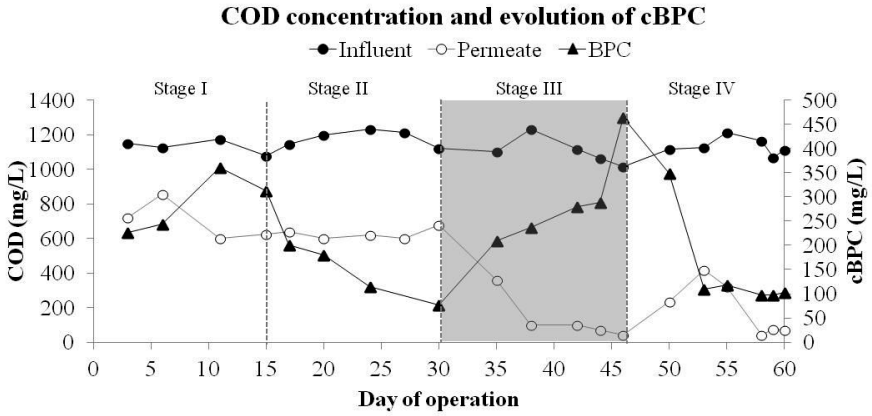

Figure 5-4 COD and BPC effluent concentrations during 60 days of AnMBR operation. Stage where aerobic sludge was continuously fed into the reactor is marked in grey.

5.4.4 Fouling properties of the sludge during AnMBR operation

Even though the results of the batch tests suggested that the introduction of aerobic sludge may worsen filterability properties, this was not confirmed during the operation of the AnMBR reactor. On the contrary, both sludge filterability and specific resistance to filtration showed an improvement after a few days of operation with aerobic activated sludge as an additional feed (figure 5-5), indicating that fouling potential of the sludge was somewhat reduced. The critical flux was around 20 $L \cdot m^{-2} \cdot h^{-1}$.

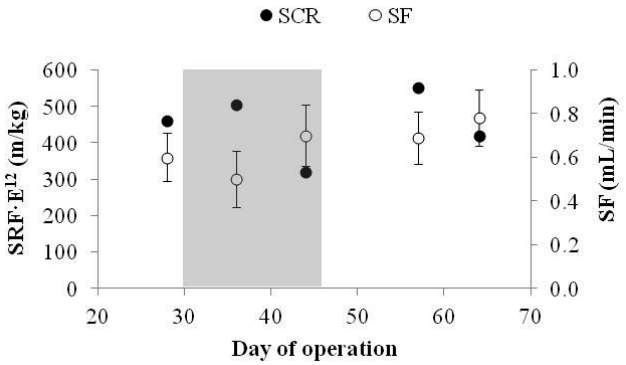

Figure 5-5 Results of supernatant filterability (SF) and SRF measurements during the operation of AnMBR.

The results of continuous AnMBR operation suggest an important role of the soluble compounds in fouling potential of the AnMBR sludge, as previously described by Wisniewski & Grasmick (1998). Part of the total resistance might be ascribed to the presence of soluble compounds such as carbohydrates and proteins and their interaction with the membrane and/or cake-layer (figure 5-6). This tendency was not observed for the bound EPS (data not shown). Results in figure 5-6 indicate that SCR is apparently positively correlated with proteins and negatively correlated with polysaccharides fraction of SMP, whereas reversed correlations were found for the sludge filterability. It would suggest that while proteins have a negative impact on fouling properties of sludge, carbohydrates seem to improve them. These results are contradictory to the batch SMA tests and the common findings (Rosenberger *et al.*, 2006; Wu & Huang, 2009) which indicate strong fouling potentials of the carbohydrate fraction of SMP rather than the protein fraction. However, according to Drews *et al.* (2008) polysaccharides and proteins are equally important. Meng *et al.* (2006) found that proteins significantly contributed to membrane fouling, whereas the carbohydrate fraction of EPS had moderate correlation with filtration resistance due to low concentrations. The authors also highlighted that apart from EPS, the MLSS concentration and sludge particle size are major factors affecting membrane fouling. Finally, Le Clech *et al.* (2003) suggested that synthetic wastewater tends to present significantly higher proteinaceous EPS levels, compared to real sewage, and therefore account for the higher fouling rate recorded for this matrix. In any case, the increase in total EPS would cause an increase in dynamic viscosity of mixed liquor,

causing a higher degree of accumulation of polymers and sludge particles on membrane surface (Meng *et al.*, 2006) and, therefore, decrease in membrane permeability.

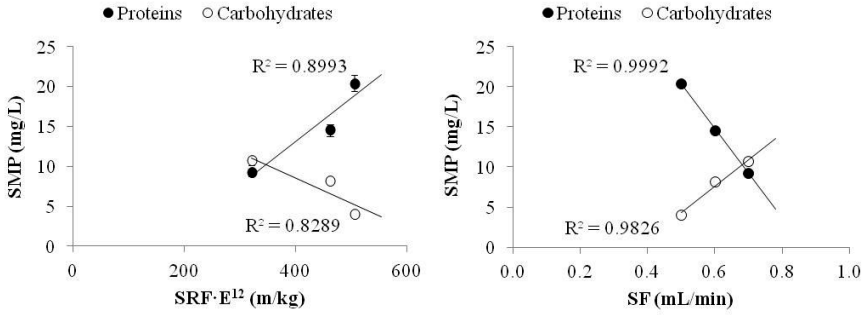

Figure 5-6 Correlation between SMP: (●) Proteins and (○) Carbohydrates concentration, and SRF (left) as well as supernatant filterability (right) found during AnMBR operation.

Finally, the TMP evolution was followed during 60 days of AnMBR operation. Figure 5-7 depicts that the mean TMP value was constant till day 37, after which a mild increase in TMP was observed till the end of Stage III. This peak coincides with the increase in cBPC concentration, which could indicate that cBPC had a negative impact on the membrane performance. The latter observation is in agreement with the findings presented by Sánchez *et al.* (2013), who observed a very good correlation of cBPC concentration peaks with the worsening of in-situ membrane permeability and increase in TMP. Moreover, this tendency is similar to that observed in the sludge from the batch SMA tests presented in this work, where the increase in cBPC concentration correlated well with the increase in fouling potential of the sludge (expressed as SRF and SF, section 5.4.2). Furthermore, the trend observed for the maximum TMP values that were reached daily also shows an increasing tendency with the increase in cBPC concentration. It was therefore postulated that cBPC might be a fouling indicator of interest.

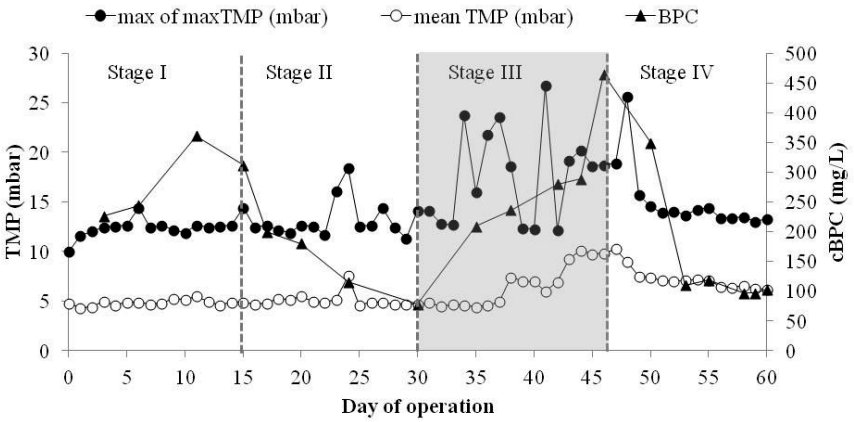

Figure 5-7 TMP over time during 60 days of AnMBR operation. (●) Maximum value of maximum TMP recorded per day, (○) Mean value of TMP recorded per day, (▲) BPC concentration.

Although cBPC concentration correlates with TMP, it might be a short term effect, because the experiment was performed only during the experimental period of 60 days. Moreover, a temperature increase (from 20 to 22 °C) that occured in period IV could have accelerated bacterial metabolism leading to the decomposition of cBPC. SMP and EPS concentration decreased gradually when the excess aerobic biomass addition was stopped and protein was the dominant fraction. Long term experiments using different feedings (e.g. glucose, more complex synthetic wastewater) with continuous addition of aerobic sludge would be advisable, since EPS and SMP composition depends, among others, on the substrate used.

5.5 CONCLUSIONS

Addition of small amounts of aerobic sludge to methanogenic biomass leads to an increased SMA and increased membrane fouling potential. However, additions exceeding a ratio of 0.10 of aerobic sludge relative to the anaerobic sludge mass caused more than 20% SMA decrease.

Aerobic sludge hydrolysis results in increased cBPC, EPS and SMP concentrations at the end of SMA test and in AnMBR.

Increase in cBPC concentration is positively correlated to SFR in SMA tests, and negatively correlated to SF, indicating that increase in aerobic sludge hydrolysis results in increased cBPC release and more resistance to filtration.

During AnMBR operation proteins significantly contributed to sludge filterability decrease (expressed as SRF and SF), while the carbohydrate fraction of SMP was only present in low concentrations and apparently of less importance. The carbohydrates concentration was positively correlated to supernatant filterability and reciprocally correlated to the sludge SFR.

cBPC increase caused an increase in mean TMP during the AnMBR operation.

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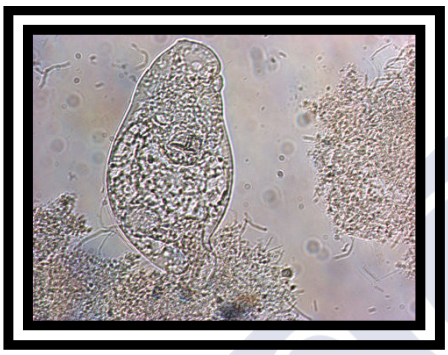
Chapter 6



Biomass characteristics in combined UASB-MBR system[1]

[1] Parts of this chapter are under preparation for publication:

D. Buntner *et al.* (2013). *Biomass characteristics in combined UASB-MBR system*. (in preparation).

SUMMARY

In this Chapter biomass present in the combined UASB-MBR system (previously described in Chapter 3 and Chapter 4) was characterized, by using a wide spectrum of analytical techniques. Among others, morphology description of granular, suspended and biofilm biomass, size distribution and composition of granular sludge, and FISH analysis were applied. To obtain more detailed information about the bacterial populations present in the combined UASB-MBR, DNA extraction, PCR, DGGE and sequencing were performed. From the application of these molecular techniques a heterogeneous distribution of microorganisms present in the granular, suspended and biofilm biomass was revealed. Among *Proteobacteria* phylum, a subclass of *Betaproteobacteria* was the most dominant, followed by the *Gammaproteobacteria*. *Alphaproteobacteria* was scarce and appeared in coccoid form, while bacteria belonging to *Deltaproteobacteria* were not observed at all. The predominance of members of *Betaproteobacteria* was associated with abundance of nitrifying and denitrifying bacteria. Apart from these microorganisms, *Bacteroidetes*, nitrite-oxidizing bacteria (NOB), *Acidobacteria*, *Firmicutes* and filamentous bacteria belonging to *Chloroflexi* were also detected. Finally, the

appearance of some Anammox bacteria, belonging to *Planctomycetales*, was observed during the first operation periods.

Apart from molecular techniques, the role of protozoa in the MBR stage was evaluated. It was revealed that the presence of plastic support and thus development of predators is crucial for stable operation and high flux achievement in the MBR stage.

6.1 INTRODUCTION

In wastewater treatment plants (WWTP) the communities of prokaryotic microorganisms present in activated-sludge or biofilm reactors are responsible for most of the carbon and nutrient removal from sewage. However, excessive occurrence of certain bacterial species can also negatively influence the operation of the plant, e.g. by worsening the settling properties of activated-sludge in the secondary clarifiers, by contributing to the formation of foam or simply by outcompeting microorganisms required for nutrient removal. Consequently, a comprehensive knowledge of the ecology of the microbial communities is required to reveal factors influencing the efficiency and stability of biological WWTPs (Wagner & Loy, 2002).

6.1.1 Main microbial groups present in the aerobic sludge and their characteristics

Beta-, *Alpha-* and *Gammaproteobacteria* as well as the *Bacteroidetes* and the *Actinobacteria* (figure 6-1) are the most frequently found phylum in activated sludge samples analysed using 16S rDNA approach. Among them, the bacteria might be further divided into following groups:

6.1.1.1 Ammonia oxidizing bacteria (AOB)

AOB are chemolitotrophic microorganisms responsible for the first (and rate-limiting) stage of nitrification. All AOBs belong to the phylum *Proteobacteria* and can be divided into two classes: *Gammaproteobacteria*, which includes strains of *Nitrosococcus oceani* and *Nitrosococcus*

halophilus, and *Betaproteobacteria*, which includes the genera *Nitrosomonas* and *Nitrosospira* (figure 6-1). In activated sludge samples of most nitrifying WWTPs the most common AOB are related to *Nitrosomonas.* In suspended and biofilm biomass they form almost spherical compact cell aggregates, with well visible single cell, with diameter between 10 and 50 μm (Figueroa, 2011).

6.1.1.2 Nitrite oxidizing bacteria

All known nitrite oxidizers belong to the genera *Nitrobacter*, *Nitrospira*, *Nitrospina* and *Nitrococcus* (figure 6-1). They catalyze the second step of the nitrification process (Chapter 1, section 1.1.1.2). Nitrite oxidizers usually form irregularly shaped cell aggregates, with diameters reaching 100 μm or more, but also can occur as single cells surrounded by biofilm matrix (Figueroa, 2011).

6.1.1.3 Denitrifying bacteria

Among others, heterotrophic microorganisms belonging to genera *Zooglea* and *Thauera*. (figure 6-1) are attributed to perform the denitrification process. However, the community composition of denitrifying bacteria will depend on the carbon source applied (Morgan-Sagastume *et al.*, 2008). Moreover, *Zooglea spp*. is also known for possessing the ability of producing exopolymeric substances (EPS), mostly polysaccharides, and floc formation. It was also stated, that these bacterial species most likely play an important role in the formation and stabilization of the granule structure (Figueroa, 2011). Other denitrifying organisms may be found in genera *Pseudomonas*, *Paracoccus*, *Rhizobium*, *Bacillus* or *Alcaligens*, although these bacteria are not specific for activated sludge. Since microorganisms capable of performing the denitrification process are not limited to one phylogenic group, they may be found among the *Proteobacteria*, *Firmicutes* and the *Bacteroidetes* (figure 6-1), covering more than 50 genera (Figueroa, 2011).

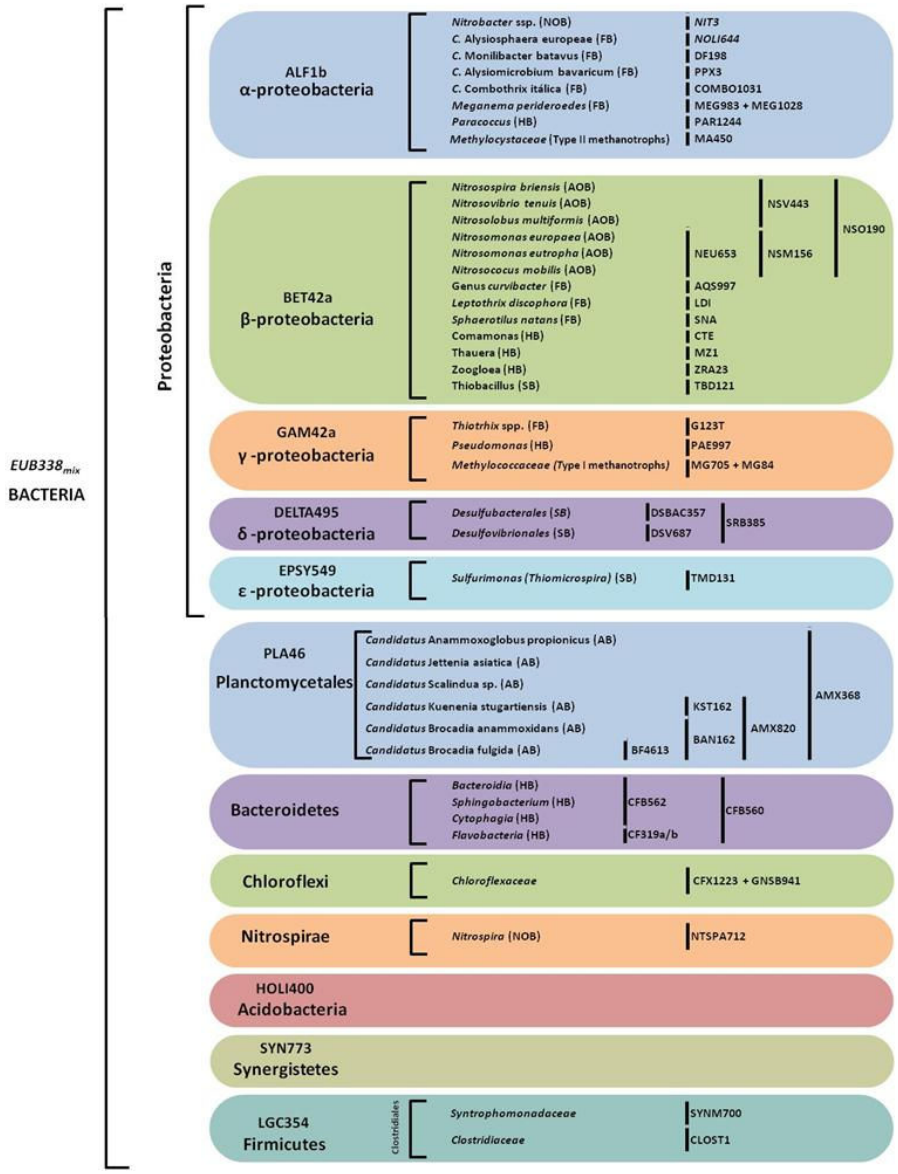


Figure 6-1 Bacteria domain and 12 phyla typical for wastewater treatment biomass with corresponding families and FISH probes (adapted from Figueroa, 2011).

6.1.1.4 Methane oxidizing bacteria

Aerobic methane oxidation is driven by the wide group of bacteria, *Methanotrophs*. These are aerobic organisms that oxidize methane (CH_4) using it for both an electron donor and as a source of cell carbon They are divided into three major phylogenetic groups: type I and type X, which belongs to *Gammaproteobacteria*, and type II, which belongs to *Alphaproteobacteria* (Jiang *et al.*, 2010). The main differences between both types include carbon assimilation pathway (ribulose for type I; serine for type II), number of internal membranes (1 for type I; 2 for type II), and N_2 fixation ability (yes in the case of type II, no in the case of type I, except from *Methylococcus*). More detailed information on methanotrophic bacteria is given in Chapter 7, section

6.1.1.5 Anammox bacteria

Anaerobic ammonia oxidation (Anammox) process is driven by the bacteria which belong to the group *Planctomycetes* (figure 6-1). These bacteria contain a peculiar membrane-bound organelle (Anammoxosome) in which ammonium and nitrite are converted to nitrogen gas. Anamox bacteria have a coccoid shape and usually occur as small to large clusters (Figueroa, 2011). Further information on the Anammox process and its characteristics will be given in Chapter 7, section 7.1.2.

6.1.2 Anaerobic biomass and biogenic methane production

Biogenic methane production, or methanogenesis, is a microbial process carried out by a unique class of prokaryotes. Although methane-producing microbes exhibit prokaryotic biochemical and morphological features, studies pioneered by Carl Woese recognized these organisms as phylogenetically distinct from other prokaryotes and eukaryotes (Barber & Ferry, 2001). Indeed, the existence of the methanogens, now called methanoarchaea, led to the present day three domain concept of phylogeny (Archaea, Bacteria, and Eukarya; figure 6-2).

The domain Archaea is divided into two kingdoms, Euryarchaeota and Crenarchaeota, based on ribosomal RNA sequence comparisons (figure 6-3). Methanoarchaea, along with halophiles and thermoacidophiles, represent the main constituency of the Euryarchaeota. Within this kingdom, 8 different classes were derived (figue 6-3), with *Methanomicrobia* and *Methanobacteria* being the most commonly found in the anaerobic digesters. Going further, within these classes methanoarchaea are subdivided into five main orders (figure 6-3), each with distinctive characteristics (Boone *et al.*, 1993).

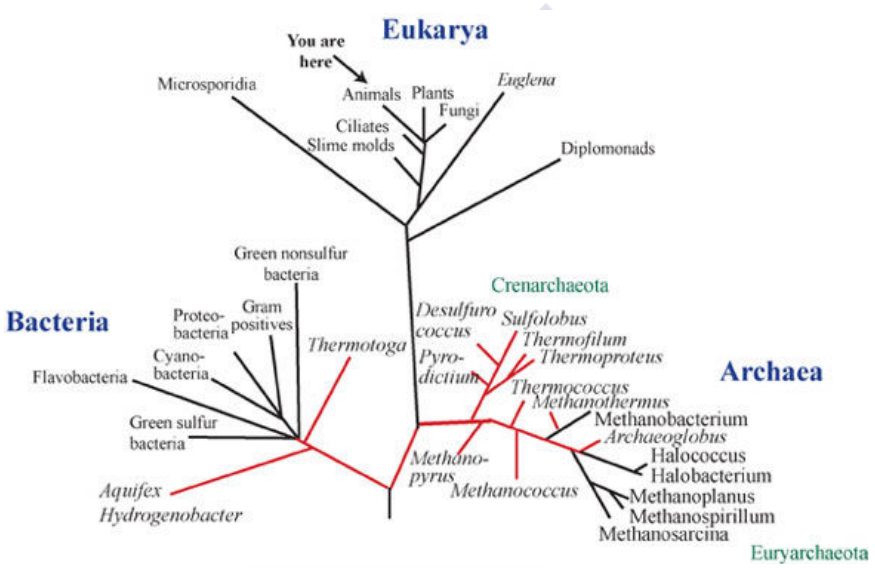


Figure 6-2 The universal phylogenetic tree based on the ribosomal RNA. The lines highlighted in red lead to organisms that are heat-loving.

The order *Methanobacteriales* comprises two families, *Methanobacteriacea* and *Methanothermacea*. *Methanobacteriacea* is a diverse family, which includes the genera *Methanobacterium*, *Methanothermobacter*, *Methanobrevibacter* and *Methanosphaera*. Strains within *Methanothermacea* can be distinguished from other members of this order by their high temperature optimal for growth of 83–88 °C. Optimal growth temperatures are often used as a criterion in microbial descriptions. Organisms exhibiting a preference for midrange growth temperatures (i.e. 30–40 °C) are termed mesophiles, while those with a decidedly lower optimal growth temperature are described as psychrophiles (or cold-

loving). Organisms with higher optimal growth temperatures are called thermophiles (or heat-loving) with those exhibiting a preference for extreme heat (90–100 °C) being denoted as hyperthermophiles.

Methanococcales is an order of coccoid, marine species comprised of two families, *Methanococcaceae* and *Methanocaldococcus*. All species within this order are slightly halophilic and most are chemolithotrophic, using hydrogen gas (H_2) or formate to reduce CO_2 to CH_4. Most strains of this order are mesophilic. However, a few thermophilic organisms are present, including *Methanococcus jannaschii*, the first archaeal species whose genomic sequence was determined.

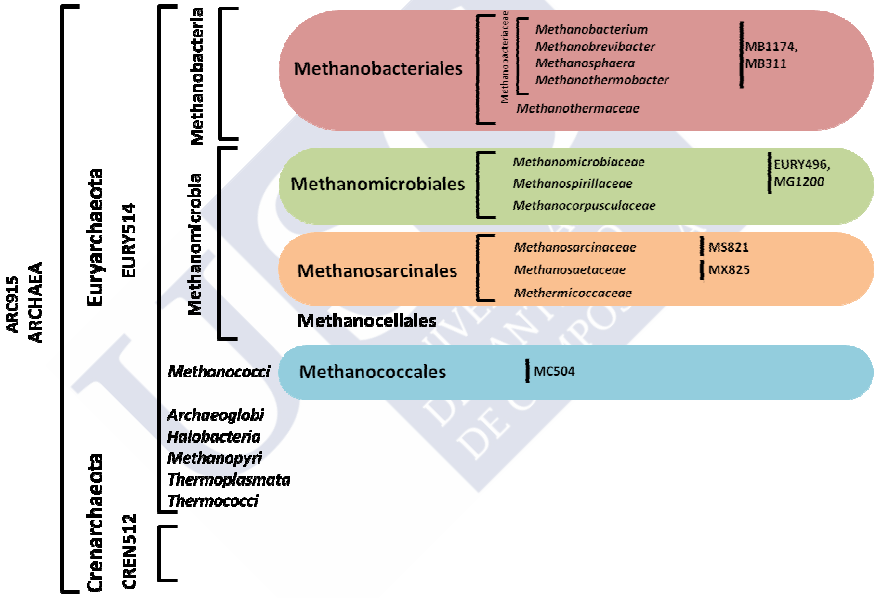


Figure 6-3 Phylogenetics of Methanoarchaea with corresponding families and FISH probes (adapted from Figueroa, 2011).

Organisms of the order *Methanomicrobiales* reduce CO_2 to CH_4 using H_2, formate, or in some instances alcohols. Nearly all species of *Methanomicrobiales* require acetate as a source of cell carbon and have additional, complex nutritional requirements. The family *Methanocorpusculaceae* contains only a singular coccoid, H_2-utilizing species (*Methanocorpusculum*). In contrast, *Methanomicrobiaceae*, the other family of *Methanomicrobiales*, contains several genera that are

diverse in morphology, physiology and phylogeny, including the deeply branching genus with spiral morphology, *Methanospirillum*.

The order *Methanosarcinales* comprises two families *Methanosarcinaceae* and *Methanosaetaceae*. Members of the *Methanosarcinaceae* are among the most metabolically versatile of all methanoarchaea regarding carbon and energy sources. Certain species of this family are able to utilize as many as seven distinct substrates for carbon and energy generation. Another unique aspect of this family is the presence of gas vesicles, possibly used as a mechanism for cell motility, in certain members such as *Methanosarcina vacuolata* and *Methanosarcina barkeri*. The family *Methanosaetaceae* contains a single acetate-degrading genus, *Methanosaeta*, also described as *Methanothrix*. Finally, the order *Methanopyrales* contains a single rod-shaped species, *Methanopyrus kandlerii*, which grows at or above the boiling point of water, using only H_2+CO_2 as carbon and energy sources.

6.1.3 Protozoa and metazoa

The presence of particular types of protozoans is related to effluent quality and plant performance. Protozoan play secondary but important role in purification of aerobic wastewater.

The protozoans in the activated sludge treatment process fall into four major classes: amoebae, flagellates, and ciliates (free-swimming, crawling, and stalked).

6.1.3.1 Amoebae

Amoebae (figure 6-4a) are the most primitive, single-celled protozoans. They move by false feet. They are frequently present in raw influent, and they are only dominant in the aeration basin for a short time. Amoebae can only multiply when there is an abundance of nutrients in the aeration tank. They move very slowly and it is difficult for them to compete for food if there is a limited amount available.

They feed on small organic particulates. When amoeba are present in large numbers in the aeration basin this usually indicates that there has been some sort of shock loading to the plant (there must be a lot of food

available). Their presence may also indicate that there is a low DO environment in the aeration basin, because they can tolerate very low amounts of DO.

6.1.3.2 Flagellates

Most flagellates (figure 6-4b) absorb dissolved nutrients. They appear soon after amoebae begins to disappear and while there is still high concentrations of soluble food. Flagellates and bacteria both feed on organic nutrients in the sewage so as the nutrient level declines they have difficulty out competing the bacteria and therefore, their numbers begin to decrease.

If large amounts of flagellates are present in the later stages of the activated sludge development this usually indicates that the wastewater still contains a large amount of soluble organic nutrients.

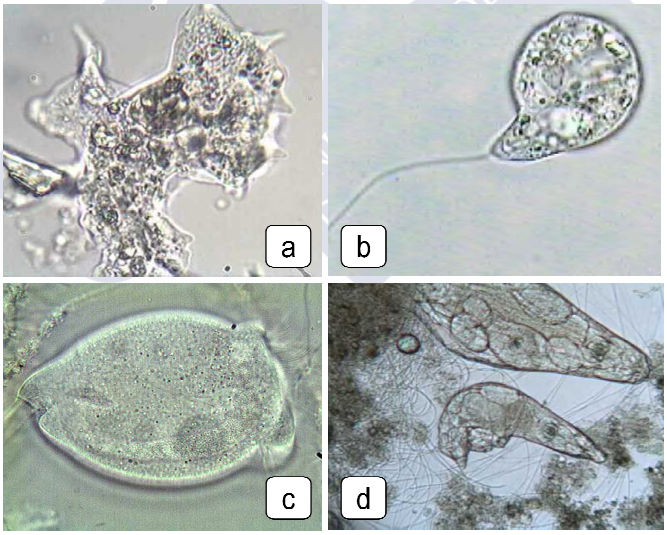


Figure 6-4 Examples of most common protozoa (microscopic observations): a) Amoebae, b) Flagellates, c) Ciliates, d) Rotifiers.

6.1.3.3 Ciliates

Ciliates (figure 6-4c) feed on bacteria not on dissolved organics. While bacteria and flagellates compete for dissolved nutrients, ciliates compete

with other ciliates and rotifers for bacteria. The presence of ciliates indicate a good sludge, because they dominate after the floc has been formed and after most of the organic nutrients have been removed.

- *Free-swimming ciliates* – These ciliates appear as flagellates begin to disappear. As the bacterial population increases, a lot of dispersed bacteria is available for feeding and as a lightly dispersed floc appears, free-swimming ciliates begin to dominate and feed on the increased numbers of bacteria.
- *Crawling ciliates* – As floc particles enlarge and stabilize, crawling ciliates graze on floc particles. Crawling ciliates out compete free-swimming ciliates for food because they can find food within the floc.
- *Stalked (sessile) ciliates* – Stalked ciliates appear in the mature sludge. Within the mature sludge the crawling and stalked ciliates compete for dominance.

6.1.3.4 Rotifers

Rotifers (figure 6-4d) are rarely found in large numbers in wastewater treatment processes. The principal role of rotifers is the removal of bacteria and the development of floc. Rotifers contribute to the removal of effluent turbidity by removing non-flocculated bacteria. Mucous secreted by rotifers at either the mouth opening or the foot aids in floc formation. Rotifers require a longer time to become established in the treatment process. Rotifers indicate increasing stabilization of organic wastes.

6.1.3.5 Factors Influencing Protozoa

- Temperature

Most protozoans can survive and reproduce in a temperature range at which activated sludge processes are carried out. They grow best in ambient temperatures (15-25 °C).

- pH

Protozoans are more sensitive to pH than floc-forming bacteria. They have an optimum pH range of 7.2-7.4 and a tolerance range of 6.0-8.0.

- Dissolved Oxygen

Like bacteria, protozoan must have oxygen to survive. Thus lack of DO will severely limit both the kind and number of protozoans.

- Nutrition

Most municipal wastewater treatment plants, however dilute, contains sufficient nutrients to support most of the protozoan associated with wastewater.

6.2 OBJECTIVES

The aim of this study was to make micro-scale observations on the structure and microbial composition of granular (anaerobic), suspended and biofilm biomass present in the combined UASB-MBR system. The structure and microbial populations were characterized using a wide range of molecular techniques, including FISH analysis (including the application of specific probes for detection of particular bacterial and archaeal groups), PCR, DGGE, cloning and sequencing. On the other hand, the necessity of the presence of Kaldnes support was explained, indicating its potential in slow-growing biomass development, as well as analysing the role of protozoa in high membrane flux maintenance.

6.3 MATERIALS AND METHODS

6.3.1 Biomass morphology

The morphology and size distribution of anaerobic granules were measured regularly using an image analysis procedure (Tijhuis *et al.*, 1994) with a

stereomicroscope (Stemi 2000-C, Zeiss), incorporating a digital camera (Coolsnap, Roper Scientific Photometrics). For the digital image analysis the programme Image Pro Plus was used.

The composition of the granule was analysed by the EVO® LS 15 environmental scanning electron microscope (SEM).

6.3.2 FISH analysis

Microscopic observation of anaerobic, aerobic and attached biomass was performed. Bacterial populations were identified by the Fluorescence in Situ Hybridization (FISH) technique. Biomass samples from the reactor were collected, disrupted and fixed according to the procedure described by Amann *et al.* (1995) with 4% paraformaldehyde solution. Hybridization was performed at 46 °C for 90 min. The used probes for in situ hybridization were 5´ labelled with the fluorochromes fluorescein isothiocyanate (fluos, green) and Cy3 (red). Fluorescence signals were recorded with an acquisition system (Coolsnap, Roper Scientific Photometrics) coupled with an Axioskop 2 epifluorescence microscope (Zeiss, Germany). FISH probes used for the microorganisms identification are collected in table 6-1.

Table 6-1 FISH probes used for the identification of different microorganisms present in the combined UASB-MBR system. %F refers to the percentage of Formamide.

Probe	Citoc.	Probe sequence (5' → 3')	%F	Target organisms
ALF1B	fluos	CGT TCG YTC TGA GCC AG	20	*Alphaproteobacteria, some Deltaproteobacteria, Spirochaetes*
Amx368	Cy3	CCTTTCGGGCATTGCGAA	15	*All Anammox bacteria*
ARC915	Cy3	GTGCTCCCCCGCCAATTCCT	20-35	*Archaea*
BET42a	Cy3	GCCTTCCCACTTCGTTT	35	*Betaproteobacteria*
CF319ab	Cy3	TGG TCC GTG TCT CAG TAC	35	*most Flavobacteria, some Bacteroidetes, some Sphingobacteria*
CFB560	fluos	WCCCTTTAAACCCART	40	*subgroup of Bacteroidetes*

Probe	Citoc.	Probe sequence (5' → 3')	%F	Target organisms
CFB562	Cy3	TACGYWCCCTTTAAACCCA	30	*subgroup of Bacteroidetes*
CFX1223	Cy3	CCA TTG TAG CGT GTG TGT MG	35	*Phylum Chloroflexi (green nonsulfur bacteria)*
Cte	Cy3	TTCCATCCCCCTCTGCCG	20	*Comamonas sp., Acidovorax sp., Hydrogenophaga sp., Aquaspirillum sp.*
DARCH872	fluos	GGCTCCACCCGTTGTAGT	30	*Various Euryarchaeota including ANME groups*
DBACT 1027	Cy3	TCTCCACGCTCCCTTGCG	30	*Bacteria belonging to NC10 phylum*
DBACT193	Cy3	CGCTCGCCCCCTTTGGTC	30	*Bacteria belonging to NC10 phylum*
DELTA495a	fluos	AGT TAG CCG GTG CTT CCT	35	*Most Deltaproteobacteria and most Gemmatimonadetes*
EUB338mix	fluos	GC(T/A)GCC(T/A)CCCGTAGG(A/T)GT	...	*Bacteria domain, Planctomycetales and Verrucimicrobiales*
EUB338mix	fluos	GC(T/A)GCC(T/A)CCCGTAGG(A/T)GT	...	*Bacteria domain, Planctomycetales and Verrucimicrobiales*
GAM42a	Cy3	GCCTTCCCACATCGTTT	35	*Gammaproteobacteria*
GAOQ431	Cy3	TCC CCG CCT AAA GGG CTT	35	*Candidatus "Competibacter phosphatis"*
GAOQ989	Cy3	TTC CCC GGA TGT CAA GGC	35	*Candidatus "Competibacter phosphatis"*
GNSB941	Cy3	AAACCACACGCTCCGCT	35	*Phylum Chloroflexi (green nonsulfur bacteria)*
MA450	Cy3	ATCCAGGTACCGTCATTATC	20	*Type II methanotrophs (Methylosinus/Methylocystis spp.)*
MG705	fluos	CTGGTGTTCCTTCAGATC	20	*Type I methanotrophs*
MG84	fluos	CCACTCGTCAGCGCCCGA	20	*Type I methanotrophs*
MX825	fluos	TCGCACCGTGGCCGACACCTAGC	50	*Some Methanosaetaceae*
MZ1	Cy3	TCTGCCGTACTCTAGCCTT	45	*Thauera spp. Mzt1t*

Probe	Citoc.	Probe sequence (5' → 3')	%F	Target organisms
NEU653	Cy3	CCCCTCTGCTGCACTCTA	40	*Most halophilic and halotolerant Nitrosomonas spp.*
NSO190	Cy3	CGATCCCCTGCTTTTCTCC	55	*Betaproteobacterial ammonia-oxidizing bacteria*
Ntspa712*	Cy3	CGCCTTCGCCACCGGCCTTCC	50	*Most members of phylum Nitrospirae*
PAO462	Cy3	CCGTCATCTACWCAGGGTATTA AC	35	*Candidatus "Accumulibacter phosphatis"*
PAO651	Cy3	CCC TCT GCC AAA CTC CAG	35	*Most members of the Candidatus "Accumulibacter" cluster*
PAO846	Cy3	GTT AGC TAC GGC ACT AAA AGG	35	*Candidatus "Accumulibacter phosphatis"*
PAR1244	Cy3	GGA TTA ACC CAC TGT CAC C	20	*Genus Paracoccus*
SYN773	Cy3	CTAGCTTTCGCACATGAG	15	*Synergistetes*
SYNM700	Cy3	ACTGGTNTTCCTCCTGATTTCTA	30	*mesophilic members of the family Syntrophomonadaceae (Gram-positive bacteria)*
ZRA23a	Cy3	CTGCCGTACTCTAGTTAT	35	*Most members of the Zoogloea lineage, not Z. Resiniphila*

6.3.3 DNA extraction

Granular, suspended and biofilm biomass was harvested directly from UASB and MBR stages and sonicated for 1 minute (5 minutes in the case of anaerobic granular biomass) at 65% of amplitude using an ultrasonic device (UP200s, Dr. Hielscher) to achieve homogenous samples. DNA was extracted using the MoBio Power Soil™ kit (MoBio Laboratories) according to the manufacturer´s protocol. DNA was then suspended in 50 μL ultrapure water, and kept at 4 °C until further analysis.

6.3.4 PCR amplification

PCR mixes used in the reactions were prepared in a laminar flow cabinet to avoid contamination of the samples. All material was previously sterilized.

The extracted material was PCR amplified using an automated thermal cycler (Applied Biosystems). The amplification products were used as targets for a second amplification using bacterial primers F968GC (GC indicates the presence of a GC-clamp at the 5'-end) and R1401 (Nübel *et al.*, 1996) for subsequent analysis in DGGE. The primers used in this work were synthesized and purified by Thermo Hybaid (Germany). The concentration used was 10 μL. The amplification was verified by electrophoresis by loading 5 μL of the reaction product in 1% agarose gel and fragment sizes were estimated using a GeneRuler^{TM} DNA Ladder Mix (Fermentas). Further, the DNA was precipitated following the protocol:

- For 45 μL of PCR product, add 4.5 μL CH_3COONa 3M and 90 μL of cold 100% ethanol (-20 °C).
- Mix and incubate at -20 °C for 60 minutes.
- Centrifuge at 12000 rpm for 30 minutes (4 °C) and discard very carefully the supernatant by using a micropipette.
- Re-dissolve the pellet in 200 μL of ethanol (70%), centrifuge again at 12000 rpm for 10 minutes and discard very carefully the supernatant by using a micropipette.
- Incubate the pellet at 37 °C until it dries.
- The dry pellet can be either stored at -20 °C or re-suspended in 15 μL of water or buffer.

6.3.5 Agarose gel electrophoresis

Horizontal electrophoresis in agarose gels was performed for both the analysis of PCR or restriction products, and in some cases for purification of DNA preparations. The agarose gels were prepared by dissolving agarose powder (Pronadisa) in TAE 0.5x buffer. Electrophoresis device used was MIDIGEL XL (Apelex). The electric source was PS-304 (Apelex). Agarose concentration was 1.0% agarose.

6.3.6 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is an electrophoretic method to distinguish among DNA sequences having the same length but differing in the base composition (Muyzer *et al.*, 1993). This method allows the electrophoretic separation and screening of heterogeneous PCR products mixtures.

The amplified sequences were separated by DGGE using a Ingenty phorU system (Ingenty).

For DGGE analysis 6% polyacrilamide gel was prepared using 40-80% urea-formamide denaturing gradient. Distribution of the samples is given in table 6-2. M represents a mixture of DNA used as a marker.

Table 6-2 Characterization of the samples used for DGGE, DNA extraction and sequencing.

DGGE track	Sample origin	DNA concentration (nanog/microL)
1.	UASB P1	20.2
2.	UASB P2	25.9
3.	UASB P3	28.6
4.	UASB P4	24.4
5.	UASB P5	24.5
6.	Kaldness	23.8
7.	Aerobic/anoxic	35.3
8.	Membrane	27.9

Reagents preparation

TAE buffer 50x: dissolve 242 g of Tris buffer in 800 mL of Milli-Q water and 100 mL od EDTA 0.5 M. Add 57.1 mL of glacial acetic acid, fill up to a final volume of 1L, and autoclave.

Stock solutions: aliquots with different percentages of urea/formamide were prepared and kept refrigerated. For each run of the gel it is necessary to take 24 mL of the low concentrated solution (i.e. 20%) and the high concentrated solution (i.e. 80%). Reagent volumes needed are given in table 6-3.

Table 6-3 DGGE stock concentration and reagent volumes for 6% polyacrilamide gels.

	0%	20%	30%	40%	50%	60%	70%	80%
TAE 50x (mL)	1	1	1	1	1	1	1	1
Polyacrilamide 40% (mL)	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Formamide (mL)	0	4	6	8	10	12	14	16
Urea (g)	0	4.2	6.3	8.4	10.5	12.6	14.7	16.8
Water	TO 50 mL							

Making a DGGE gel

- Clean the glasses, assemble and clamp the front and back glass plates with the spacers in between and then place the comb according to the Ingeny instructions. Wash out the gradient former.
- Prepare the low and high concentrated solutions, and add 5 μL of TEMED (N,N,N´,N´-tetramethylenediamine) and 50 μL of 20% APS (Ammonim Persulfate).
- Mix in the tube by inverting it a couple fo times and pour the content into the open side of the gradient former. With the help of the gradient former and a peristaltic pump, the two solutions are introduced into the gel sandwich.
- Allow the gel to polymerize for about 1-2 hours at room temperature.
- Add 6 μL of TEMED and 60 μL of APS to 6 mL of 0% DGGE solution. Mix by inverting several times and ppour into the gradient former.

Sample loading, gel running and visualization

- Put the polymerized gel sandwich inside the electrophoresis tank preheated to 60 °C wit TAE 1X buffer, and check if the upper chamber is filled up.
- Prepare the samples with ¼ (vol/vol) of loading buffer.
- Run the gel at 100 V for about 17 hours at 60 °C.

- Gels were stained with SYBR-Gold solution (Invitrogen) in TAE 1x buffer for 30 minutes and visualized under UV transillumination in the LIAS Xlite system (Avegene).

After visualization of the gel, in order to identify the main microorganisms forming part of the microbial community, most intense bands were cut with a sterilized scalpel, dissolved in 30 μL Milli-Q water and stored at 4 °C overnight to further reamplification by PCR using the same primers of first PCR without GC clamp.

In this work 36 bands were cut and send for sequencing and the results were aligned with the previously published sequences from the NCBI database. Among 36 bands send for sequencing 25 samples came back with positive results (extended methodology was presented by Figueroa, 2011).

6.4 RESULTS AND DISCUSSION

The combined UASB-MBR system was operated during more than 3 years. Within these period, stable microbial population has developed. The system was used to treat low- to medium-strength wastewater (Chapter 3 and Chapter 4, respectively) and was operated at ambient temperature (17 – 25 °C), meaning that wastewater temperature changed with seasons. The pH of the effluent from UASB stage was around 6.7. Aerobic/anoxic chamber of MBR stage and permeate pH varied from 6.7 to 7.7 and from 7.0 to 8.2, respectively, depending on the system performance.

6.4.1 General biomass characteristics

In the UASB stage the MLVSS concentration measured at the middle-bottom part (sampling port P3) was maintained at 30-35 g/L. The overall biomass yield calculated for the entire system was 0.094 and 0.14 $gVSS \cdot gCOD^{-1} \cdot d^{-1}$, for start-up and dairy wastewater treatment, respectively. Both values are much lower than the ones typically determined for aerobic MBRs (0.25 – 0.61 $gVSS \cdot gCOD^{-1}$) (Judd, 2006), and close to those observed

for the anaerobic wastewater treatment systems, that are in the range between 0.11 and 0.14 gVSS·gCOD^{-1} (van Haandel & Lettinga, 1994).

6.4.1.1 Anaerobic biomass characteristics

Anaerobic granules used as a seed were well retained in the anaerobic chamber and its growth was observed during the whole experimental period. Even though the system was purged to avoid wash-out of granules from the UASB stage, biomass concentration measured in the 4th sampling port (figure 2-1, P4) increased from around 21 gVSS·L^{-1} measured at the beginning of the operation to 34 gVSS·L^{-1}. The average ratio between MLVSS and MLTSS was constant among the whole operation and equal to 0.92, indicating that no inert material accumulation occurred in the methanogenic step and the sludge was stable (Mahnmoud *et al.*, 2004). Moreover, microscopic observation of the anaerobic biomass allowed to examine the formation of new layers of biomass on the surface of existing granules. In figure 6-5 the evolution of the anaerobic biomass is shown: on the left, inoculum biomass forming dark, smooth granules, with very homogenous size distribution. The middle figure shows whitish, ragged granules after more than 200 days of operation of the system and finally, on the right, the cross-section of the anaerobic granule, with the nuclei formed by the inoculum granule and subsequent layers of biomass is shown. The nuclei is surrounded by a spacious coat, probably formed by the gas produced by methanogenic and hydrogenic bacteria. The spatial arrangement of cells in the aggregated biomass was also observed by Beeftink & Staugaard (1986). The authors demonstrated the existence of mass transfer effects in anaerobic microbial aggregates growing on carbohydrates. Active cells were located in the outside portion of the aggregates, while the centre of the aggregates was composed of lysed cells and exopolymers. On the other hand, this stratification is tipical for granular biomass – in the case of anaerobic environments the special distribution of microorganisms is as follows: hydrolytic and acidogenic bacteria occupy the outher layers of the granule, while acetogenic bacteria and methanogenic archaea are place inside the granule.

Figure 6-5 Left – innoculum anaerobic granules; middle – anaerobic granules observed durig the operational periods; right – cross-section of an anaerobic granule. Scale bar corresponds to 1 mm.

Moreover, the composition of the anaerobic granules was analysed. In this sense three layers of biomass were tested, as indicated in figure 6-6.

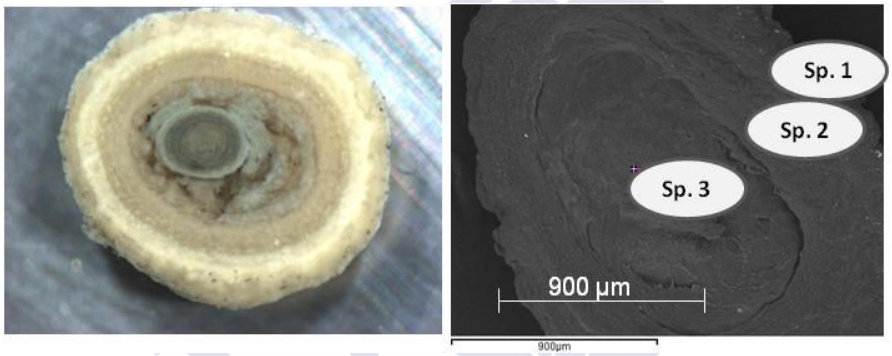


Figure 6-6 Cross-section of the anaerobic granule of the UASB stage and the location of each spectrum taken for the composition analysis.

Carbon and oxygen accounted for more than 90% of the mass of the anaerobic granules, independently of the layer analysed, indicating organic origin (table 6-4). However, few differences were observed within the depth of the granule concerning Cl, Ti and Fe. 0.13% of chloride and 0.24% of titanium were found in the centre part of the granule, while Fe was present either in the centre, or in the outer layer, accounting for 1.17 and 0.17%, respectively. Moreover, considering that the proposed UASB-MBR system was fed with diluted milk, surprisingly low amounts of Ca, P, K, Na, Mg, Cl and S were found, with a little higher concentration of Mg, P and K in the outer layer, and Na, S, Ca and Cl in a centre. All elements, except from Al, Si, Cl and Ti, are typical composition of methanogenic (and not only) bacteria.

In addition particle size distribution of the anaerobic sludge was done on day 123, 149 and 190 of operation taking samples from sampling ports P1, at the bottom, P3, in the middle, P4 (days 149 and 190) and P5, at the top of the sludge blanket of the UASB chamber (figure 2-1). The average granules sizes are collected in table 6-5, showing a successive decrease of the size of the granules with the height of the reactor. The most developed granules were found in the lower part of the UASB chamber, while the upper part was characterized by a mixture of disrupted granules and flocculent sludge recycled from aerobic chamber of the MBR and accumulated in this part of the UASB stage. Finally, the size distribution measurement on day 190, when the system was operated without recirculation, shows the enlargement of anaerobic granules, especially in ports P4 and P5. This fact could be easily explained, since no flocculent sludge was introduced to the UASB stage.

Table 6-4 Elemental composition of the anaerobic granules. Spectrum 1, 2 and 3 correspond to Sp.1, 2 and 3 in figure 6-6.

Element	Spectrum 1		Spectrum 2		Spectrum 3	
	Weight%	Atomic%	Weight%	Atomic%	Weight%	Atomic%
C	53.01	62.14	54.79	63.12	47.01	55.92
O	39.58	34.83	39.77	34.39	45.56	40.69
Na	0.37	0.23	0.52	0.31	0.63	0.39
Mg	0.39	0.23	0.32	0.18	0.36	0.21
Al	0.22	0.11	0.53	0.27	1.18	0.63
Si	0.28	0.14	0.72	0.36	0.42	0.21
P	1.81	0.82	1.22	0.55	1.22	0.56
S	1.02	0.45	0.97	0.42	1.40	0.62
Cl	-	-	-	-	0.13	0.05
K	0.78	0.28	0.16	0.06	0.12	0.04
Ca	1.37	0.48	1.00	0.35	1.55	0.55
Ti	-	-	-	-	0.24	0.07
Fe	1.17	0.29	-	-	0.17	0.04

Grotenhuis *et al.* (1991) studied the effect of substrate concentration on methanogenic granule size distribution. The authors found out that the methanogenic activity is directly proportional to influent substrate concentration. In this sense, granule size increases until substrate

limitations occurs in the centre of the granule. When this happens, the substrate limited biomass in the centre begins to decay, weakening the structure of the granule and making it susceptible to shear forces.

Table 6-5 Summary of the size distribution of anaerobic granules from UASB stage of the system during Period III and IV.

Day of operation	Sampling port	Comments	Mean volume (mm^2)	Mean radius (mm^2)	Mean diameter (mm^2)
123 (start of Period III)	P1	COD in the influent was increased and the system was operated at stable conditions. No biomass purges.	13.99	1.49	2.99
	P3		9.11	1.29	2.59
	P5		3.21	0.91	1.83
149 (end of Period III)	P1	Start-up of nitrification in aerobic biofilm chamber. Slight decrease of methane production. Biomass purges from sampling port P5.	12.40	1.43	2.87
	P3		4.48	1.02	2.04
	P4		4.86	1.05	2.10
	P5		2.18	0.80	1.61
190 (Period IV)	P1	No recirculation from aerobic biofilm chamber to UASB unit. Biomass purges from the sampling port P5. Stable operation. Nitrification in biofilm chamber.	12.64	1.44	2.89
	P3		8.83	1.28	2.56
	P4		11.74	1.41	2.82
	P5		4.51	1.02	2.05

Specific Methanogenic Activity (SMA) measured at 20 °C at the end of Period IV of the start-up period (Chapter 3), was 5.26 and 13.54 $mLCH_4 \cdot g^{-1} \cdot VSS^{-1} \cdot d^{-1}$, for sampling ports P2 and P4, respectively. When the second feeding was applied, activities were slightly higher, reaching 9.02 and 15.42 $mLCH_4 \cdot g^{-1} \cdot VSS^{-1} \cdot d^{-1}$ for P2 and P4, respectively. These values are similar to those presented by Álvarez *et al.* (2006), for granular anaerobic biomass fed with domestic wastewater at ambient temperatures, and were maintained during the whole operation of the UASB-MBR system. However, the maximum apparent capacity of the reactor observed taking into account a maximum methane production (approx. 97.5 $L \cdot d^{-1}$), CH_4 dissolved in the effluent (approx. 6.6 $L \cdot d^{-1}$, taking into account the average flow of 270 $L \cdot d^{-1}$ and that 24 $mgCH_4 \cdot L^{-1}$ was dissolved in the UASB effluent) and granular

biomass concentration of approx. 30 gVSS $\cdot L^{-1}$ occupying the volume of approx. 100 L of the UASB, the maximum SMA would be around 35 $mLCH_4 \cdot g^{-1} \cdot VSS^{-1} \cdot d^{-1}$. This value is 2-3 times higher than that obtained in SMA tests. On the other hand, if the average daily methane production is taken into account, being 60 $L \cdot d^{-1}$, the estimated SMA would be 20 $mLCH_4 \cdot g^{-1} \cdot VSS^{-1} \cdot d^{-1}$. This value is closer to that obtained in SMA tests.

6.4.2 Aerobic biomass characteristics

In both aerobic and filtration chamber of MBR stage the MLVSS concentration was kept around 4 $g \cdot L^{-1}$, except during certain moments of the system operation, when punctual excessive solids accumulation occurred (reaching around up to 12 $g \cdot L^{-1}$ in the aerobic and membrane chamber, respectively), or, on the contrary, biomass concentration diminished to 0.5 – 1 $g \cdot L^{-1}$. Biofilm growing on the support carrier in aerobic chamber was well developed within the experiment (figure 6-8), and its concentration was 22.81 gVSS m^{-2}.

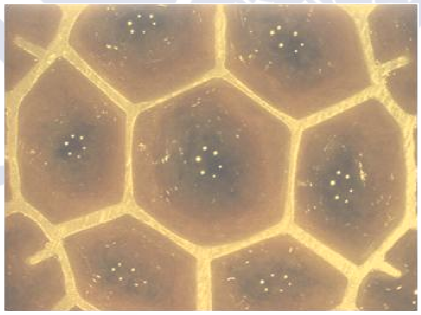


Figure 6-7 Biofilm developed on plastic carriers in aerobic chamber during the start-up of the system

6.4.2.1 Biofilm development

Microscopic observations of aerobic and attached biomass were performed. In the case of Kaldnes support, biofilm development was monitored during the start-up period by microscopic observations. Figure 6-8 shows the biofilm layer during the crucial events of the system operation. At the beginning, rapid development of attached biomass was observed, mostly because of the low organic load introduced to the aerobic chamber and no

competition with suspended sludge, which was recycled to the anaerobic UASB stage with ratio equal to 0.15. First diminution of biofilm layer was observed after methanol was added (day 99, Chapter 3), leading to successive loss of attached biomass in favour of suspended sludge.

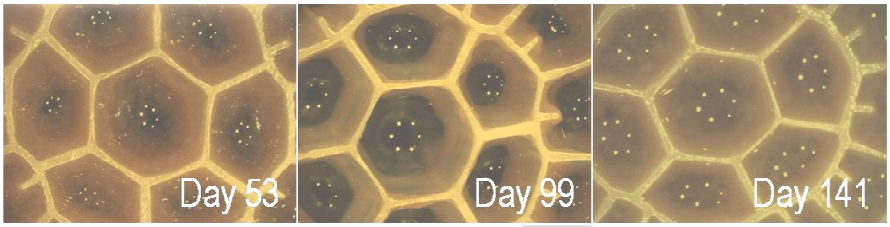


Figure 6-8 Development of the biofilm growing on Kaldnes support. Day 53 – full development of the biofilm; day 99 - biofilm depletion, day 141 – recovery of biofilm layer

Due to the punctual overload flocculent bacteria became dominant and suspended biomass concentration increased. This phenomenon was observed also after day 123 (data not shown), when almost complete biofilm depletion was observed. However, when the purges of the biomass were started, biofilm layer was recovered (day 141). After that, the available food to the MBR stage was relatively constant, however when $F/M_{biofilm}$ ratio increased (e.g. due to the recirculation of suspended biomass from MBR stage to the UASB), the thicker biofilm layer was formed. On the contrary, when F/M ratio decreased, biofilm detachment and its slower apparent growth was observed.

6.4.2.2 Protozoa

After 570 days of operation (Sánchez *et al.*, 2013) the Kaldnes support was removed from the system to see its influence on membrane performance. Remaining biomass was subjected to microscopic analysis and brief comparison of microorganisms present in the aerobic chamber with and without Kaldnes rings was done. In the first case biofilm layer formed on Kaldnes´ surface was characterized by a certain structure (figure 6-9a): the inner layer was composed of the compact film of bacteria, while the outer layer was occupied by the protozoa and metazoa (mostly sessile ciliates and rotifers). A large number of protozoa (most likely free swimming ciliates) was also present in the mixed liquor, together with the flocs formed by bacteria growing in suspension (figure 6-9b).

Curiously, it was observed that in the case of the biomass samples taken when the system operated without Kaldnes, the amount of ciliates decreased significantly. Moreover, rotifers were not observed. As mentioned in section 6.1.3, the principal role of ciliates and rotifers is to remove bacteria and, in the case of the later, the development of flocs via mucous secretion. Apart from low loading rates, both microorganisms require a longer time to become established in the treatment process, therefore Kaldnes support seems to promote their growth. As an advantage, the colonies of ciliates and rotifers feed on non-flocculated bacteria and colloids, decreasing the turbidity of the liquid phase and controlling excessive bacterial growth. Hypothetically, the absence of these filtering organisms provokes the increase of colloidal biopolymer concentration (cBPC). This in turn was proved to have a strong effect on membrane fouling properties of aerobic chamber broth in the studied UASB-MBR system (Sánchez *et al.*, 2013).

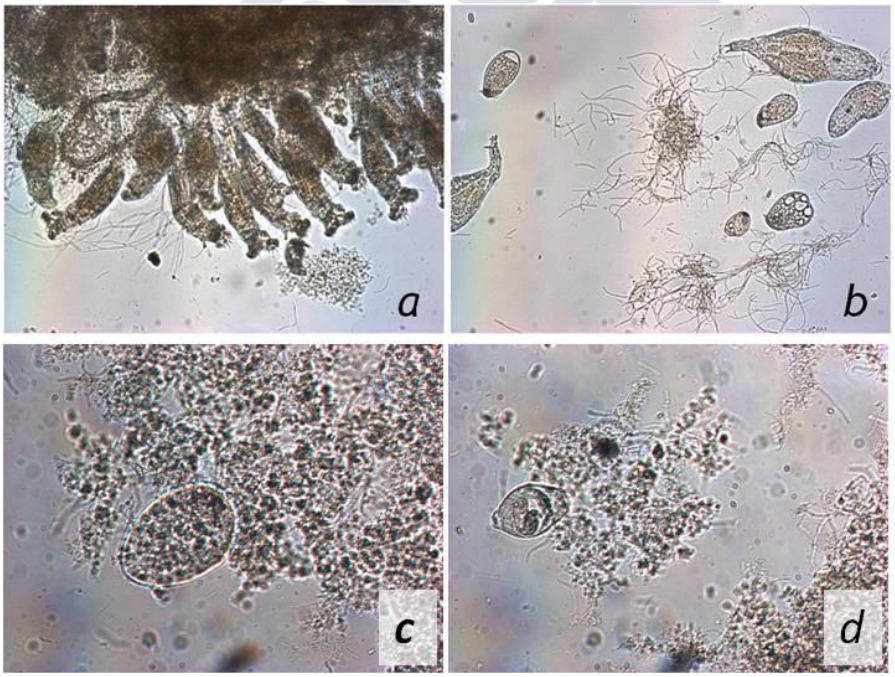


Figure 6-9 Microscopic observations of biomass with Kaldnes rings: (a) biofilm and (b) suspended biomass; and suspended biomass without Kaldnes support: (c), (d). Photos were taken with 100x augmentation

Although digestion of detrital colloids by protozoans is not fully understood, assimilation of some forms of colloidal exopolymers by protozoans has been reported (Sherr, 1988; Barbeau *et al.*, 1996; Posch & Arndt, 1996). On the other hand, exoplymer reduction was also observed in worm reactors used to reduce biological excess sludge. In recent years, the influence of coupling a worm reactor with an MBR on fouling potential of soluble microbial products (SMP) was studied (Tian *et al.*, 2013). The authors found that the worm predation weakened fouling potential of SMP generated in the MBR, and this effect was mainly attributed to the difference in the nature of the fouling layer. Their conclusion was that these changes of fouling layer could be explained by the lower hydrophobic interaction resulting from the decrease in the number of aromatic rings in SMP due to worm predation. This fact indicates important role of predatory organisms in fouling reduction in MBR systems.

Finally, Derlon *et al.* (2013) suggested, that for wastewaters reach in particulate organic carbon (POC) the use of worms is suitable to enhance POC removal before ultrafiltration units. They observed, that in the absence of predation, the hydraulic permeability of the biofilm is governed by the TOC content only. Under these conditions, the permeate flux is inversely related to TOC content due to higher accumulation of biofilm. These results are in agreement with the work presented by Sánchez *et al.* (2013), who found negative correlation of colloidal fraction of BPC (measured as TOC of filtrated mixed liquor and permeate) with membrane flux.

Derlon *et al.* (2013) also highlighted the role of metazoan in biofilm formation, structure and fouling properties. According to their work, metazoan organisms (in particular nematodes and oligochaetes) built-up a protective environment, which results in the formation of open and spatially heterogeneous biofilm composed of biomass scraps. Such biofilm has increased hydraulic permeability and therefore allows to achieve higher fluxes. In the absence of metazoa, a flat and compact biofilm develops. It was concluded that the activity of metazoan organisms balances the detrimental effect of a high biofilm accumulation, thus positively influencing filtration properties.

Therefore, in the case of proposed UASB-MBR system, the presence of plastic support and thus development of predators is crucial for stable operation and high flux achievement in the MBR stage, especially when the

MBR is operated at low MLVSS. These strategy could be also helpful in start-up procedure.

6.4.3 Characteristic of microbial populations in anaerobic biomass

The FISH technique was applied to visually estimate the share of archaea and bacteria in the samples of granular anaerobic biomass taken from UASB stage. As can be seen in figure 6-10, the archaea were very abundant, accounting for approximately 50±5% of DAPI stained microorganisms (according to photo image analysis performed with DAIME programme).

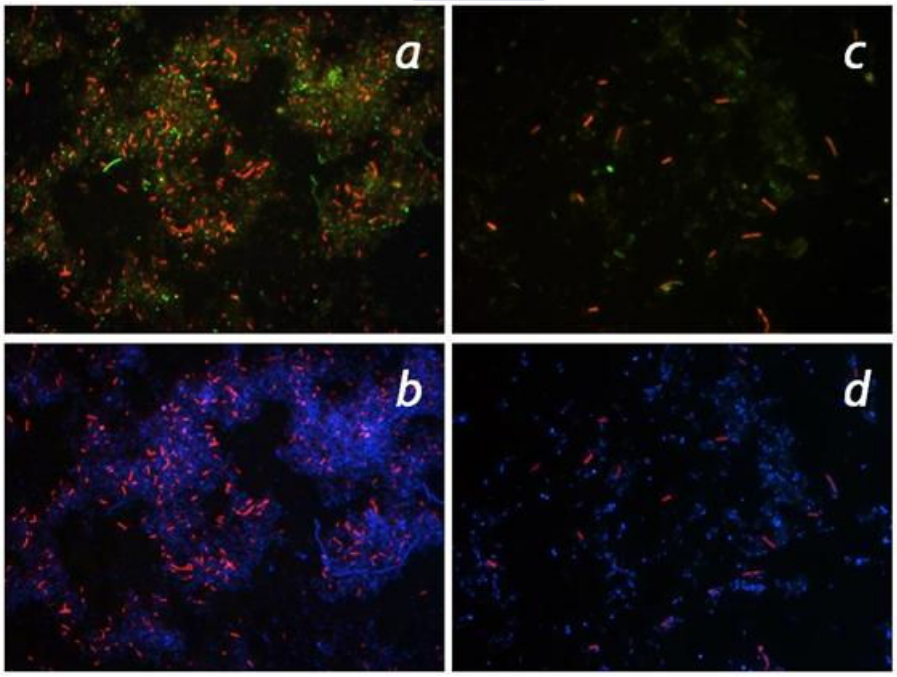


Figure 6-10 (a) Archaea: ARC915 cy3 (red) and Bacteria domain: EUB338mix fluos (green), x40; (b) ARC915 cy3 (red) and DAPI (blue) x40; (c) ARC915 cy3 (red) and EUB338mix fluos (green), x100; (d) ARC915 cy3 (red) and DAPI (blue) x100. Biomass was taken from the sampling port P1 on 92 day on operation (campaign 1).

Further analysis was dedicated to determine the presence of particular methanogenic archaea. Firstly, the occurrence of *Methanobacteriales* was checked. It is an order of anaerobic, coccoid to rod-shaped methanogens, in the kingdom Euryarchaeota (section 5.1.2). They are widely distributed in

nature and can use as a substrate H_2 + CO_2, formate and CO (which may also be oxidized), methanol (which may be reduced), and secondary alcohols + CO_2 (Dworkin *et al.*, 2006). However, no *Methanobacteriales* were found.

Additionally, the presence of *Methanosarcina* and some *Methanosaetaceae* was checked. Both *Methanosarcina* and *Methanosaeta* (which belongs to *Methanosaetaceae*) exhibit natural tendencies to aggregate and therefore are commonly found in anaerobic sludge granules. Figure 6-11 (a and b) shows the confirmation of the presence of *Methanosaetaceae*. However, the determination of *Methanosarcina* came negative (figure 6-11c) and no confirmation was found combining MS821 probe with DAPI (figure 6-11d).

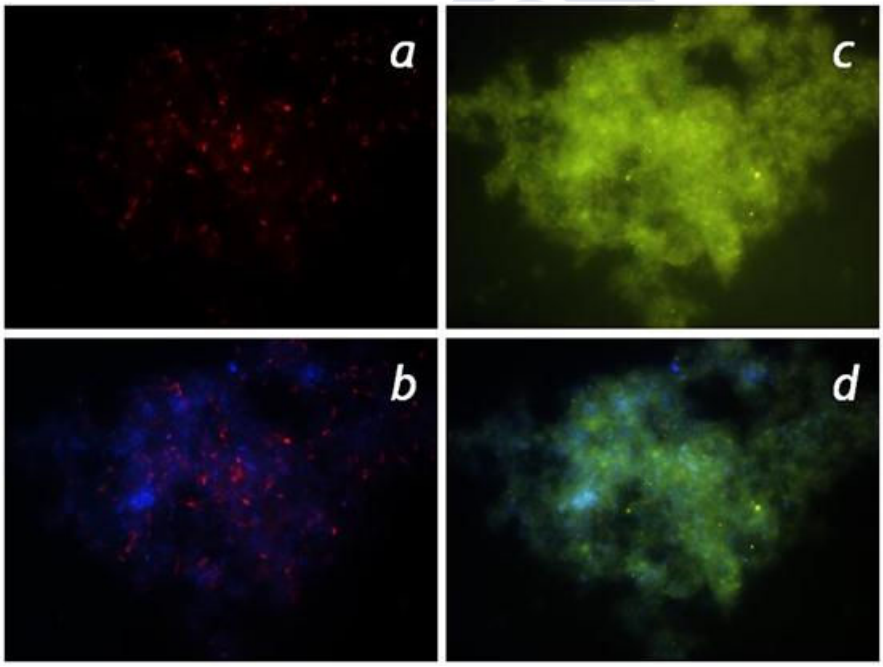


Figure 6-11 (a) Methanosaetaceae: MX825 cy3 (red), x100; (b) MX825 cy3 (red) and DAPI (blue) x100; (c) Methanosarcina: MS821 fluos (green), x100; (d) MS821 fluos (green) and DAPI (blue), x100. Biomass was taken from the sampling port P3 on 181 day on operation (campaign 1).

Dworkin *et al.* (2006) and Smith & Ingram-Smith (2007) highlighted that in environments such as anaerobic digesters *Methanosaeta* prevail over *Methanosarcina* due to their high affinity for acetate. *Methanosaeta* usually out-compete *Methanosarcina* in environments in which turnover is slow

and acetate concentrations are low. This could happen in the case of anaerobic biomass studied in this work, since the organic load applied to UASB chamber is low in comparison with typical anaerobic digesters. Moreover, the system is operated at ambient temperatures (17.3 – 23.5 °C), which also narrows the pool of methanogens able to acclimate and grow under this conditions, since the optimum temperature for the majority of methanogenic bacteria is within the mesophilic range. Additionally, acetoclastic methanogens are known to be a key microorganisms in psychrophilic anaerobic digesters and their presence is significant as the maintenance of granular sludge, even at low-temperature operation during treatment of challenging low-strength wastewaters, such as domestic sewage (McHugh *et al.*, 2003).

6.4.4 Characteristic of microbial populations of biomass from MBR stage

The FISH technique was also applied to characterize the main populations present in the MBR stage of the combined UASB-MBR system. Biomass samples taken from aerobic/anoxic chamber as well as biofilm biomass growing on Kaldnes support were analysed. Table 6-6 shows a compilation of the results obtained by the analysis of all types of biomass applying the indicated FISH probes. Among *Proteobacteria* phylum, a class of *Betaproteobacteria* was the most dominant, followed by the *Gammaproteobacteria*. *Alphaproteobacteria* was quite scarce and appeared in coccoid form, while *Deltaproteobacteria* were not observed at all, except some samples of biofilm biomass, were some weak positive signals were detected. The predominance of members of *Betaproteobacteria* was associated with abundance of nitrifying and denitrifying bacteria.

Table 6-6 Comparison of bacterial populations observed in aerobic, membrane and biofilm (Kaldnes) biomass during the operation of the combined UASB-MBR system. The amount of microorganisms identified is described as: (+) some, (++) quite abundant, (+++) very abundant.

Probe	Microorganisms	Aerobic	Membrane	Kaldnes
Alf1b	*Alfaproteobacteria*	+/-	+/-	+/-
Amx368	*All Anammox bacteria*	+	+	+
Beta42	*Betaproteobacteria*	+++	+++	++

Probe	Microorganisms	Aerobic	Membrane	Kaldnes
CFB560 + CF319	*Subgrup of Bacteroidetes Flavobacteria, some Bacteroidetes and Sphingobacteria*	+	+	+
CFB562	*Subgrup of Bacteroidetes*	++	++	+
CFX1223 + NSB941	*Chloroflexi (green non-sulfur bacteria)*	++ (2 types)	+ (2 types)	+
Cte	*Comamonas, Acidovorax, etc*	+	++	++
DARCH872	*Various Euryarchaeota including ANME groups*	+	+	+
DBACT1027 +DBACT193	*Bacteria belonging to NC10 phylum*	+	+	+
Delta	*Deltaproteobacteria*	no	no	+/-
Gam42	*Gammaproteobacteria*	+	++ (tetraedros)	+
GAOmix	*Competibacter phosphatis Glycogen accumul. organisms*	+ (3 types)	+ (2 types)	+ (2 types)
MA450	*Type II methanotrophs*	no	no	no
MG705 + MG84	*Type I methanotrophs*	+++	++	+++
MX825	*Some Methanosaetaceae*	+++ (all Arc915)	+++ (all Arc915)	+++ (all Arc915)
MZ1	*Thauera spp.*	+	++	+
NEU653	*AOB Nitrosomonas spp.*	+ (?)	+ (?)	+ (?)
Nit3	*Nitrobacter*	no	no	no
Nso190	*AOB Betaproteobacteria*	++	++	++
NTSPA712	*Nitrospira*	++	++	+++
PAOmix	*Accumulibacter phosphatis*	no	no	no
Par	*Paracoccus*	no	no	no
Syn700	*Mezophilic Syntrophomonadaceae*	no	no	no
Syn773	*Synergistes*	no	no	no
Zra23	*Zooglea*	+	+	+/-

During the first periods of operation some Anammox bacteria (probe Amx368) were found in granular, suspended and biofilm biomass. The appearance and significance of these bacteria will be further discussed in Chapter 7. By application of FISH technique it could be observed that these bacteria were grouped in cauliflower-type clusters, which are typical form for these bacteria. On the other hand, microscopic observation revealed the presence of a little number of orange granules (Chapter 7, section 7.4.4, figure 7-8), which were attributed to Anammox bacteria. This orange

(sometimes red) colour is due to the heme c group of the protein cytochrome c, that plays an important role in the anammox metabolism.

Probes CFB560, CF319 and CFB562 which target the group of *Bacteroidetes*, gave positive results revealing that most of the microorganisms were long and small, bacillus-type. However, in figure 6-12f it can be seen, that in the case of biofilm biomass bacteria that gave positive results for specific *Bacteroidetes* probes were small and round rather than long. Kragelund *et al.* (2008) revealed that these bacteria are specialized in degradation of sugars, e.g. glucose, and may participate in the conversion of lipopolysaccharides and peptidoglycan liberated by decaying cells. In their study the authors observed that many surface-associated exo-enzymes were excreted (e.g. chitinase, glucuronidase, esterase and phosphatise) supporting conversion of polysaccharides and possibly other released cell components. In this sense Bacteria belonging to *Bacteroidetes* may play an important role in fouling properties of sludge in membrane filtration chamber.

Some microorganisms belonging to *Chloroflexi*, detected by the application of probes CFX1223 and NSB941, were found (table 6-6, figure 6-12b). These filamentous bacteria are typical for municipal sludge. Kragelund *et al.* (2006) revealed that *Chloroflexi* is a specialized group of filamentous bacteria only active under aerobic conditions consuming primarily carbohydrates. The authots observe the excretion of many exo-enzymes, e.g. chitinase, glucuronidase and galactosidase, suggesting growth on complex polysaccharides. Several other studies also shows potential of bacteria beolonging to *Chloroflexi* in the degradation of carbohydrates (Kindaichi *et al.*, 2004; Ariesyady *et al.*, 2007). Therefore, these microorganisms may also contribute in the regulation of soluble microbial products (SMP) concentration present in the bulk of membrane filtration chamber, and thus have an impact on membrane performance.

The application of Cte probe, used to identify the family *Comamonadaceae*, produced positive signal both in suspended and biofilm biomass. These denitrifying bacteria belonging to *Betaproteobacteria* are very common in the activated sludge. Apparently, they were more abundant in suspended biomass located in membrane filtration chamber, where aeration was constant, and in biofilm. They appeared to cover a wide range of microorganisms, including rod-shaped and filamentous bacteria.

Bacteria belonging to NC10 phylum were identified by using the combination of probes DARCH872 and DBACT1027 + DBACT193 (Chapter 7, section 7.4.3, figure 7-7). These bacteria are able to use methane as the sole carbon and energy source in the process of anaerobic denitrification. Their significance in the nitrogen and dissolved methane removal will be further discussed in Chapter 7, section 7.4.3.

GAOmix probe used for detection of glycogen accumulating organisms gave positive signal in suspended and biofilm biomass (table 6-6, figure 6-12i). However, in aerobic/anoxic chamber three types of cluster formation were found: cluster, diplococcus and multi-tetrad, while in membrane chamber and biofilm biomass these microorganisms were present as streptococcus or clusters (figure 6-12i).

FISH analysis of bacteria belonging to *Methanotrophs* revealed that type I was abundant (table 6-6, figure 6-12j), while specific probe for type II (MA450) gave negative results. Type I *Methanotrophs* belong to *Gammaproteobacteria* and are responsible for aerobic oxidation of methane present in dissolved form in the bulk liquid of MBR stage. Dissolved methane can be estimated considering that UASB effluent will be, at least, in equilibrium with the biogas formed by using the Henry´s law. In the case of present work, the UASB effluent is oversaturated with methane, reaching between 13.4 and 20.8 $mgCH_4 \cdot L^{-1}$, operating at 17-25 ºC. Further discussion on potential of dissolved methane oxidation by methanotrophic bacteria will be presented in Chapter 7, sections 7.4.1 and 7.4.3.

All archaea present both in suspended and biofilm biomass were identified as *Methanosaeta* (table 6-6). Since these microorganisms were dominant in the anaerobic UASB stage of the system (section 6.4.3), their appearance in MBR stage is reasonable and could be explained simply by the occasional wash-out of the anaerobic biomass.

Some *Thaurea spp.* (probe MZ1) and *Zooglea* (probe Zra23) were detected in all types of biomass analysed (anaerobic granular, suspended and biofilm) (table 6-6). However, microorganisms identified as *Zooglea* were more abundant in suspended biomass (figure 6-12e). Bacteria identified as *Thaurea spp.* appeared as small clusters dispersed within the sample (figure 6-12d).

Nitrifying bacteria belonging to *Nitrosomonas spp.* and *Nitrospira* (table 6-6, figure 6-12l) were detected using probes NEU653 and NTSPA712, respectively. While microorganisms belonging to *Nitrospira* were quite abundant, hybridization with probe NEU653 gave some uncertain results. Ammonia oxidizing bacteria belonging to *Betaproteobacteria* and detected with probe Nso190 were quite abundant in both suspended and biofilm biomass (table 6-6, figure 6-12k).

Biomass samples analysed with FISH technique indicated the presence of both *Bacteria* and *Archaea* domains. Due to application of membrane technology all the microorganisms are retained within the system which allow their further development, if the growth favouring conditions are met.

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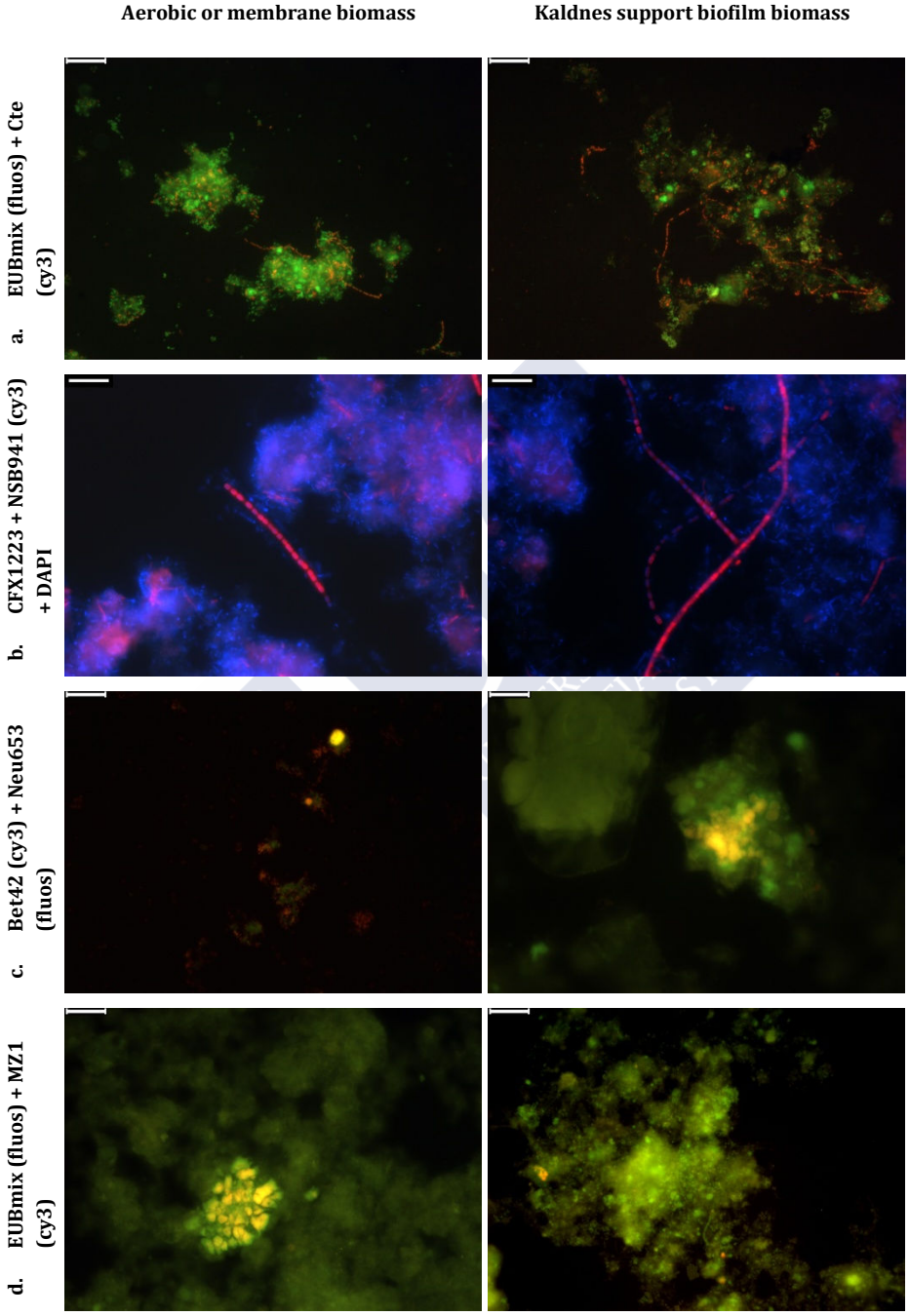
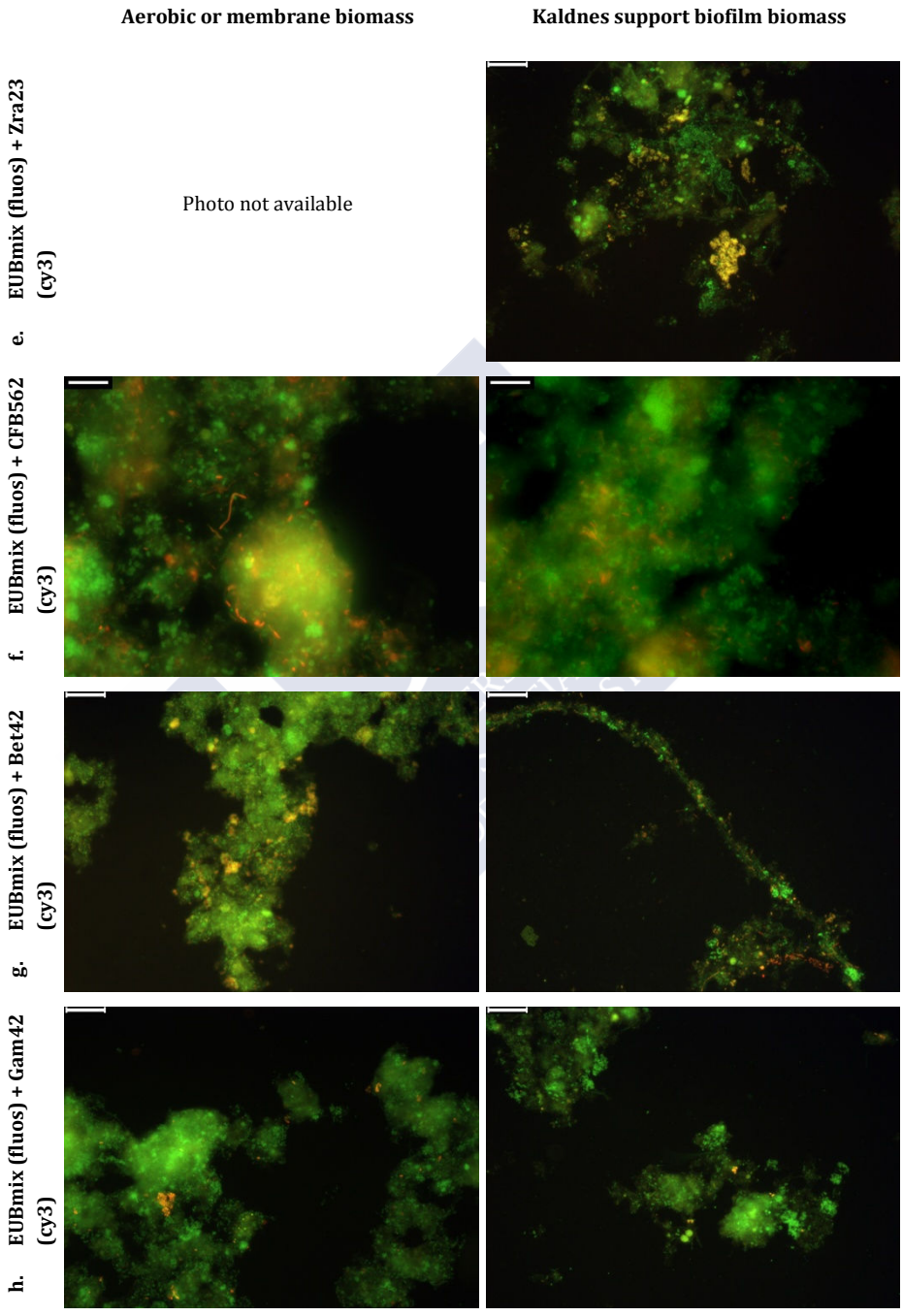

Aerobic or membrane biomass
Kaldnes support biofilm biomass
a. EUBmix (fluos) + Cte (cy3)
b. CFX1223 + NSB941 (cy3) + DAPI
c. Bet42 (cy3) + Neu653 (fluos)
d. EUBmix (fluos) + MZ1 (cy3)


Aerobic or membrane biomass
Kaldnes support biofilm biomass
e. EUBmix (fluos) + Zra23 (cy3)
Photo not available
f. EUBmix (fluos) + CFB562 (cy3)
g. EUBmix (fluos) + Bet42 (cy3)
h. EUBmix (fluos) + Gam42 (cy3)

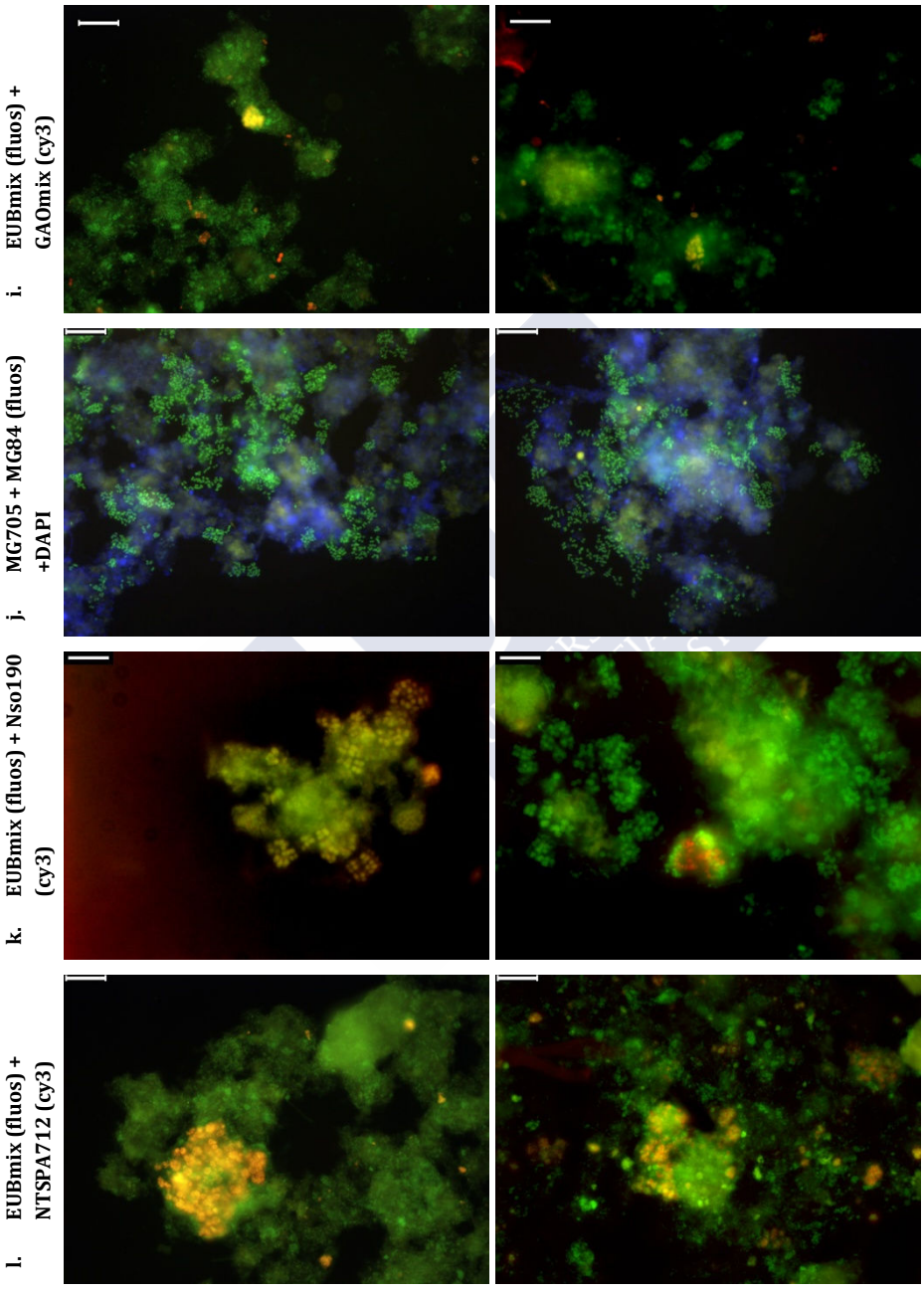

Aerobic or membrane biomass
Kaldnes support biofilm biomass
i. EUBmix (fluos) + GAOmix (cy3)
j. MG705 + MG84 (fluos) +DAPI
k. EUBmix (fluos) + Nso190 (cy3)
l. EUBmix (fluos) + NTSPA712 (cy3)

Figure 6-12 On previous pages: FISH analysis with the specific probes (indicated on the left) for suspended and biofilm biomass form MBR stage of combined UASB-MBR system. Fluorochromes used were fluos (green) and Cy3 (red). Scale bars are equal to 25 μm in photos (a), (c), (d), (e), (g), (h), (i) left, (j) and (l); and 10 μm in photos (b), (f), (i) right and (k).

Finally, molecular DGGE profile of PCR amplified bacterial rDNA 16s genes revealed the distribution of microbial populations within a sludge profile in the UASB stage (figue 6-13, samples 1 – 5) and bacterial populations in suspended and biofilm biomass of MBR stage (figue 6-13, samples 6 – 8). The DGGE profiles of the samples taken from different sampling ports revealed that the microbial composition of anaerobic biomass was quite homogenous; bacteria belonging to *Clostridium* and *Firmicutes* family, as well as *Propionibacterium*, were present along the anaerobic sludge profile (table 6-7). These microorganisms are common for the anaerobic sludge digesters. *Acido-* and *Actinobacteria* related microorganisms were not found in the sampling port located at the bottom of the reactor (P1), but detected in sampling ports P2, P3, P4 and P5. On the other hand, *Verrucomicrobia* were found in the upper parts of UASB reactor (table 6-7).

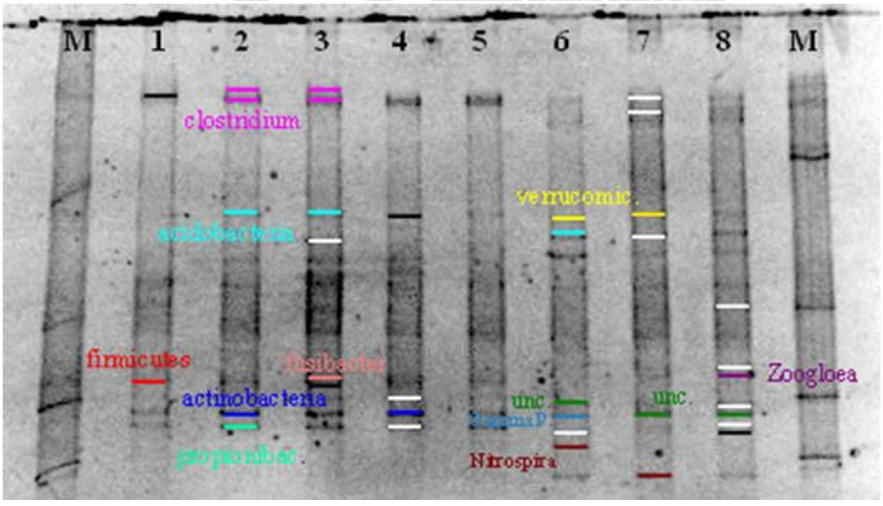


Figure 6-13 DGGE gel with corresponding bands send for sequencing. Numbers from 1 to 8 corresponds with the biomass samples described in table 6-2.

In the case of aerobic/anoxic biomass (MBR stage), bacteria belonging to subclass *Gammaproteobacteria* (most probably methanotrophic bacteria), *Verrucomicrobia* and various microorganisms related to *Nitrospira* family were found both in suspended and biofilm biomass. However, *Zooglea* was

only detected in suspended biomass samples, while some clones related to *Acidobacteria* were found only in biofilm biomass.

Table 6-7 Results of the affiliation of the bacterial 16S sequences retrieved from DGGE gel. In DGGE band number given as X.Y, X corresponds to the samples as indicated in figure 6-13, and Y corresponds to the band cut for the sequencing within the sample (the higher number the further location of the band).

DGGE band	Best match	Name	% pair-wise	Closest described	Phylogenic similarity
1.2	DQ676350	Uncultured firmicute clone MVP-51	99.2	GU389907 Uncultured bacterium clone JF747966 Uncultured bacterium clone	*Firmicutes*
2.1	AB298726	Clostridiaceae bacterium WN011	100	HM445969 Uncultured bacterium clone HM445979 Uncultured bacterium clone W-16S-52	*Clostridium*
2.2	AB298726	Clostridiaceae bacterium WN011	100	HM445969 Uncultured bacterium clone W-16S-42 CR933122 Uncultured bacterium	*Clostridium*
2.3	EU266820	Uncultured Acidobacteriaceae bacterium clone D12_13	99.7	GQ406202 Uncultured Acidobacteria bacterium clone GQ406182 Uncultured Chloroflexi bacterium	*Acidobacteria*
2.4	CU926578	Uncultured Actinobacteria bacterium clone QEDN4AB1	99.7	Uncultured Actinobacteria bacterium clone EDN4AB1 HQ093448 Uncultured Tessaracoccus sp.	*Actinobacteria*
2.5	HQ232445	Uncultured bacterium clone 11	99.7	GQ853793 Uncultured bacterium clone GU470895 Propionibacterium propionicum	*Propionibacterium*
3.1	AB298726	Clostridiaceae bacterium WN011	99.1	CR933122 Uncultured bacterium HM445969 Uncultured bacterium clone	*Clostridium*
3.2	HM445969	Uncultured bacterium clone W-16S-42	99.5	HM445979 Uncultured bacterium clone W-16S-52 AB298726 Clostridiaceae bacterium WN011	*Clostridium*
3.3	EU266820	Uncultured Acidobacteriaceae bacterium clone D12_13	99.4	GQ406182 Uncultured Chloroflexi bacterium clone GQ406202 Uncultured Acidobacteria	*Acidobacteria*
3.5	GU389907	Uncultured bacterium clone SGE110H	99.2	JF747966 Uncultured bacterium EU809707 Uncultured Fusibacter	*Firmicutes*
3.6	No data	No data	<95	No data	*Actinobacteria*

DGGE band	Best match	Name	% pair-wise	Closest described	Phylogenic similarity
4.2	No data	No data	<95	No data	*Verruco-microbia*
4.3	FJ189560	Uncultured bacterium clone EUB_39	99.7	CU926578 Uncultured Actinobacteria GU111567 Tessaracoccus	*Actinobacteria*
6.1	AJ507505	Uncultured Verrucomicrobium sp.	98.9	EU051659 Uncultured Verrucomicrobia AY216730 Uncultured soil bacterium clone	*Verruco-microbia*
6.2	DQ676807	Uncultured bacterium clone E1d10	99.7	DQ676806 Uncultured bacterium AB500053 Uncultured Acidobacteria	*Acidobacteria*
6.3	DQ363612	Uncultured bacterium clone Jul-eub50	99.4	HQ856361 Uncultured bacterium clone	*Nitrospira*
6.4	AB252885	Uncultured gamma proteobacterium	100	AF428697 Uncultured bacterium clone EF019980 Uncultured Bacteroidetes	*Gamma-proteobacteria*
6.6	GQ249372	Candidatus Nitrospira defluvii clone B14	100	JN679171 Uncultured Nitrospira sp. AF155154 Nitrospira cf. moscoviensis	*Nitrospira*
7.3	EU051659	Uncultured Verrucomicrobia bacterium clone G1c	99.1	GQ158454 Uncultured bacterium	*Verruco-microbia*
7.5	DQ363612	Uncultured bacterium clone Jul-eub50	99.7	HQ856361 Uncultured bacterium clone Exudates	*Nitrospira*
7.6	AB500063	Uncultured Nitrospira sp.	100	AF155154 Nitrospira cf. Moscoviensis EU104347 Uncultured bacterium clone	*Nitrospira*
8.3	NR041286	Zoogloea oryzae strain A-7	99.9	JN679163 Uncultured Zoogloea	*Zoogloea*
8.5	DQ363612	Uncultured bacterium clone Jul-eub50	100	HQ856362 Uncultured bacterium clone Exudates HQ856361 Uncultured bacterium clone Exudates	*Nitrospira*

The results of sequencing were somewhat coinciding with the results of FISH analysis. However, in the case of present work FISH technique gave more descriptive and complete image of biomass populations in combined UASB-MBR system.

6.5 CONCLUSIONS

Anaerobic granules growth was observed during the whole experimental period, reaching concentration of 34 $gVSS \cdot L^{-1}$ and the average diameter of around 3 mm. Moreover, microscopic observations of the anaerobic biomass allowed to examine the formation of new layers of biomass on the surface of existing granules.

The presence of Kaldnes support in the aerobic chamber of the system is crucial for its stable operation due to the development of proto- and metazoa. The colonies of ciliates and rotifiers attached to Kaldnes rings feed on non-flocculated bacteria and colloids, decreasing the turbidity of the liquid phase and controlling excessive bacterial growth. Moreover, they probably have a positive impact on fouling properties of the mixed liquor.

FISH analysis revealed that among *Proteobacteria* phylum, a subclass of *Betaproteobacteria* was the most dominant, followed by the *Gammaproteobacteria*. *Alphaproteobacteria* was scarce and appeared in coccoid form, while bacteria belonging to *Deltaproteobacteria* were not observed at all. The predominance of members of *Betaproteobacteria* was associated with abundance of nitrifying and denitrifying bacteria. Apart from these microorganisms, *Bacteroidetes*, nitrite-oxidizing bacteria (NOB), *Acidobacteria*, *Firmicutes* and filamentous bacteria belonging to *Chloroflexi* were also detected.

Finally, some Anammox and bacteria belonging to NC10 phylum were detected indicating the wide range of microorganisms that could develop in the proposed combined UASB-MBR system.

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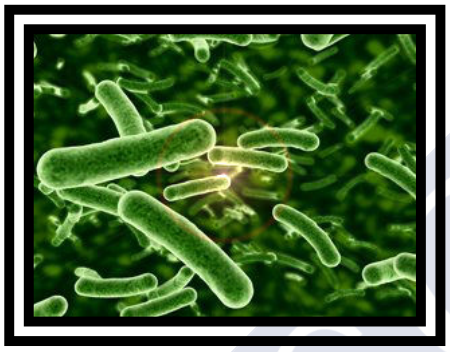
Chapter 7



Microbiological curiosities of combined UASB-MBR system – an open gate to future investigations[1]

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J.M. Garrido, **D. Buntner**, A. Sánchez, J.M. Lema Rodicio (2013). *Sistema integrado de reactor anaerobio metanogénico y biorreactor de membranas para la eliminación de materia orgánica y nitrógeno en aguas residuales*. Número de solicitud: ES-P201330118.

SUMMARY

The combined UASB-MBR system was operated during more than 3 years. Since the system was subjected to many modifications (OLR, temperature, aerobic/anoxic conditions in MBR chambers, recirculation ratio, etc) within this period a development of big variety of microorganisms was observed. Here, two interesting and intriguing processes will be described: methane

oxidation coupled to denitrification and anammox, which were observed at the end and at the beginning of the operation of the system, respectively.

The presence of dissolved methane, especially at low temperature, represents an important environmental concern in terms of greenhouse gas (GHG) emissions of wastewaters treated using methanogenic bioreactors. Methane has a global warming potential 25 times higher than carbon dioxide. For low strength wastewaters, dissolved methane might account up to 50% of the produced methane. The dissolved methane is easily desorbed from the effluents, especially if these are either released in the environment or post-treated using aerobic bioreactors. Thus the use of anaerobic technology could increase GHG emissions of wastewater treatment. The use of this dissolved methane as a carbon source for biological denitrification has been proposed as an alternative to reduce both GHGs emissions and nitrogen content of the treated wastewater. In this study the effluent of a UASB reactor was post-treated in an MBR with a first anoxic chamber in order to use dissolved methane as carbon source for denitrification. Up to 60% and 95% nitrogen removal and methane consumption were observed, respectively. The stripping of the dissolved methane present in the UASB effluent led to a worsening of nitrogen removal in the MBR system. Batch experiments confirmed the presence of microorganisms capable of denitrifying using the dissolved methane as carbon source. Recirculation ratio between the anoxic and aerobic chambers of the MBR system, and either the presence or absence of dissolved methane were shown as the main important parameters governing the denitrification process.

On the other hand, the presence and activity of Anammox bacteria in the combined UASB-MBR system shows its potential to develop a wide variety of populations of microorganisms, depending on the effluent requirements.

7.1 INTRODUCTION

Anaerobic treatment processes have been widely applied to various types of wastewaters because of advantages such as lower energy consumption, energy recovery as methane, and less excess sludge production compared with conventional aerobic treatment systems. Anaerobic technology is widely used in template and warm climate countries for the treatment of

municipal wastewaters. Nevertheless, anaerobic treatment produces methane, a greenhouse gas (GHG) with a warming potential 25 times higher than that of carbon dioxide. A fraction of the methane generated is present in the effluent. Dissolved methane can be estimated considering that effluents are, at least, in equilibrium with the biogas formed by using the Henry´s law. Thus, methane concentrations in the UASB effluent between 13.4 and 20.8 $mg \cdot L^{-1}$ may be expected operating at 17-25 ºC, with 60-80% methane composition in the biogas at operating pressure of 1 atm.

Methane may be lost by stripping, if the effluents are either aerobically post-treated or discharged in the environment without further post-treatment, increasing the environmental impact of anaerobic wastewater treatment due to GHG emissions.

On the other hand, methane present in the effluents of methanogenic bioreactors may be used also as an inexpensive electron donor for denitrification. The use of dissolved methane as carbon source for denitrification might be a way to reduce GHGs emissions after anaerobic wastewater treatments, even in those locations in which nitrogen removal is not considered as an environmental concern.

7.1.1 Biological methane oxidation

Biological methane oxidation proceeds either via aerobic or anaerobic pathway. Very recently however, the new metabolic pathway has been found (Ettwig *et al.*, 2008; Wu, 2012), in which methane oxidation is coupled with denitrification. In this process a newly discovered bacteria belonging to NC10 phylum produces its own supply of oxygen by metabolizing nitrite via nitric oxide into oxygen and N_2 (Wu, 2012). This oxygen is later used for the oxidation of methane in the classical aerobic methane oxidation process.

7.1.1.1 Aerobic Methane Oxidation

Aerobic methane oxidation is driven by the wide group of bacteria, *Methanotrophs*. These bacteria are very important in the overall carbon cycle, since they oxidize most of the methane generated in the anaerobic environments before in reaches the atmosphere and therefore contribute significantly in global warming prevention (Hanson and Hanson, 1996).

Methanotrophic bacteria are aerobic and till very recently (Ettwig *et al.*, 2008, Wu, 2012) unique in their ability to utilize methane as a single carbon and energy source and formaldehyde assimilation (Hanson & Hanson, 1996). They belong to methylotrophic bacteria, which utilize one-carbon compounds such as methane, methanol, methylated amines, halomethanes, and metylated compounds containing sulphur. Methanotrophic bacteria were divided into three groups: type I, type II and type X, which initially belonged to type I. The main difference among these groups are compiled in table 7-1.

Table 7-1 Main characteristics of Type I, Type II and Type X mathanotrophs (adapted from Hanson & Hanson, 1996).

Characteristic	**Type I**	**Type II**	**Type X**
Cell morphology	Short rods, usually single; some cocci or ellipsoids	Crescent-shaped rods, rods, pear-shaped cells, sometimes occur in rosettes	Cocci, often found as pairs
Growth at 45 °C	No	No	Yes
G+C content of DNA (%)	49-60	62-67	59-65
Membrane arrangement:			
Bundles of vesicular discs	Yes	No	Yes
Paired membranes aligned to periphery of cells	No	Yes	No
Nitrogen fixation	No	Yes	Yes
Ribulose pathway	Yes	No	Yes
Serine pathway	No	Yes	Sometimes
Proteobacterial subdivision	Gamma	Alpha	Gamma
Genera	*Methylococcus, Methylomicrobium, Methylomonas, Methylobacter*	*Methylosinus, Methylocistis*	*Methylococcus capsulatus* and similar
PHB accumulation	Yes	Yes	Yes

Defining characteristic of *Methanotrophs* is the use of specific enzymes, methane monooxygenases (MMO), to catalyse the oxidation of methane to

methanol. Two types of MMO were found in methanotrophic bacteria: soluble (sMMO), present in type Ⅱ and type X; and particulate or membrane-bound (pMMO), which can be formed by all known *Methanotrophs* (Hanson & Hanson, 1996). What is interesting, is that MMO is not substrate-specific, which allows the use of a variety of different compounds, however sMMO has a broader substrate specificity than pMMO. The whole metabolic pathway may be simplified as follows:

$$\begin{array}{c} TYPE\ I\ METHANOTROPHS \\ Ribulose\ pathway \\ \uparrow \\ CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2 \\ \downarrow \\ Serine\ pathway \\ TYPE\ II\ METHANOTROPHS \end{array} \qquad \text{eg-7-1}$$

As could be seen above, the central role in metabolism of methanotrophic bacteria plays formaldehyde, which is assimilated either by ribulose or by serine pathway. That in turns distinguishes two types of *Methanotrophs*: Type I (and X) and Type Ⅱ.

Type I (and X) seems to be adapted to grow at low methane concentrations and when copper is present in the medium, whilst Type Ⅱ grows rather with higher CH_4 levels, when both nitrogen and oxygen concentrations are low (Hanson & Hanson, 1996). Both types of methanotrophs have also different survival strategies: type Ⅰ are characterized by rapid growth under favourable conditions and rapid die-off under stress conditions. Type Ⅱ grow slower, but survive better. Moreover, they outcompete type Ⅰ methanotrophs under oxygen- and nitrogen-limiting conditions.

Methanotrophs can oxidize methane to methanol or acetate at low oxygen concentrations. These products can be subsequently utilized as carbon source for denitryfication.

Another characteristic of methanotrophic bacteria is the formation of poly-B-hydroxybutyrate (PHB), especially at low oxygen conditions (Costa *et al.*, 2000). PHB is formed by condensation of acetate molecules and its synthesis is initiated and strongly depends on the applied nutrient

deficiency and is not associated with cell reproduction. Wendland *et al.* (2010) indicated that the best results are achieved when nitrogen and phosphorus are depleted, reaching up to 51% of polymer content after 24h. PHB produced from methane and methane-containing gases has beedn protected by the brand name Methanomer®. Since PHB formation is a common property among methylotrophic bacteria, an intracellular PHB degradation can be used as a reserve energy source by methanotrophs under anoxic conditions (Vecherskaya *et al.*, 2001).

7.1.1.2 Aerobic Methane Oxidation coupled to denitrification

Denitrification is a respiratory process in which an electron donor is needed as energy source (Chapter 1, section 1.1.1.3). Denitrifying bacteria are mostly heterotrophic and need organic carbon for the reduction of nitrate to nitrogen gas (N_2). If the wastewater to be treated has insufficient amount of electron donors, the endogenous energy source has to be added, such as glucose, methanol, acetate etc. Dissolved methane could be used as sole electron donor in denitrification under two different environmental conditions (Islas-Lima *et al.*, 2004): (1) anoxic methane oxidation under denitrifying conditions (section 7.1.1.4), or methane oxidation and nitrite reduction under low oxygen partial pressure. In this last case, methanotrophic bacteria are responsible for methane oxidation, producing organic carbon consumed by denitrifiers (Costa *et al.*, 2000). Methanol and formaldehyde have been detected in methanotrophic cultures, but polysaccharides and proteins can also be produced and excreted (Modin *et al.*, 2007).

7.1.1.3 Anaerobic Methane Oxidation

In 2000 Boetius *et al.* gave evidence for the existence of structural consortium of anaerobic methanogenic archaea (ANME) and sulphate-reducing bacteria (SRB) present in the methane-rich sediments of the Hydrate Ridge. Structurally, the consortium consisted of a dense aggregates of coccoid archaea (approx. 100 cells) surrounded by sulphate-reducing bacteria (approx. 200 cells). In this consortium, the ANME organisms, which are currently known to fall into three distinct phylogenetic groups (ANME I-III) (Knittel & Boetius, 2009) reverse the process of

methanogenesis. The stoichiometry of anaerobic methane oxidation coupled with sulphate reduction is as follows:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \text{eq. 7-2}$$

Members of *Methanosarcinales* were dominant group in the consortium (Boetius *et al.*, 2000). As mentioned in section 6.1.1, they can synthesize methane not only form CO_2 and H_2, but also from simple methyl-group-containing compounds such as acetate, methanol, methylamines and methyl sulphide. On the other hand, SRB from *Desulfosarcina/Desulfococcus* branch oxidize organic compounds completely to CO_2, while several species can grow autotrophically with CO_2, H_2 and sulphate.

7.1.1.4 Anoxic methane oxidation coupled to denitrification

In 2006 Raghoebarsing *et al.* found a microbial consortium that couples anaerobic methane oxidation to denitryfication. This consortium consisted of two microorganisms: a bacterium representing a phylum (NC10) without any cultured species and an archaeon distantly related to marine methanotrophic Archaea. The authors successfully enriched the consortia from the sediment of the Twentekanaal (The Netherlands). About 80% of the population of the enrichment co-culture consisted of a bacteria from NC10 phylum while a smaller fraction (up to 10%) was made of archaeal species phylogenetically positioned between *Methanosaeta* and ANME-II. However, recent studies (Ettwig *et al.*, 2008) shows that a single bacteria, named "*Candidatus* Methylomirabilis oxyfera, is able to oxidize methane in anoxic conditions without an archaeal partner. Ettwig *et al.* (2010) and Wu (2012) explained the mechanism of anoxic methane oxidation coupled to denitrification, which involves the dismutation of nitric oxide (NO) into O_2 and N_2. Stoichiometric representation of anoxic methane oxidation coupled to denitrification using either nitrite or nitrate is as follows:

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad \text{eq. 7-3}$$

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad \text{eq. 7-4}$$

Since its discovery, anaerobic oxidation of methane coupled with denitrification was further studied by growing number of authors (Islas-

Lima *et al.*, 2004; Waki et al., 2009; Leusken et al., 2011; Kampman et al., 2012) and proved to be feasible in full-scale applications.

7.1.2 Anaerobic Ammonia Oxidation (Anammox)

In the nitrogen cycle molecular nitrogen (N_2) is fixed biologically or industrially to ammonium (NH_4^+), the main fertilizer for plants. When ammonium is released to the environment, it may be oxidized by aerobic, nitrifying bacteria and archaea to nitrite (NO_2^-) and nitrate (NO_3^-), respectively, which can be used by plants as an additional nitrogen source. Under anaerobic conditions, nitrate and nitrite may be reduced back to ammonium, or to nitrogen gas through dentrification. Nitrite can also be combined with ammonium to give nitrogen gas in the anammox reaction. The anammox process is responsible for at least 50% of the nitrogen turnover in marine environments (Strous & Jetten, 2004) and occurs in nature at both low and high temperatures and salinities. It is a shortcut in the nitrogen cycle that was discovered quite by accident at Gist Brocades denitrifying pilot plant (Delft, The Netherlands) and developed in the early 1990s at TU Delft.

The Anammox bacteria, which belong to the group *Planctomycetes*, contain a membrane-bound organelle (Anammoxosome) in which ammonium and nitrite are converted to nitrogen gas via the toxic and extremely energy-rich hydrazine intermediate (Kartal *et al.*, 2010). Special lipids found in these bacteria, ladderanes, are believed to assist in keeping the hydrazine within this organelle. The bacteria use CO_2 as their carbon source for growth and hence do not require organic carbon. The nitrite required for their growth may be provided by aerobic ammonium-oxidizing bacteria or archaea. The anammox (I) and partial nitrification (II) reactions are as follows:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (I) \qquad \text{eq. 7-5}$$

$$NH_4^+ + 1\tfrac{1}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (II) \qquad \text{eq. 7-6}$$

Therefore, together yield is as follows:

$$2NH_4^+ + 1\tfrac{1}{2}O_2 \rightarrow N_2 + 2H^+ + 3H_2O \qquad \text{eq. 7-7}$$

A key advantage of anammox technology is that it consumes far less energy than conventional treatment techniques due to fact than no external

carbon source is added, as would be the case for nitrification-denitrification process. This makes the application of the Anammox process a promising biological treatment, especially for strong nitrogen loaded wastewaters with low C/N ratio (Lopez *et al.*, 2008). The main disadvantage is slow growth of Anammox bacteria (generation times of 10 to 12 days at 35°C), however this could be overcome by formation of granules and therefore maintaining high concentration of biomass within the reactor.

In recent years, there have been several reports stating that nitrogen removal can be observed in an anaerobic process, for example in a denitrifying-fluidized bed reactor treating the effluent from a methanogenic reactor (Mulder *et al.*, 1995). Because the Anammox process requires nitrite as an electron donor, a pre-partial nitrification process should be initiated. On the other hand, this situation could result in competition between autotrophic Anammox bacteria and heterotrophic denitrifying bacteria in the process of biological nitrogen removal, while treating carbonaceous waste (Ahn *et al.*, 2004).

The NH_4^+-N load introduced to the aerobic biofilm chamber of the system studied in this work during start-up (Chapter 3) was relatively low (up to 35 $mg \cdot L^{-1}$), however, since the COD concentration in the UASB effluent was also low (around 80 $mgCOD \cdot L^{-1}$), the C/N ratio could meet the requirements for the development of Anammox bacteria. Moreover, the existence of anoxic zones within aerobic biofilm chamber could give place for – at least partial – nitrification.

7.1.2.1 Anammox at low temperatures

Temperature is one of the most important factors influencing the operation of all biological processes. Several authors (Strous *et al.*, 1999; Egli *et al.*, 2001; Toh *et al.*, 2002; Yang *et al.*, 2006) found that optimum temperature for the operation of the Anammox process was around 30–40 ºC. In all these works the Anammox bacteria involved belonged to the species which can be found in wastewater treatment plants or fresh water. However, in recent years various authors proved that successful operation of Anammox at low temperatures in feasible. Cema *et al.* (2007) proved that a rotating biological contactor (RBC) with the established Anammox process could be effectively operated at temperatures around 20 ºC. Similar results were

reported by Isaka *et al.* (2007), who operated a fixed bed Anammox reactor which treated 8.1 $gN \cdot L^{-1} \cdot d^{-1}$ at temperature range between 20 and 22 ºC. Also Isaka *et al.* (2008), who worked with Anammox bacteria entrapped into a gel carrier, reported nitrogen conversion rates of 2.8 and 0.36 $gN \cdot L^{-1} \cdot d^{-1}$ at 22 and 6.3 ºC, respectively. Moreover, several works done with marine anammox samples reported measurable activities at very low temperatures (slightly below 0 ºC). Finally, Dalsgaard *et al.* (2002) reported anammox activity from bacteria present in marine sediments between 6 and 43 ºC. On the other hand, Fernandez (2010) proved that operation of Anammox systems at relatively low temperatures (about 18-20 ºC) is possible, despite the fact that the optimum has been found at 35-40 ºC. In this sense, the application of Anammox process for the UASB effluent at ambient temperatures seems possible.

7.2 OBJECTIVES

The use of methane as an electron donor in denitrification reaction could be an alternative to reduce GHGs emissions and total nitrogen of those wastewater treatment plants using a first methanogenic stage. Since in previous chapters the risk of uncontrolled methane emissions to the atmosphere by stripping was pointed out, one of the objectives of this chapter was to study the possibility of denitrification using dissolved methane present in the effluent of an anaerobic UASB stage. This strategy would allow simultaneous nitrogen elimination – another bottleneck of the combined UASB-MBR system. Moreover, FISH analysis were employed to try to explain the possible mechanisms of methane oxidation coupled with denitrification.

On the other hand, Anammox activity was discovered during the start-up of the UASB-MBR system. SAA tests, microscopic observations as well as FISH analysis were performed in order to confirm the presence of Anammox bacteria.

7.3 MATERIALS AND METHODS

7.3.1 Reactor description and operational conditions

The first chamber of MBR in the combined UASB-MBR system was converted into anoxic chamber in order to study the possibility of nitrogen removal using dissolved methane as carbon source. The operational periods are described in table 7-2. Stripping of methane present in the UASB effluent was realized in period IV in order to check its influence on denitrification rate.

The reactor was operated at ambient temperature (17-23 ºC) and fed using synthetic wastewater composed of diluted skimmed milk, $NaHCO_3$ and trace elements. COD concentration in the feeding varied between 800 and 1300 $mg \cdot L^{-1}$.

Table 7-2 Operational periods of combined UASB-MBR system.

Period	days	First MBR chamber	Rec. ratio	CH_4 stripping
I	0-84	Aerobic	1.0	no
II	85-120	Anoxic	3.0	no
III	121-150	Anoxic	1.0	no
IV	151-169	Anoxic	1.0	yes
V	170-198	Anoxic	0.5-1.0	no
VI	199-233	Anoxic	1.5-2.0	no

7.3.2 Denitrification batch experiments

Two different batch denitrification assays using methane and/or acetate as electron donor were performed using 500 mL flasks. In the first assay denitrifying activity of both biomass in suspension and biofilm was tested. Four flasks were filled with 400 mL of 2 $gMLVSS \cdot L^{-1}$ suspended biomass and 20 plastic carriers Kaldnes K3 (40% of apparent volume). In the second assay only biofilm activity was measured and therefore four bottles were filled with 50 plastic carriers Kaldnes K3 and 400 mL of phosphate buffer (0.143 $g \cdot L^{-1}$ of KH_2PO_4 and 0.740 $g \cdot L^{-1}$ of K_2HPO_4).

Both biofilm and suspended biomass were taken from the anoxic chamber of the UASB-MBR system, settled for at least 12 h and washed three times with phosphate buffer in order to assure the absence of organic matter or nitrogen. The absence of any soluble carbon source in the supernatant was

confirmed by COD measurement. After inoculation, the flasks were flushed for 5 min using nitrogen or methane depending on the conditions (table 7-3), to guarantee anaerobic atmosphere.

Table 7-3 Conditions in denitrification batch tests.

Flask	Headspace	Carbon Source
Blank	N_2	None
Methane	CH_4	CH_4
Acetate	N_2	Acetate
Methane+acetate	CH_4	Acetate + CH_4

5 mL of $NaC_2H_3O_2 \cdot 3H_2O$ 0.9M were spiked as a carbon source in the corresponding flasks (table 7-3). 1 mL of KNO_3 0.86M was spiked to each bottle at the beginning of the experiment.

The flasks were incubated at 25 ºC and stirred in a shaker at 150 rpm during five hours. 5 mL samples were taken each hour with a syringe through a septum and filtered through 0.45 µm nitrocellulose membrane filters (HA, Millipore). All control assays were performed in duplicate.

7.3.3 Specific Anammox activity assays

The batch assays used to measure the maximum Specific Anammox Activity (SAA) were performed according to the methodology described by Dapena-Mora *et al.* (2007). Completely closed vials with a total volume of 38 mL and 25 mL of liquid volume were used to perform the Anammox batch assays. The procedure was as follows:

- The biomass was washed three times with phosphate buffer (0.143 g $KH_2PO_4.L^{-1}$ and 0.747 g $K_2HPO_4 \cdot L^{-1}$). The pH value was fixed at 7.8 and the temperature was fixed at T = 35 °C.
- Gas and liquid phases were purged with an inert gas (Ar, He) to remove O_2.
- The vials were placed in a thermostatic shaker, at 150 rpm and the temperature T.

- After some minutes for thermal stabilization, substrates were added into the vials. Initial concentrations of substrates were 70 mg NH_4^+-N·$L^{-1}$and 70 mg NO_2^--N·L^{-1}.
- The production of N_2 was measured (pressure transducer Centrepoint Electronics) in the gas phase as the increment of pressure in the headspace of the vials.

Maximum Specific Anammox Activity (SAA) was estimated from the maximum slope of the curve described by the cumulative N_2 production along the time and related to the biomass concentration in the vials. Since the values of the affinity constant of the Anammox bacteria for ammonium and nitrite are lower than 10 μM and 5 μM, respectively (Strous *et al.*, 1999), it can be considered that the activity measured is the maximum activity for the range of nitrite and ammonium concentrations used.

7.3.4 Microbial population identification by FISH

The different populations of microorganisms present in the sludge samples of UASB-MBR system were researched by Fluorescent In Situ Hybridization (FISH). Three types of biomass were analysed: anaerobic granular sludge (from UASB stage), suspended biomass (either aerobic and/or filtration chamber of the MBR stage) and biofilm (aerobic chamber of MBR stage). The probes used in this chapter are collected in table 7-4.

Table 7-4 Specific probes used for the microorganism identification by FISH

Probe	Citoc.	Probe sequence (5' → 3')	%F	Target organisms
Amx368	cy3	CCTTTCGGGCATTGCGAA	15	All Anammox bacteria
ARCH915	cy3	GTGCTCCCCCGCCAATTCCT	20-35	Archaea
DARCH872	fluos	GGCTCCACCCGTTGTAGT	30	Various Euryarchaeota including ANME groups
DBACT1027	cy3	TCTCCACGCTCCCTTGCG	30	Bacteria belonging to NC10 phylum
DBACT193	cy3	CGCTCGCCCCCTTTGGTC	30	Bacteria belonging to NC10 phylum

Probe	Citoc.	Probe sequence (5' → 3')	%F	Target organisms
EUB338mix	Fluos	GC(T/A)GCC(T/A)CCCGTAGG(A/T)GT	...	Bacteria domain, Planctomycetales and Verrucimicrobiales
MA450	cy3	ATCCAGGTACCGTCATTATC	20	Type II methanotrophs (Methylosinus/Methylocystis spp.)
MG705	6-fam	CTGGTGTTCCTTCAGATC	20	Type I methanotrophs
MG84	6-fam	CCACTCGTCAGCGCCCGA	20	Type I methanotrophs

7.4 RESULTS AND DISCUSSION

7.4.1 Denitrification with dissolved methane

During the six operational periods (table 7-1) the system treated an average of 280 $L \cdot d^{-1}$ of wastewater. Membrane flux was maintained around 15 $L \cdot m^{-2} \cdot h^{-1}$, with permeabilities between 150 and 230 $L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$. Regarding MLVSS, the concentrations in the UASB reactor, the anoxic chamber and the membrane filtration chamber ranged between 28-35 $g \cdot L^{-1}$, 2-5 $g \cdot L^{-1}$ and 4-8 $g \cdot L^{-1}$, respectively. Biomass concentration in the biofilm was around 45 $gMLVSS \cdot m^{-2}$, which was equivalent to an MLVSS concentration of approximately 6 $g \cdot L^{-1}$. Sludge retention time (SRT), referred to the MBR, was maintained between 15 and 30 d during the six operational periods. Anaerobic biomass was not purged from the UASB reactor during the study. Food to microorganism (F/M) ratio applied to the MBR was around 0.03 $gCOD \cdot gMLVSS^{-1} \cdot d^{-1}$, referred to non-methane soluble COD.

Soluble COD and dissolved methane concentrations measured in the UASB effluent during the operation were very low, being 57±34 $mg \cdot L^{-1}$ and 19 – 25 $mg \cdot L^{-1}$, respectively, except from Period IV, when methane was stripped off and its concentration decreased to values between 3 and 8 $mg \cdot L^{-1}$. Moreover, the concentration of VFAs in the UASB effluent was always below minimum detection limit (20 $mg \cdot L^{-1}$). Most of the total nitrogen in this effluent was present as soluble ammonia (35.7±7.9 $mg \cdot L^{-1}$).

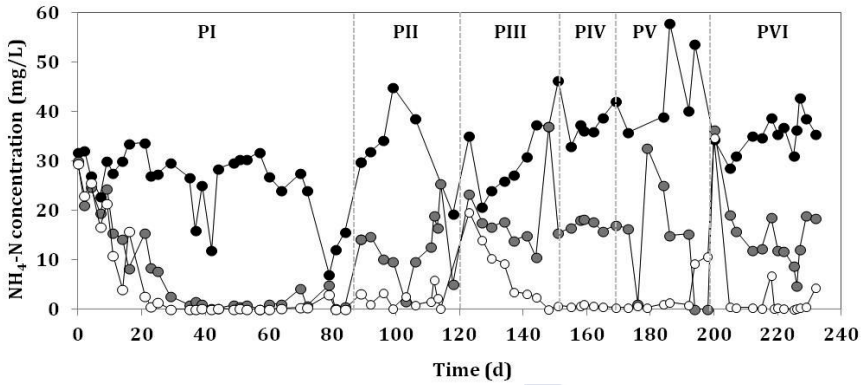

Figure 7-1 Ammonia conversion in the combined UASB-MBR system during six operational periods: (●) UASB effluent, (●) aerobic/anoxic chamber, (○) permeate.

In figures 7-1 and 7-2 the conversion of ammonia and generation of NO_X-N during the six operational periods can be followed. In Period I, full nitrification was obtained and no nitrogen removal was observed. However, from Period II onwards, when aerobic chamber was converted into anoxic one, nitrogen elimination between 20 and 65% was observed (figure 7-3). Part of the ammonia was oxidized in the anoxic chamber, while in the filtration chamber the nitrification of remaining ammonia was completed. The generated NO_X-N were recycled to the anoxic chamber and reduced to N_2. Since the UASB effluent contained (non-methane) biodegradable COD and dissolved methane, both could be used as carbon source for denitrification. In order to distinguish between the denitrification rate originating from the non-methane biodegradable COD, during Period IV dissolved methane was stripped off from the UASB effluent. Soluble COD in the UASB effluent during that period ranged between 16 and 27 $mg \cdot L^{-1}$.

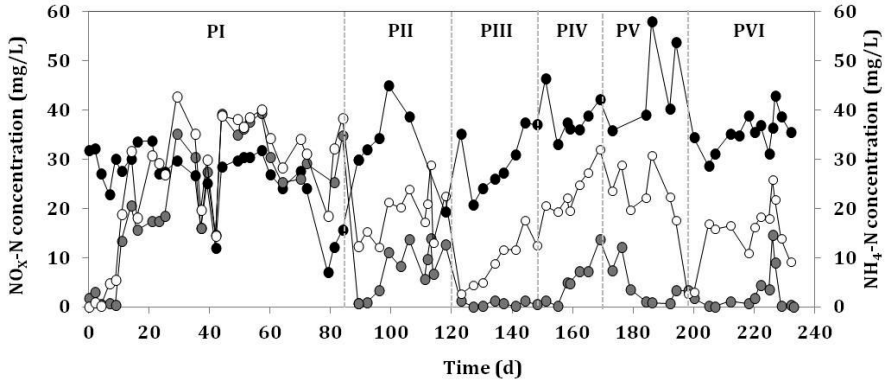

Figure 7-2 Nitrates and nitrites conversion in the combined UASB-MBR system during six operational periods: (●) Ammonia in UASB effluent, (●) nitrates and nitrites in aerobic/anoxic chamber, (○)nitrates and nitrites in permeate.

As can be seen in figure 7-2 the total nitrogen (NH_4^--N, NO_2^--N and NO^{3-}-N) concentrations gradually increased both in anoxic and filtration chamber of MBR stage. Nitrogen elimination ratio decreased progressively reaching 20% (figure 7-3) at the end of Period IV, indicating the denitrification associated with biodegradable organic matter. Since the maximum nitrogen removal obtained during the operation of the system was 65%, the total nitrogen removal due to the oxidation of methane could account up to 45 % indicating that dissolved methane played a crucial role in the denitrification process. Furthermore, when stripping of methane was stopped in Period V, nitrogen removal increased again to the previous values observed during period III (up to 60 %) confirming the significant role of methane in denitrification. On the other hand, during Period IV NO_X-N concentration was almost zero in the anoxic chamber. Thus, denitrification was limited by nitrate availability. Nevertheless, during Period IV, the absence of dissolved methane led to a progressive increase of nitrate in the anoxic chamber, indicating that the limiting factor in this period was the carbon source (figures 7-1, 7-2 and 7-3).

On the other hand, the recirculation ratio applied during the operation of the system also played an important role in TN elimination. The highest recirculation rates were applied during Period II (R=3), resulting with a progressive increase of NO_X-N concentrations in the anoxic chamber and permeate (figure 7-2). The TN elimination dropped to around 35%. When the recirculation ratio was diminished to R=1 (beginning of Period III) this elimination was recovered and reached 65% at the end of Period III.

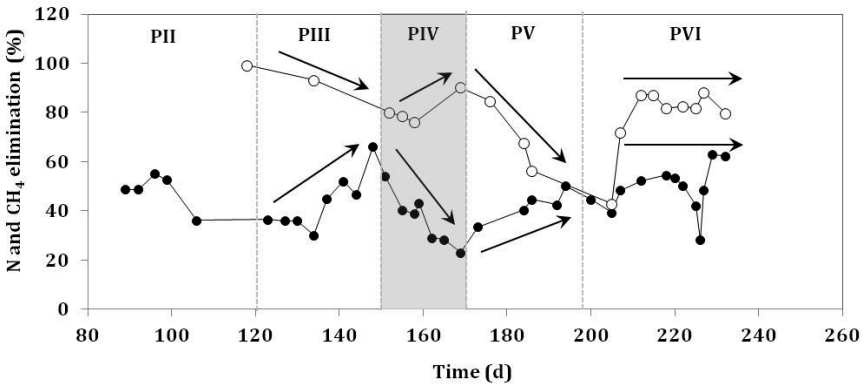

Figure 7-3 Partial elimination of nitrogen (●) and elimination of dissolved methane (○) during periods II – VI. The shaded area indicates the period when dissolved methane was stripped out.

The results show that denitrification with methane as a carbon source is effectively possible and feasible. Soluble COD concentration in the UASB effluent was used for conventional heterotrophic denitrification. On the other hand, this low COD concentration promoted the use of dissolved methane as a complementary carbon source to denitrify.

7.4.2 Denitrification batch assays

The results of six periods of operation of UASB-MBR system show that denitrification using methane as a complementary carbon source (in the presence of the oxygen) was possible. Nevertheless, the denitrification mechanism might be complex, involving different pathways (Modin, 2007). To determine the main denitrification mechanisms batch assays were performed. In order to prove if anaerobic methane oxidation coupled to denitrification was feasible, the batch assays were performed in anaerobic conditions. A further objective of batch assays was to determine either denitrification took place in the biofilm and/or suspended biomass.

In figure 7-4 the NO_3-N consumption is depicted depending on the substrate used. Interestingly, the mixed (biofilm and suspended) biomass showed relatively high endogenous denitrification rate, being 20.0±14.3 $mgN \cdot gMLVSS^{-1} \cdot d^{-1}$ for the blank. In any case, batch experiments showed higher denitrification rates for the flasks fed with acetate, independently of the presence of methane, being 57.1±19.1 $mgN \cdot gMLVSS^{-1} \cdot d^{-1}$ (figure 7-4). Nevertheless, this activity was only three times higher than the activity of

the blank and significantly lower than those reported for activated sludge at 20 °C, being 250 mgN·gMLVSS^{-1}·d^{-1} (Henze *et al.*, 2002). In any case, the apparent specific denitrification rates observed during the operation of the combined UASB-MBR system were lower than observed in batch experiments, being around 30 mgN·gMLVSS^{-1}·d^{-1}.

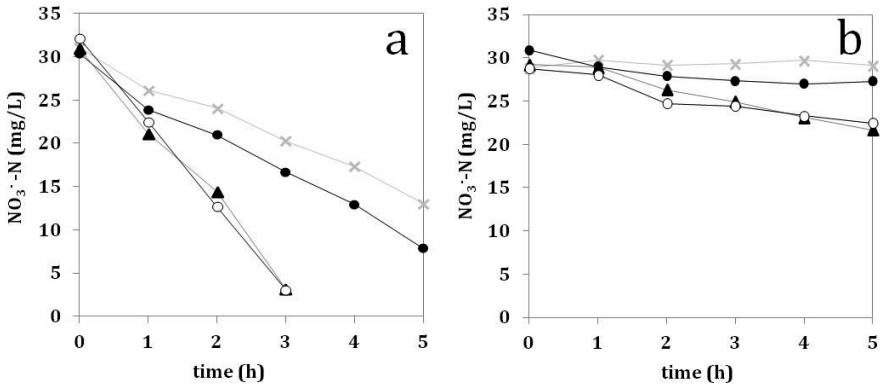

Figure 7-4 Batch denitrification assays with the presence of both suspended and biofilm biomass (a) and only biofilm biomass (b) as inocula. Carbon sources employed were: blank test (X), acetate (▲), methane (●) and methane and acetate (○).

Additionally, some activity was also observed where methane was used as a sole carbon source. This activity was slightly higher than the one measured for the biomass without any substrate and reached 28.2±11.2 mgN·gMLVSS^{-1}·d^{-1}. This could indicate that anaerobic methane oxidation coupled to denitrification might have taken place.

7.4.3 Microorganisms responsible for biological methane oxidation

FISH analyses were performed to determine the potential denitrification mechanisms and confirm the possibility of nitrite-driven methane oxidation. Abundant methanotrophs type I were found (figure 7-5) in both suspended and biofilm biomass. These bacteria can oxidize methane to methanol or acetate at low oxygen concentrations, which can be subsequently utilized by heterotrophs as carbon source for denitrification (Hanson & Hanson, 1996).

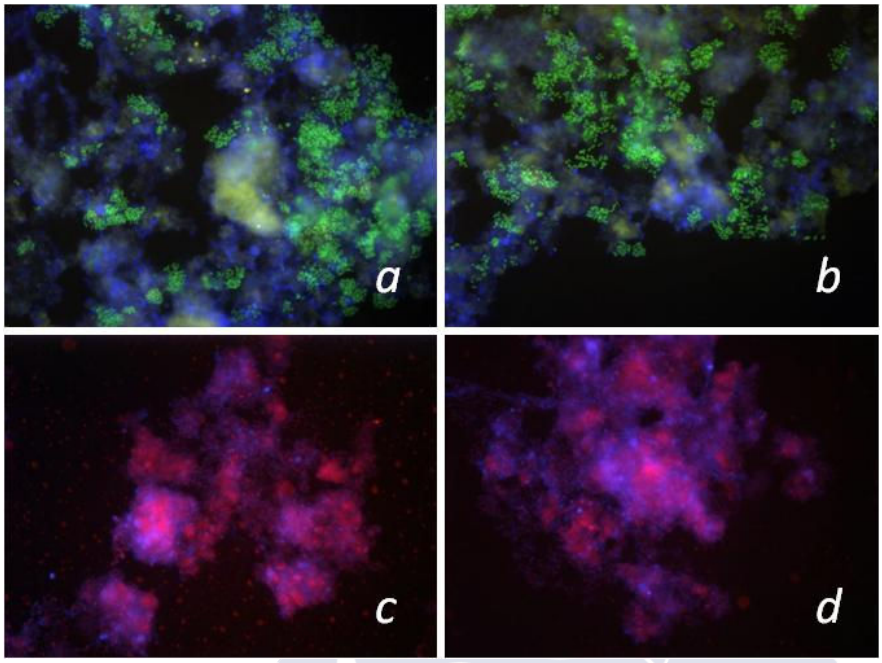

Figure 7-5 FISH analysis of methanotrophs: (a), (b) Type I methanotrophs MG705 and MG84 Fluos (green) in combination with DAPI (blue), x40; (c), (d) Type II methanotrophs (Methylosinus/Methylocystis spp.) MA450 cy3 (red) in combination with DAPI (blue), x40.

Taking into account the abundance of methanotrophic bacteria, it might be assumed that aerobic methane oxidation coupled to conventional heterotrophic denitrification was probably the dominant process in the presented system especially when the first aerobic chamber worked as aerobic chamber, and when the recirculation ratio was high (e.g. R=3). This assumption would be in accordance with the literature (Rhee & Fuhs, 1978; Thalasso *et al.*, 1997).

On the other hand, FISH analyses confirmed the presence of some archaeal species phylogenetically positioned between Methanosaeta and anaerobic methanogenic archaea (ANME) (figure 7-6), which are normally found in anaerobic environments (Nauhaus *et al.*, 2005). Therefore, the presence of these bacteria in MBR was probably caused by wash out of a fraction of anaerobic biomass from the UASB. ANME are known to be able to carry out reversed methanogenesis (Knittel & Boetius, 2009; Valentine & Reeburgh, 2000), where methane (and optionally CO_2) is converted into

acetic acid (or acetate, if CO_2 is involved) and H_2. This acetic acid/acetate could serve as an electron donor for nitrate-reducing bacteria.

Table 7-5 Average denitrification, methane and oxygen apparent specific consumption rates and CH_4:O_2 molar ratio in the anoxic chamber during the operation of the UASB-MBR system.

Period	R	mgN/gMLVSS·d	$mgCH_4$/gMLVSS·d	mgO_2/gMLVSS·d	mol CH_4/mol O_2
V	0.5	14.3	40.1	4.9	16.4
III, IV, V	1.0	22.5	43.7	10.9	8.0
II, VI	2.0	16.8	15.9	13.0	2.4

Reverse methanogenesis might occur in the anoxic chamber either during the low recirculation period (i.e. R=0.5) and/or deep inside the biofilm growing on the plastic support, where anaerobic conditions would be maintained. If this is true, it could explain methane oxidation observed in the reactor even though the experimental molar ratio between the oxidized methane and the oxygen consumed was always higher than theoretical molar relationship of the aerobic methane oxidation pathway (table 7-5), suggesting a combination of both, aerobic and anaerobic methane oxidation.

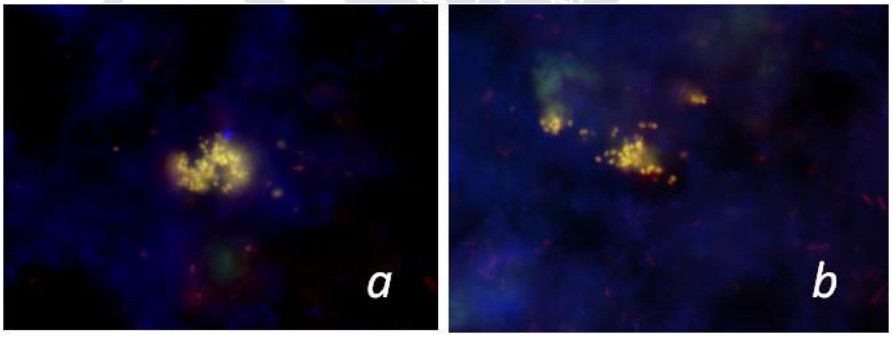

Figure 7-6 FISH analysis of archaeal species: (a), (b) DARCH872 fluos (green) in combination ARC915 cy3 (red) and DAPI (blue), x100.

On the other hand, Raghoebarsing *et al.* (2006) demonstrated that the consortium of archaeal species with bacteria belonging to NC10 phylum could couple anaerobic methane oxidation to denitrification. In this process the reverse methanogenesis and electron shuttling to the denitrifying partner would be analogue to ANME and SRB syntrophic realtion. Later,

however, it was found that the process of nitrite-driven methane oxidation could be carried out without an archaeal partner (Ettwig *et al.*, 2008; Wu, 2012).

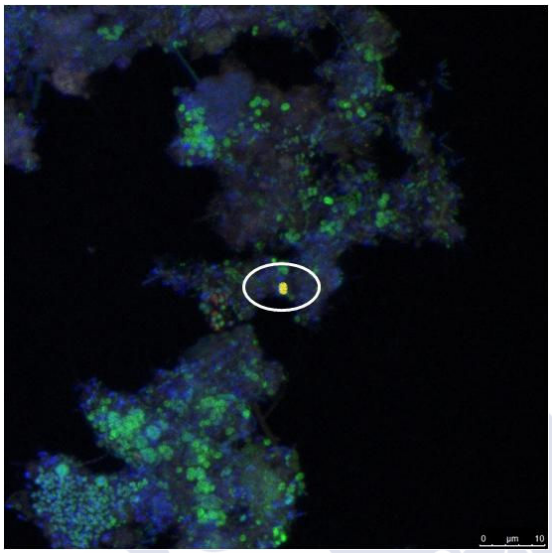

Figure 7-7 FISH analysis of bacteria belonging to the NC10 phylum: DBACT193 and DBACT1027 fluos (green) in combination ARC915 cy3 (red) and DAPI (blue), x100. White circle marks a bacterium/group of bacteria that exhibited a positive signal with all the probes.

In the case of present work, FISH analysis confirmed the presence of single bacteria belonging to NC10 phylum (figure 7-7) and believed to be responsible for nitrite-driven methane oxidation. Their activity might be reflected by denitrification observe in the flasks with methane as the sole carbon source in batch assays (section 7.4.2). However, oxidation of methane is one of the most scientifically intriguing and controversial processes and there is no single mechanism of coupling it to denitrification. Apart from archaeal anaerobic activity, in most cases, nitrogen removal in the presence of CH_4 and O_2 is a mixture between methanotrophic, denitrifying, ammonia-oxidizing and Anammox activity (Waki *et al.*, 2009). In the case of proposed UASB-MBR system anaerobic/anoxic denitrification with methane was proved to be possible. Moreover, the application of membrane technology could be a solution to problems related with wash-out of extremely slow-growing bacteria, such as denitrifying methanotrophs (Kampman *et al.*, 2012), and avoid the loss of methanogenic bacteria that reaches the MBR from the UASB reactor.

7.4.4 Anammox bacteria

Curiously, during the operation of the system, Anammox bacteria were found (figure 7-8). Their presence is difficult to explain, since their activity was not observed during the very first days of the operation of the system and no information on their presence in the inoculum taken from the IC reactor was available.

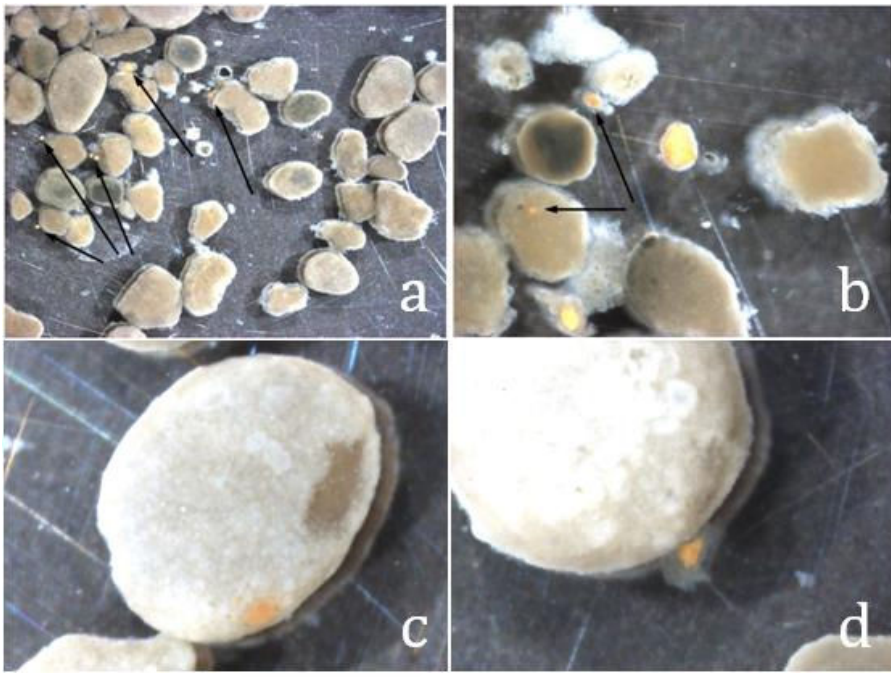

Figure 7-8 Orange granules of Anammox bacteria observed in the anaerobic stage of the system on 149 (photos a and b) and 190 (photos c and d) day of operation (left x6.5, right x20, bottom x50).

On the other hand, the combined UASB-MBR system was not designed for anaerobic ammonia oxidizing bacteria development. However, their occurrence in both anaerobic (figure 7-8) and MBR stage of the reactor was observed, and in the case of the later, confirmed by FISH analysis. In figure 7-8 very small orange granules, typical for Anammox bacteria, can be observed. This orange (sometimes red) colour is one of the features of Anammox bacteria and occurs due to the heme c group of the protein cytochrome c, that plays an important role in metabolism of these bacteria

(Strous *et al.*, 1999). As can be seen in figure 7-8, Anammox bacteria were forming either independent aggregates (photos a and b), or were attached to the outer layer of anaerobic granules. In both cases, these bacterial formation were detected in the upper parts of UASB stage (sampling ports P4 and P5, occasionally P3).

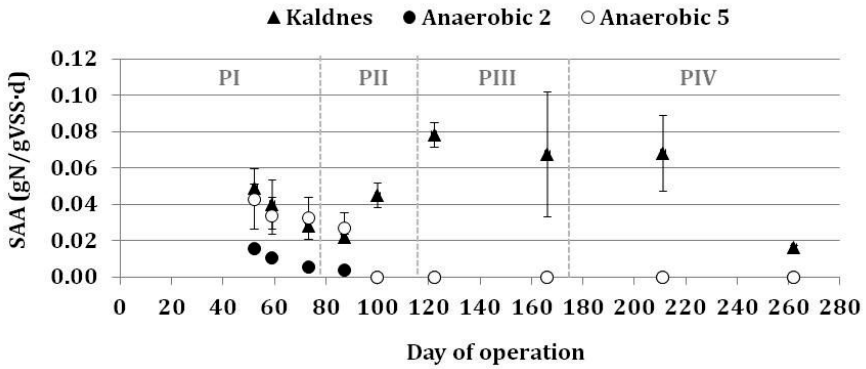

Figure 7-9 Average specific Anammox activity (SAA) of biofilm growing on Kaldnes support (▲), anaerobic granules taken form sampling port P2 (●) and anaerobic granules taken from sampling port P5 (○) during the start-up period of combined UASB-MBR system operation.

Anammox activity was observed and followed in the attached biomass of aerobic chamber and in anaerobic granules (figure 7-9), taken from two sampling ports of the UASB stage: P2 and P5. The specific anammox activity values reached up to 0.10 gN·gVSS·d^{-1} in the case of biofilm, and up to 0.06 gN·gVSS·d^{-1} in the case of anaerobic granular biomass taken form P5 (figure 7-9). This values stands for 20 and 12% of the reference anammox activity (0.5 gN·gVSS·d^{-1}, measured in the "Mother" reactor for Anammox bacteria cultivation at University of Santiago de Compostela, Spain), respectively. However, since the system operated at ambient temperatures (17 – 25 °C, while the SAA activity is performed at 35 °C), the anammox activity in-situ was most probably significantly lower. Fernández (2010) studied the short- and long-term effects of temperature on the anammox biomass activity. These authors discovered that by lowering the temperature from 30 to 20 °C the SAA was decreased by more than 55%. However, adaptation of biomass to low temperatures was also observed.

In table 7-6 the average SAA values corrected by the temperature factor (SAA at 20 °C is equal to around 40% of the SAA measured at 35 °C;

Fernández, 2010) are presented. As can be seen, the maximum potential values of nitrogen elimination that could be achieved in aerobic chamber of MBR stage (biofilm biomass) reached not more than 7.5 $gN \cdot d^{-1}$. On the other hand, the amount of nitrogen required for cell synthesis during the operation periods when anammox was observed was 2.81 $gN \cdot d^{-1}$ (taking into account the overall biomass yield calculated in Chapter 3, being 0.14 $gVSS \cdot gCOD^{-1} \cdot d^{-1}$, and that 0.122 $gN \cdot gVSS^{-1}$ of new cells is necessary; Tchobanoglous *et al.*, 2004). However, neither is reflected in the TN balance of the reactor (figure 7-10).

Table 7-6 Average SAA values for biofilm and anaerobic biomass. (a) indicates the specific Anammox activity corrected by the temperature factor, being 0.4 for 20 °C (according to Fernández, 2010), (b) SAA values corrected by factor 0.8, since at the end of SAA test 20% of gas phase was formed by methane.

Biomass	SAA ($gN \cdot gVSS^{-1} \cdot d^{-1}$)	Corrected SAA ($gN \cdot gVSS^{-1} \cdot d^{-1}$)	Potential N elimination ($gN \cdot d^{-1}$)
Biofilm	0.059±0.043	0.023±0.017[a]	4.30±3.17
Anaerobic P2	0.011±0.007	0.003±0.002[a, b]	6.71±4.45
Anaerobic P5	0.039±0.021	0.012±0.007[a, b]	24.88±13.13

Additionally, the potential elimination of nitrogen calculated for anaerobic biomass would suggest complete N removal, however adequate substrate availability was not maintained in the UASB stage for Anammox bacteria development. Moreover, since about 20% of methane was detected at the end of SAA test with anaerobic granular biomass (as indicated in table 7-6), these results might had been overestimated. Even though in these tests ammonia was completely consumed, indicating that anammox activity occurred, the undesired activity of methanogenic bacteria and/or even denitrifying activity could interfere with the SAA estimation. However, whatever the fraction of anammox activity was, it disappeared after the experiment with methanol addition (figure 7-9).

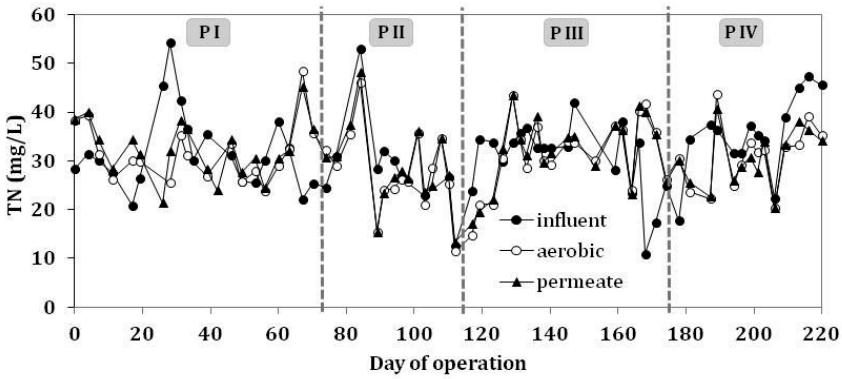

Figure 7-10 Total nitrogen concentration during four operational periods of the start-up of the combined UASB-MBR system: (●) influent, (○) aerobic MBR chamber and (▲) permeate.

As can be seen in figure 7-9, the average anammox activity measured in biofilm and anaerobic biomass decreased gradually during the first operating period (days 0 – 77), characterized by high COD load variations (Chapter 3, section 3.4.2). Only in Period II, when the addition of methanol was stopped and better control of incoming COD was achieved, SAA of biofilm biomass increased, reaching the highest values in Period III, being 0.07±0.034 gN·gVSS·d^{-1} (figure 7-9). However, in the case of anaerobic granules anammox activity was no longer detected. This could be explained either by the fact that methanol addition (performed at the beginning of Period II) directly inhibited anaerobic ammonia oxidation. Irreversible inhibition of anaerobic ammonium oxidation by methanol was reported before (Isaka *et al.*, 2008). Another reason could be the fact that Anammox bacteria were outcompeted by other microorganisms, such as, for example, hydrolytic or denitrifying heterotrophs. On the other hand, the recirculation ratio between aerobic chamber of MBR stage and UASB stage was relatively high (R=0.15), which might have caused a gradual wash-out of extremely slow-growing Anammox bacteria. Even though due to the application of membrane filtration all microorganisms are retained in the system (except from biomass purges), in the mixed aerobic/anoxic/anaerobic conditions and presence of biodegradable organic matter Anammox bacteria are unlikely to survive. Therefore, eventually the SAA activity was only measured in biofilm, where anoxic conditions and optimal substrate availability could be obtained. Nevertheless, after 260 day of operation of

the combined UASB-MBR system the SAA measured in biofilm biomass decreased to 0.02 gN·gVSS·d^{-1} and was no longer taken into account.

Nonetheless, anammox activity should not be discarded, whenever there are gaps in nitrogen removal (figure 7-10, Chapter 3, Chapter 5). Moreover, since Anammox process was possible in combined UASB-MBR, optimization of these kind of systems towards Anammox development could be another solution for the facilities where nitrogen removal is an issue. Moreover, since Anammox was proved to adapt to temperatures as low as 15 °C (Vázquez-Padín *et al.*, 2009; Fernandez, 2010), these systems could also be applicable in the mild region countries. Nevertheless, further study on optimization of the operational conditions would be crucial.

On the other hand, as previously mentioned by Waki *et al.* (2009) if anammox activity could be increased, the coupling of anammox to NO_3^- reduction with CH_4 oxidation, NH_4^+ oxidation, or both, would also be a feasible post-treatment after anaerobic digestion. In current one-stage full-scale autotrophic nitrogen removal bioreactors in which anammaox bacteria are present, nitrite is supplied through partial nitrification by aerobic ammonium-oxidizing bacteria. In these systems, aerobic and anaerobic ammonium-oxidizing microorganisms convert inorganic nitrogen compounds under oxygen-limited conditions in a single-stage reactor. In similar oxygen-limited systems, such as could be the anoxic chamber of proposed in this Thesis UASB-MBR system (section 7.4.1), the nitrite-dependent anaerobic methanotrophs (such as *M.* oxyfera) would have to compete with both aerobic methane-oxidizing and heterotrophic denitrifying bacteria, and anaerobic ammonia-oxidizing (anammox) bacteria (Leusken *et al.*, 2011). However, the same authors also stated, that most likely, if ammonium is present in excess, Anammox bacteria would probably outcompete anaerobic methanotrophs, suggesting that Anammox bacteria have a higher affinity for nitrite.

Finally, the confirmation of the presence of Anammox bacteria was done by FISH analysis. In this sense, the biomass attached to Kaldnes rings (where the SAA was observed) was analysed. As it can be seen in figure 7-11, strong evidence of the presence of Anammox bacteria was found; firstly, because of the positive signal obtained via FISH analysis, and secondly,

because of the specific colony formation, which in case of Anammox bacteria resembles cauliflower (Schmid *et al.*, 2003).

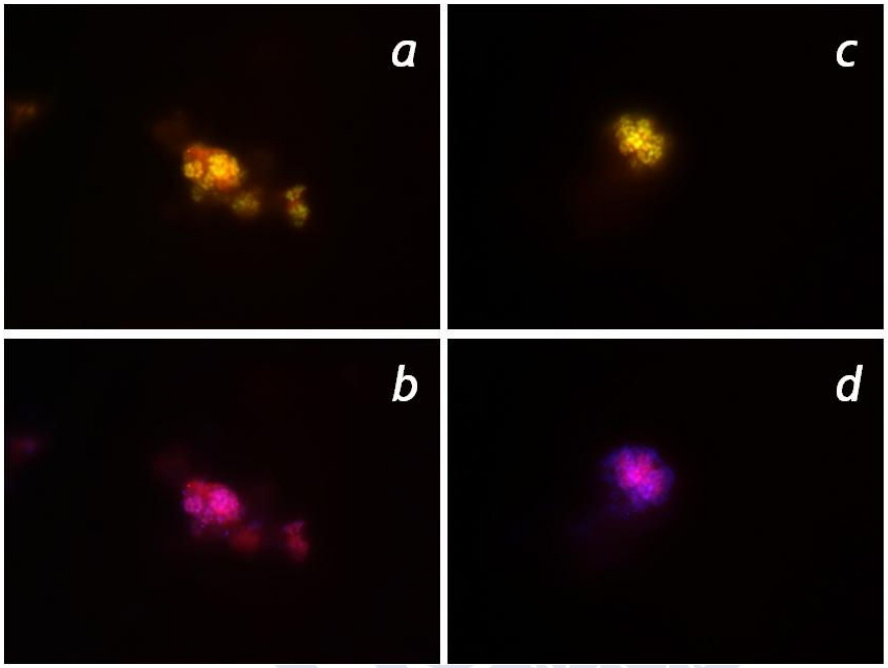

Figure 7-11 (a), (c) AMX368 cy3 Anammox (red) and combination of EUB338I + EUB338II Fluos Bacteria domain and Planctomycetales (green), x100; (b), (d) AMX368 cy3 Anammox (red) and DAPI confirmation (blue), x100.

In any case, the presence and activity of Anammox bacteria in the system presented in this work shows its potential to develop a wide variety of populations of microorganisms, depending on the effluent requirements. However, to establish optimal conditions for growth and maintenance of selected bacteria, further study would be necessary.

7.5 CONCLUSIONS

Membrane bioreactors (MBR) might be the suitable technology as a post-treatment for an anaerobic digester effluent. The use of membranes would produce not only a high quality effluent, suitable for reuse, but will also allow a complete retention of the microorganisms in the system, which is very important in the case of methanogenic and methanotrophic bacteria.

The use of methane as an electron donor in denitrification reaction could be an alternative to reduce total nitrogen and GHGs emissions of those wastewater treatment plants using a first methanogenic stage. Denitrification in an MBR stage using dissolved methane present in the effluent of an anaerobic UASB system was proved to be possible and feasible.

FISH analysis revealed a broad spectrum of microbial populations that may develop in the proposed system. Among them, a newly discovered bacteria belonging to NC10 phylum, capable to denitrify with methane as a sole carbon and energy source was found.

The presence of Methanotrophs and activity of Anammox bacteria in the system presented in this work shows its potential to develop a wide variety of populations of microorganisms, depending on the effluent requirements. However, to establish optimal conditions for growth and maintenance of selected bacteria, further study would be necessary.

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General conclusions

The present study is a step forward into the development of combined UASB-MBR technology, since it is an attempt to resolve problems related to the main drawbacks of such a solution, related with the need of post-treatment of anaerobic effluents, the operation of anaerobic MBR (fouling, low membrane fluxes) and aerobic MBR (high energy consumption and sludge production) and the identification of some of the microorganisms involved in the processes.

1. Operation effectiveness

The combined UASB-MBR system achieved excellent COD removal performance, comparable with aerobic MBRs treating domestic- and dairy-type wastewater at ambient temperatures. On average, the permeate COD was less than 6 $mg \cdot L^{-1}$ and the s-COD removal was above 95%, reaching 99% during the stable operation. Additionally, the effluent was free of suspended solids. Moreover, UASB-MBR system presented a high tolerance to organic loading changes (up to 3.9 $kgCOD \cdot m^{-3} \cdot d^{-1}$) and temperature fluctuations (17 – 25 °C).

Very low COD concentration and the level of nutrients in the effluent allows reusing purified wastewater (e.g. in agriculture). Moreover, application of the membrane module guarantees bacteria free permeate.

Conversion of nitrogen compounds was observed in the combined UASB-MBR system, with nitrification being the dominant process. On the other hand, denitrification in the MBR stage using dissolved methane present in the effluent of UASB stage was proved to be possible and feasible.

High biogas production was detected during the whole operation of the system, with an average methane content of 73% and the highest production rate, measured was 130 $L \cdot d^{-1}$.

Aerobic MBR post-treatment of the UASB effluent in general serves as a buffer; in the case when the anaerobic COD removal efficiency decreases, the remaining organic matter is oxidized in the aerobic MBR chamber. However, longer HRT assures almost complete elimination of COD in the methanogenic step.

Comparison of the results obtained during the first stages of the present work, using LCA analysis, indicated that eutrophication was a potential bottleneck of combined UASB-MBR system, with the main contributor being direct release of nutrients present in the effluent. For global warming effects the avoided production of energy from the generated biogas was the reason behind the negative values, which implies beneficial consequences. However, this assumption did not take into account the impact of dissolved methane stripped off the UASB effluent. However, in the case of combined UASB-MBR system the use of methane as an electron donor in denitrification reaction could be an alternative to reduce total nitrogen and GHGs emissions of those wastewater treatment plants using a first methanogenic stage.

2. Membrane performance

The membrane operated with fluxes of 15 $L \cdot m^{-2} \cdot h^{-1}$, lower than those achieved in aerobic MBRs treating municipal wastewater, but higher than fluxes obtained in anaerobic MBRs.

With respect to the membrane performance, both MLVSS concentration and recirculation between aerobic and anaerobic stages were the main factors affecting membrane fouling. Therefore, in this system it would be necessary to assure a minimum OLR in the aerobic stage in order to minimize fouling rate.

The predominant fouling that took place in the membrane was reversible fouling, since permeability was recovered with mechanical cleaning. cBPC concentration was reported as a reliable parameter related with fouling rate, in batch-, lab- and pilot-scale experiment. Since determination of cBPC is much easier and less costly than other fouling indicators (i.e. SMP and EPS), this parameter was recommended for the follow-up of the fouling potential of the liquid broth of MBR stage.

The surplus aerobic sludge hydrolysis in UASB stage (due to its recirculation from MBR stage) resulted in increased cBPC release, and thus, strongly affected membrane operation. This tendency and was observed especially in periods when the MLVSS concentration in the MBR stage was low. Therefore, the recommended concentration of MLVSS in filtration stage of proposed UASB-MBR system should not be lower than 3 $g \cdot L^{-1}$. Positive correlation of cBPC and fouling properties of sludge was also confirmed in batch assays.

3. Biomass characterization and microbiology issues

Anaerobic granules growth was observed during the whole experimental period, reaching concentration of 34 gVSS·L^{-1} and the average diameter of around 3 mm. Moreover, microscopic observations of the anaerobic biomass allowed to examine the formation of new layers of biomass on the surface of existing granules used as seed.

The presence of Kaldnes support in the aerobic chamber of the system was crucial for its stable operation due to the development of proto- and metazoa. The colonies of ciliates and rotifers attached to Kaldnes rings feed on non-flocculated bacteria and colloids, decreasing the turbidity of the liquid phase and controlling excessive bacterial growth. Moreover, they probably have a positive impact on fouling properties of the mixed liquor.

FISH analysis revealed that among *Proteobacteria* phylum, a subclass of *Betaproteobacteria* was the most dominant, followed by the *Gammaproteobacteria*. *Alphaproteobacteria* was scarce and appeared in coccoid form, while bacteria belonging to *Deltaproteobacteria* were not observed at all. The predominance of members of *Betaproteobacteria* was associated with abundance of nitrifying and denitrifying bacteria. Apart from these microorganisms, *Bacteroidetes*, nitrite-oxidizing bacteria (NOB), *Acidobacteria*, *Firmicutes* and filamentous bacteria belonging to *Chloroflexi* were also detected.

FISH analysis confirmed that a broad spectrum of microbial populations may develop in the proposed UASB-MBR system. Among them, a newly discovered bacteria belonging to NC10 phylum, capable to denitrify with methane as a sole carbon and energy source was found in the MBR stage.

Finally, some Anammox and bacteria belonging to NC10 phylum were detected indicating the wide range of microorganisms that could develop in the proposed combined UASB-MBR system.

4. Precautions, tips and future perspectives

Since MLVSS concentration and recirculation between aerobic and anaerobic stages were the main factors affecting membrane fouling, it is crucial to assure a minimum OLR in the aerobic stage for biomass development and therefore minimizing fouling rate.

The application of the internal recirculation (from MBR stage to UASB stage) allows to avoid a loss of methanogenic biomass in the case of its wash-out from UASB stage of the system. On the other hand, it assures lower overall sludge production, since part of the surplus aerobic sludge is hydrolysed in anaerobic stage. Moreover, non-readily biodegradable compounds can be subjected to further degradation. However, caution has to be taken since excess aerobic sludge hydrolysis in UASB stage results in increased cBPC, EPS and SMP concentrations and will negatively affect membrane performance.

Regarding phosphorus removal, chemical precipitation could be used if required

Membrane bioreactors (MBR) might be the suitable technology as a post-treatment of (existing) anaerobic digesters effluents, especially at ambient temperatures. The use of dissolved methane as an electron donor in denitrification reaction could be an alternative to reduce total nitrogen and GHGs emissions of those wastewater treatment plants using a first methanogenic stage. Moreover, application of membranes would produce not only a high quality effluent, suitable for reuse, but will also allow a complete retention of microorganisms in the system, which is very important in the case of slow growing rate microorganisms.

The presence of *Methanotrophs* and activity of Anammox bacteria, as well as discovery of microorganisms belonging to newly described NC10 phylum shows the UASB-MBR system potential to develop a wide variety of populations of microorganisms, depending on the effluent requirements. However, to establish optimal conditions for growth and maintenance of selected bacteria, further study would be necessary.

Conclusiones generales

El presente estudio es un paso adelante en el desarrollo de la tecnología combinada UASB-MBR, ya que es un intento de resolver los problemas relacionados con los principales inconvenientes de esta solución, en relación con la necesidad de post-tratamiento de los efluentes anaerobios, la operación del MBR anaerobio (ensuciamiento, flujos bajos de membrana) y del MBR aerobio (alto consumo de energía y producción de lodos) y la identificación de algunos de los microorganismos implicados en los procesos.

1. Eficacia de la operación

El sistema combinado UASB-MBR logró una excelente eliminación de la DQO, comparable con los MBR aerobios tratando aguas residuales domésticas y de lechería a temperatura ambiente. En promedio, el DQO del permeado fue inferior a 6 $mg \cdot L^{-1}$ y la eliminación de DQO-s estuvo por encima de 95%, llegando a 99% durante la operación estable. Adicionalmente, el efluente estaba completamente libre de sólidos en suspensión. Además, el sistema UASB-MBR tuvo una gran tolerancia a los cambios de carga orgánica (hasta 3,9 $kgDQO \cdot m^{-3} \cdot d^{-1}$) y a las fluctuaciones de temperatura (17 – 25 ° C).

La baja concentración de DQO y el nivel de nutrientes en el efluente permiten reutilizar las aguas residuales purificadas (por ejemplo, en la agricultura). Además, la aplicación del módulo de membrana garantiza un permeado libre de bacterias.

Se observó conversión de compuestos de nitrógeno en el sistema combinado UASB-MBR, siendo la nitrificación el proceso dominante. Por otra parte, se ha demostrado que era posible llevar a cabo la desnitrificación en la etapa MBR usando el metano disuelto presente en el efluente.

Se observó una elevada producción de biogás durante toda la operación del sistema, con un contenido de metano promedio de 73%. La velocidad de producción más alta medida fue de 130 $L \cdot d^{-1}$.

El post-tratamiento del efluente del UASB mediante el MBR aerobio, en general sirve como un tampón. En los casos en que la eficiencia de eliminación

anaerobia de DQO disminuye, la materia orgánica restante se oxida en la cámara del MBR aerobio. Sin embargo, los TRH largos aseguran la eliminación casi total de la DQO en la etapa metanogénica.

La comparación de los resultados obtenidos durante las primeras etapas del presente trabajo, utilizando el ACV, indicó que la eutrofización era un cuello de botella potencial del sistema combinado UASB-MBR, siendo el contribuyente principal la liberación directa de nutrientes presentes en el efluente. En el caso de los efectos del calentamiento global, la producción de energía evitada gracias al biogás generado fue la razón de los valores negativos, lo que implica consecuencias beneficiosas. Sin embargo, esta hipótesis no tuvo en cuenta el impacto del metano disuelto liberado por el efluente del UASB. De todos modos, en el caso del sistema combinado UASB-MBR, el uso de metano como donador de electrones en la reacción de desnitrificación puede ser una alternativa para reducir las emisiones de nitrógeno total y gases de efecto invernadero de las plantas de tratamiento de aguas residuales que usan una primera etapa metanogénica.

2. Rendimiento de la membrana

La membrana operó con flujos de 15 $L \cdot m^{-2} \cdot h^{-1}$, inferiores a los alcanzados en MBRs aerobios tratando aguas residuales municipales, pero superiores a los flujos obtenidos en MBRs anaerobios.

Con respecto al rendimiento de la membrana, tanto la concentración de SSV en el licor de mezcla como la recirculación entre la etapa aerobia y anaerobia fueron los factores principales que afectaron al ensuciamiento de la membrana. Por lo tanto, en este sistema sería necesario asegurar una mínima VCO en la etapa aerobia con el fin de minimizar la velocidad de ensuciamiento.

El ensuciamiento predominante que tuvo lugar en la membrana fue ensuciamiento reversible, ya que la permeabilidad se recuperó con la limpieza mecánica. La concentración de cBPC fue un parámetro fiable para seguir la velocidad de ensuciamiento, en los experimentos discontinuos, de laboratorio y a escala piloto. Dado que la determinación de cBPC es mucho más fácil y menos costosa que otros indicadores de ensuciamiento (i.e. SMP y EPS), este parámetro se recomienda para el seguimiento del potencial de ensuciamiento del licor de mezcla de la etapa MBR.

La hidrólisis del excedente de lodo aerobio en la etapa UASB (debido a su recirculación desde la etapa MBR) resultó en una mayor liberación de cBPC, y por lo tanto, afectó de forma importante a la operación de la membrana. Esta tendencia ya se observó especialmente en los períodos en los que la concentración de SSV en la etapa de MBR era baja. Por lo tanto, la concentración recomendada de SSV en el licor de mezcla en la etapa de filtración en el sistema propuesto UASB-MBR no debe ser inferior a 3 $g \cdot L^{-1}$. La correlación positiva entre cBPC y el potencial de ensuciamiento del lodo también se confirmó en ensayos discontinuos.

3. Caracterización de la biomasa y microbiología

El crecimiento de gránulos anaerobios se observó durante todo el período experimental, alcanzando una concentración de 34 $gSSV \cdot L^{-1}$ y un diámetro promedio de alrededor de 3 mm. Además, las observaciones microscópicas de la biomasa anaerobia permitieron examinar la formación de nuevas capas de biomasa sobre la superficie de gránulos existentes utilizados como inóculo.

La presencia de soportes Kaldnes en la cámara aerobia del sistema fue crucial para su funcionamiento estable debido al desarrollo de proto-y metazoos. Las colonias de ciliados y rotíferos unidos a los anillos Kaldnes se alimentaron de bacterias no-floculadas y coloides, disminuyendo la turbidez de la fase líquida y controlando el crecimiento bacteriano excesivo. Por otra parte, es probable que tengan un impacto positivo en las propiedades de ensuciamiento del licor de mezcla.

El análisis FISH reveló que dentro del filo *Proteobacteria*, la más dominante era una subclase de *Betaproteobacteria*, seguida por la *Gammaproteobacteria*. La presencia de *Alphaproteobacteria* fue escasa y apareció en forma de cocoides, mientras que las bacterias que pertenecen a *Deltaproteobacteria* no se observaron en absoluto. El predominio de los miembros de *Betaproteobacteria* se asoció con la abundancia de bacterias nitrificantes y desnitrificantes. Aparte de estos microorganismos, se detectaron Bacteroidetes, bacterias oxidantes de nitrito, *Acidobacteria*, *Firmicutes* y bacterias filamentosas pertenecientes a *Chloroflexi*.

El análisis FISH confirmó que en el sistema propuesto UASB-MBR se puede desarrollar un amplio espectro de poblaciones microbianas. Entre estas poblaciones, una bacteria recién descubierta perteneciente al filo NC10, capaz de

desnitrificar con metano como única fuente de carbono y de energía, fue detectada en la etapa de MBR.

Por último, se detectaron algunos Anammox y bacterias que pertenecen al filo NC10, lo cual indica la amplia gama de microorganismos que pueden desarrollarse en el sistema combinado UASB-MBR propuesto.

4. Precauciones, consejos y perspectivas de futuro

Dado que la concentración de SSV en el licor de mezcla y la recirculación entre las etapas aerobia y anaerobia fueron los factores principales que afectaron al ensuciamiento de la membrana, es crucial asegurar una mínima VCO en la etapa aerobia para el desarrollo de la biomasa y, por lo tanto, para minimizar la velocidad de ensuciamiento.

La aplicación de la recirculación interna (de la etapa MBR a la etapa UASB) permite evitar una pérdida de biomasa metanogénica en el caso de su lavado de la etapa UASB del sistema. Por otra parte, se asegura una menor producción de lodos en general, ya que parte del lodo aerobio excedente se hidroliza en la etapa anaerobia. Ademas, los compuestos no fácilmente biodegradables pueden ser sometidos a una mayor degradación. Sin embargo, debe tenerse precaución ya que la hidrólisis del exceso de lodo aerobio en la etapa UASB resulta en un aumento de las concentraciones de cBPC, EPS y SMP y afectará negativamente al rendimiento de la membrana.

En cuanto a la eliminación de fósforo, la precipitación química podría ser utilizada en caso de que sea necesaria.

Los biorreactores de membrana (MBR) podrían ser la tecnología adecuada como post-tratamiento de los efluentes de digestores anaerobios (existentes), especialmente a temperatura ambiente. El uso de metano disuelto como donador de electrones en la reacción de desnitrificación podría ser una alternativa para reducir las emisiones de nitrógeno total y de gases de efecto invernadero de las plantas de tratamiento de aguas residuales que emplean una primera etapa metanogénica. Además, la aplicación de las membranas produciría no sólo un efluente de alta calidad, adecuado para su reutilización, sino también permitirá una retención completa de microorganismos en el sistema, lo cual es muy importante en el caso de microorganismos de crecimiento lento.

La presencia de *Metanótrofos* y actividad de bacterias Anammox, así como el descubrimiento de microorganismos que pertenecen al recién descrito filo NC10 muestra el potencial del sistema UASB-MBR para desarrollar una amplia variedad de poblaciones de microorganismos, dependiendo de los requerimientos del efluente. Sin embargo, para establecer las condiciones óptimas para el crecimiento y el mantenimiento de las bacterias seleccionadas serían necesarios estudios adicionales.

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Conclusións xerais

O presente estudo é un paso adiante no desenvolvemento da tecnoloxía combinada UASB-MBR, xa que é un intento de resolver os problemas relacionados cos principais inconvenientes desta solución, en relación coa necesidade de post-tratamento dos efluentes anaerobios, a operación do MBR anaerobio (ensuciamento, fluxos baixos de membrana) e do MBR aerobio (alto consumo de enerxía e produción de lodos) e a identificación dalgúns dos microorganismos implicados nos procesos.

1. Eficacia da operación

O sistema combinado UASB-MBR logrou unha excelente eliminación da DQO, comparable cos MBR aerobios tratando augas residuais domésticas e de leitaría a temperatura ambiente. Como media, a DQO do permeado foi inferior a 6 $mg \cdot L^{-1}$ e a eliminación de DQO-s estivo por encima do 95%, chegando ó 99% durante a operación estable. Adicionalmente, o efluente estaba completamente libre de sólidos en suspensión. Ademais, o sistema UASB-MBR tivo unha gran tolerancia aos cambios de carga orgánica (ata 3,9 $kgDQO \cdot m^{-3} \cdot d^{-1}$) e ás flutuacións de temperatura (17 – 25 °C).

A baixa concentración de DQO e o nivel de nutrientes no efluente permiten reutilizar as augas residuais purificadas (por exemplo, na agricultura). Ademais, a aplicación do módulo de membrana garante un permeado libre de bacterias.

Observouse conversión de compostos de nitróxeno no sistema combinado UASB-MBR, sendo a nitrificación o proceso dominante. Por outra banda, demostrouse que era posible levar a cabo a desnitrificación na etapa MBR usando o metano disolto presente no efluente.

Observouse unha elevada produción de biogás durante toda a operación do sistema, cun contido de metano medio de 73%. A velocidade de produción máis alta medida foi de 130 $L \cdot d^{-1}$.

O post-tratamento do efluente do UASB mediante o MBR aerobio, en xeral serve como un tampón. Nos casos en que a eficiencia de eliminación

anaerobia de DQO diminúe, a materia orgánica restante oxídase na cámara do MBR aerobio. Con todo, os TRH longos aseguran a eliminación case total da DQO na etapa metanoxénica.

A comparación dos resultados obtidos durante as primeiras etapas do presente traballo, utilizando o ACV, indicou que a eutrofización era un pescozo de botella potencial do sistema combinado UASB-MBR, sendo o contribuínte principal a liberación directa de nutrientes presentes no efluente. No caso dos efectos do quecemento global, a produción de enerxía evitada grazas ao biogás xerado foi a razón dos valores negativos, o que implica consecuencias beneficiosas. Con todo, esta hipótese non tivo en conta o impacto do metano disolto liberado polo efluente do UASB. De tódolos xeitos, no caso do sistema combinado UASB-MBR, o uso de metano como donante de electróns na reacción de desnitrificación pode ser unha alternativa para reducir as emisións de nitróxeno total e gases de efecto invernadoiro das plantas de tratamento de augas residuais que usan unha primeira etapa metanoxénica.

2. Rendemento da membrana

A membrana operou con fluxos de 15 $L \cdot m^{-2} \cdot h^{-1}$, inferiores aos alcanzados en MBRs aerobios tratando augas residuais municipais, pero superiores aos fluxos obtidos en MBRs anaerobios.

Con respecto ao rendemento da membrana, tanto a concentración de SSV no licor de mestura como a recirculación entre a etapa aerobia e anaerobia foron os factores principais que afectaron ao ensuciamento da membrana. Polo tanto, neste sistema sería necesario asegurar unha mínima VCO na etapa aerobia co fin de minimizar a velocidade de ensuciamento.

O ensuciamento predominante que tivo lugar na membrana foi ensuciamento reversible, xa que se recuperou a permeabilidade coa limpeza mecánica. A concentración de cBPC foi un parámetro fiable para seguir a velocidade de ensuciamento, nos experimentos descontinuos, de laboratorio e a escala piloto. Dado que a determinación de cBPC é moito máis fácil e menos custosa que outros indicadores de ensuciamento (i.e. SMP e EPS), recoméndase este parámetro para o seguimento do potencial de ensuciamento do licor de mestura da etapa MBR.

A hidrólise do excedente de lodo aerobio na etapa UASB (debido á súa recirculación desde a etapa MBR) resultou nunha maior liberación de cBPC, e polo tanto, afectou de forma importante á operación da membrana. Esta tendencia xa se observou especialmente nos períodos nos que a concentración de SSV na etapa de MBR era baixa. Polo tanto, a concentración recomendada de SSV no licor de mestura na etapa de filtración no sistema proposto UASB-MBR non debe ser inferior a 3 $g \cdot L^{-1}$. A correlación positiva entre cBPC e o potencial de ensuciamento do lodo tamén se confirmou en ensaios descontinuos.

3. Caracterización da biomasa e microbioloxía

O crecemento de gránulos anaerobios foi observado durante todo o período experimental, alcanzando unha concentración de 34 $gSSV \cdot L^{-1}$ e un diámetro medio de ao redor de 3 mm. Ademais, as observacións microscópicas da biomasa anaerobia permitiron examinar a formación de novas capas de biomasa sobre a superficie de gránulos existentes utilizados como inóculo.

A presenza de soportes Kaldnes na cámara aerobia do sistema foi crucial para o seu funcionamento estable debido ao desenvolvemento de proto- e metazoos. As colonias de ciliados e rotíferos unidos aos aneis Kaldnes alimentáronse de bacterias non-floculadas e coloides, diminuíndo a turbidez da fase líquida e controlando o crecemento bacteriano excesivo. Por outra banda, é probable que teñan un impacto positivo nas propiedades de ensuciamento do licor de mestura.

A análise FISH revelou que dentro do filo *Proteobacteria*, a máis dominante era unha subclase de *Betaproteobacteria*, seguida pola *Gammaproteobacteria*. A presenza de *Alphaproteobacteria* foi escasa e apareceu en forma de cocoides, mentres que as bacterias que pertencen a *Deltaproteobacteria* non se observaron en absoluto. O predominio dos membros de *Betaproteobacteria* foi asociado coa abundancia de bacterias nitrificantes e desnitrificantes. Ademais destes microorganismos, detectáronse *Bacteroidetes*, bacterias oxidantes de nitrito, *Acidobacteria*, *Firmicutes* e bacterias filamentosas pertencentes a *Chloroflexi*.

A análise FISH confirmou que no sistema proposto UASB-MBR se pode desenvolver un amplo espectro de poboacións microbianas. Entre estas poboacións, unha bacteria recentemente descuberta pertencente ao filo NC10,

capaz de desnitrificar con metano como única fonte de carbono e de enerxía, foi detectada na etapa de MBR.

Para rematar, detectáronse algúns Anammox e bacterias que pertencen ao filo NC10, o cal indica a ampla gama de microorganismos que poden desenvolverse no sistema combinado UASB-MBR proposto.

4. Precaucións, consellos e perspectivas de futuro

Dado que a concentración de SSV no licor de mestura e a recirculación entre as etapas aerobia e anaerobia foron os factores principais que afectaron ao ensuciamento da membrana, é crucial asegurar unha mínima VCO na etapa aerobia para o desenvolvemento da biomasa e, polo tanto, para minimizar a velocidade de ensuciamento.

A aplicación da recirculación interna (da etapa MBR á etapa UASB) permite evitar unha perda de biomasa metanoxénica no caso do seu lavado da etapa UASB do sistema. Por outra banda, asegúrase unha menor produción de lodos en xeral, xa que parte do lodo aerobio excedente se hidroliza na etapa anaerobia. Ademais, os compostos non facilmente biodegradables poden ser sometidos a unha maior degradación. Con todo, debe terse precaución xa que a hidrólise do exceso de lodo aerobio na etapa UASB resulta nun aumento das concentracións de cBPC, EPS e SMP e afectará negativamente ao rendemento da membrana.

En canto á eliminación de fósforo, a precipitación química podería ser utilizada no caso de que sexa necesaria.

Os biorreactores de membrana (MBR) poderían ser a tecnoloxía adecuada como post-tratamento dos efluentes de dixestores anaerobios (existentes), especialmente a temperatura ambiente. O uso de metano disolto como donante de electróns na reacción de desnitrificación podería ser unha alternativa para reducir as emisións de nitróxeno total e de gases de efecto invernadoiro das plantas de tratamento de augas residuais que empregan unha primeira etapa metanoxénica. Ademais, a aplicación das membranas produciría non só un efluente de alta calidade, adecuado para a súa reutilización, senón tamén permitirá unha retención completa de microorganismos no sistema, o cal é moi importante no caso de microorganismos de crecemento lento.

A presenza de *Metanótrofos* e actividade de bacterias Anammox, así como o descubrimento de microorganismos que pertencen ao recentemente descrito filo NC10 mostra o potencial do sistema UASB-MBR para desenvolver unha ampla variedade de poboacións de microorganismos, dependendo dos requirimentos do efluente. Con todo, para establecer as condicións óptimas para o crecemento e o mantemento das bacterias seleccionadas serían necesarios estudos adicionais.

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List of acronyms and symbols

16S	Specific region in rRNA genes	—
23S	Specific region in rRNA genes	—
A	Adenine	—
AD	Anaerobic Digester	—
AF	Anaerobic Filter	—
AI	Intermediate Alkalinity	$mgCaCO_3 \cdot L^{-1}$
AMPTS	Automatic Methane Potential Test System	—
Anammox	Anaerobic AMMonium Oxidation	—
AnMBR	Anaerobic MBR	—
AOA	Ammonium Oxidizing Archaea	—
AOB	Ammonia Oxidizing Bacteria	—
AP	Partial Alkalinity	$mgCaCO_3 \cdot L^{-1}$
APHA	American Public Health Association	—
APS	Ammonium persulfate	—
AS	Activated Sludge	—
AT	Total Alkalinity	$mgCaCO_3 \cdot L^{-1}$
AWWA	American Water Works Association	—
BC	Before Christ	—
BCE	Before the Common Era	—
BOD	Biological Oxygen Demand	$g \cdot L^{-1}$
BPC	Biopolymer cluster	$gTOC \cdot L^{-1}$

BREF	Best Available Techniques (BAT) reference document	—
C	Cytosine	—
CAS	Conventional Activated Sludge systems	—
cBPC	Colloidal fraction of BPC	$gTOC \cdot L^{-1}$
CLSM	Confocal Scanning Microscope	—
COD	Chemical Oxygen Demand	$g \cdot L^{-1}$
COD/N	Chemical oxygen demand to nitrogen ratio	—
CW	Constructed Wetlands	—
Cy3	Cyanine 3	—
DAF	Dissolved Air Flotation	—
DAPI	4´,6-DiAmidino-2-Phenylindole	—
DGGE	Denaturing Gradient Gel Electrophoresis	—
DNA	Deoxyribo-Nucleic Acid	—
DO	Dissolved oxygen concentration	$mgO_2 \cdot L^{-1}$
EDTA	Ethylene-Diamine-Tetra-Acetic Acid	—
EGSB	Expanded Granular Sludge Blanket	—
EMBL	European Molecular Biology Laboratory	—
EPA	US Environmental Protection Agency	—
EPS	Extracellular Polymeric Substances	—
F	Formamide	—
F/M	Food to microorganism ratio	—
FA	Free ammonia	$gN \cdot L^{-1}$
FAS	Ferrous Ammonium Sulphate	—
FB	Fluidized Bed	—
FID	Flame Ionization Detector	—
FISH	Fluorescent *In Situ* Hybridization	—

G	Guanine	—
GF	Glass Fiber filters	—
GHG	GreenHouse Gas	
GLS	Gas-Liquid-Solid separator	—
HAO	Hydrohylamine oxidoreductase	—
HF	Hollow Fibre	—
HMABR	Hybrid Anaerobic Baffled Reactor	—
HRT	Hydraulic retention time	d
HyMBR	Hybrid MBR	—
HyVAB	Hybrid Vertical Anaerobic sludge – Anaerobic Biofilm reactor	—
IC	Inorganic Carbon	$g \cdot L^{-1}$
IC	Internal Circulation reactor	—
IWA	Internation Water Association	—
J	Flux	$L \cdot m^{-2} \cdot h^{-1}$
MBBR	Moving Bed Biofilm Reactor	—
MBR	Membrane Biological Reactor	—
MF	Microfiltration	—
MLTSS	Mixed Liquor Total Suspended Solids	$g \cdot L^{-1}$
MLVSS	Mixed Liquor Volatile Suspended Solids	$g \cdot L^{-1}$
N	Nitrogen	—
NCBI	National Center for Biotechnology Information	—
NED	N-(1-napththyl)-ethylenediamine	—
NF	Nanofiltration	—
NO	Nitrous Oxide	—
NOB	Nitrite Oxidizing Bacteria	—
NPGA	Neopentylglycoladipate	—
OLR	Organic Loading Rate	$kgCOD \cdot m^{-3} \cdot d^{-1}$

P	Phosphorus	—
PAC	Powdered Activated Carbon	—
PAO	Polyphosphate Accumulating Organisms	—
PBS	Phosphate Buffer Solution	—
PCR	Polymerase Chain Reaction	—
PET	Polyethylene terephthalate	—
PHA	Poly-Hydrohy-Alkanoates	—
PHB	Poly-Hydrohy-Butyrates	—
PLC	Programmable Logic Controller	—
POC	Particulate Organic Carbon	—
PS	Polysaccharides	$g \cdot L^{-1}$
PTFE	Polytetrafluoroethylene	—
PVDF	Polyvinylidene fluoride	—
R	Recirculation Ratio	—
R	The ideal gas coefficient (0.082)	$atm \cdot L \cdot mol \cdot K^{-1}$
RBC	Rotating Biofilm Contactor	—
RO	Reversed Osmosis	—
rRNA	Ribosomal Ribo-Nucleic Acid	—
SAA	Specific Anammox Activity	$gN \cdot gVSS \cdot d^{-1}$
SAM	Sequencing Anoxic/Anaerobic MBR	—
SAMBR	Submerged Anaerobic MBR	—
SAnAMBR	Staged Anaerobic and Aerobic MBR	—
SBR	Sequencing Batch Reactor	—
s-COD	Soluble COD	$g \cdot L^{-1}$
SEM	Scanning Electron Microscope	—
SF	Supernatant Filterability	$mL \cdot min^{-1}$
SMA	Specific Methanogenic Activity	$gCOD \cdot gVSS^{-1} \cdot d^{-1}$

SRF	Specific Resistance to Filtration	$m \cdot kg^{-1}$
SRT	Solids Retention Time	d
SS	Suspended Solids	$g \cdot L^{-1}$
SVI_n	Sludge Volumetric Index after n minutes of settling	$mL \cdot gVSS^{-1}$
T	Thymine	—
TAE	Tris base, acetic acid and EDTA	—
TC	Total Carbon (in this Thesis Total dissolved Carbon)	$g \cdot L^{-1}$
t-COD	Total COD	$g \cdot L^{-1}$
TEMED	N,N,N´,N´-TEtraMethylEneDiamine	—
TF	Trickling Filter	—
TMP	Transmembrane pressure	mbar
TN	Total Nitrogen	$g \cdot L^{-1}$
TOC	Total Organic Carbon (in this Thesis Total dissolved Organic Carbon)	$g \cdot L^{-1}$
Tris	Tris(hydrohymethyl)aminomethane	—
TSS	Total Suspended Solids	$g \cdot L^{-1}$
UASB	Upflow Anaerobic Sludge Blanket	—
UF	Ultrafiltration	—
UV	Ultraviolet	—
VFA	Volatile Fatty Acids	$g \cdot L^{-1}$
VSS	Volatile Suspended Solids	$g \cdot L^{-1}$
WPCF	Water Pollution Control Federation	—
WWTP	Wastewater Treatment Plant	—
Y	Yield coefficient	$gVSS \cdot gCOD^{-1}$
μ	Viscosity	$kg \cdot m^{-1} \cdot s^{-1}$

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